International Journal of Pharmaceutics 424 (2012) 76–88

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Development of intravenous lipid emulsion of tanshinone IIA and evaluation of its anti-hepatoma activity *in vitro*

Ting Chu [a], Qing Zhang [a], Hui Li [b,c], Wei-cong Ma [a], Na Zhang [a], Hui Jin [a], Sheng-jun Mao [a,*]

[a] *Key Laboratory of Drug Targeting and Drug Delivery System, Ministry of Education, West China School of Pharmacy, Sichuan University, No. 17, Section 3, Southern Renmin Road, Chengdu 610041, PR China*
[b] *Department of Hematology, Sichuan Provincial People Hospital & Sichuan Academy of Medical Sciences, Chengdu 610072, PR China*
[c] *Department of Hematology, West China Hospital & State Key Laboratory of Biotherapy, Sichuan University, Chengdu, Sichuan 610041, PR China*

ARTICLE INFO

Article history:
Received 7 September 2011
Received in revised form 3 December 2011
Accepted 25 December 2011
Available online 31 December 2011

Keywords:
Tanshinone IIA
Lipid emulsion
Formulation design
Anti-tumor
Drug development

ABSTRACT

The purpose of this study was to develop a lipid emulsion of tanshinone IIA (Tan IIA-LE) for intravenous administration and to investigate its feasibility for future clinical practice. The formulation was optimized using central composite design-response surface methodology (CCD-RSM), and the homogenization process was investigated systematically. The Tan IIA-LE was evaluated in terms of stability, safety and *in vitro* anti-hepatoma activity. The formulation of Tan IIA-LE is composed of 0.05% (w/v) Tan IIA, 20% (w/v) soybean oil–MCT mixture (1:1, w/w), 1.2% (w/v) soybean lecithin, 0.3% (w/v) F68 and 2.2% (w/v) glycerol, a high pressure homogenization at 100 MPa for 3 cycles was selected as the optimal homogenization process. The Tan IIA-LE was light-sensitive but stable for at least 12 months at room temperature in dark. The safety study demonstrated that the Tan IIA-LE did not cause venous irritation or obvious acute toxicity. Furthermore, the Tan IIA-LE displayed significant anti-tumor activity against human hepatoma cell lines *in vitro*. Overall, the Tan IIA-LE developed in this study was suggested to be a suitable and safe dosage form of Tan IIA for intravenous administration and has potential in liver cancer therapy in future.

1. Introduction

Danshen, the dried root of *Salvia Miltiorrhiza Bunge*, is a traditional Chinese medicine widely used for the prevention and treatment of cardiovascular and cerebrovascular diseases for hundred of years (Cheng, 2007; Zhou et al., 2005). Tanshinone IIA (Tan IIA, Fig. 1) as one of the major bioactive constituents isolated from Danshen, has been shown to possess multiple pharmacological activities, such as anti-oxidative (Cao et al., 1996), anti-inflammatory (Dong et al., 2009a,b; Fan et al., 2009), cardioprotective (Chang et al., 2006; Sun et al., 2011) and neuroprotective effects (Liu et al., 2010), exerting obvious effectiveness in improving cardiac function, protecting cardiomyocytes, preventing angina pectoris, reducing brain infarct volume and improving neurological functions (Dong et al., 2009a,b; Fang et al., 2010; Mao et al., 2006). More recently, some studies have revealed that Tan IIA is also a drug with cytotoxic properties against a variety of human cancer cell lines (Cheng and Su, 2010; Gong et al., 2011; Jiao and Wen, 2011; Lu et al., 2009; Shan et al., 2009; Won et al., 2010; Zhang et al., 2011), indicating its benefits to the cancer therapy. However, because Tan IIA is a substrate for P-Glycoprotein (P-gp), the P-gp mediated efflux of Tan IIA into the gut lumen, as well as a significant first-pass metabolism, often result in a low oral bioavailability of Tan IIA after administration in the dosage forms of tablet or capsule (Yu et al., 2007), subsequently a poor therapeutic efficacy in clinic. Additionally, the high lipophilicity and poor oral absorbility of Tan IIA challenge the pharmaceutists to develop suitable dosage forms for Tan IIA. A constructive advance is the development of sodium tanshinone IIA sulfonate (STS or Danshen 201, Fig. 1), a derivative of tanshinone IIA by means of conjugating a sulfonate group, which has been successfully applied to treat patients with coronary artery disease and angina pectoris for more than 30 years in China (sodium tanshinone IIA sulfonate injection, Nuoxinkang®, 2 mL:10 mg, Shanghai No. 1 Biochemical and Pharmaceutical Co., Ltd.). This intravenous injection product has solved the problem concerning water-solubility of Tan IIA and has made

Abbreviations: ANOVA, analysis of variance; CCD-RSM, central composite design-response surface methodology; DMSO, dimethyl sulphoxide; FBS, fetal bovine serum; FFA, free fatty acid; F68, Poloxamer 188; LCT, long-chain triglyceride; MCT, medium-chain triglyceride; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenylterazolium bromide; NC, negative control group; PA, phosphatidic acid; PG, phosphatidyl glycerol; P-gp, P-Glycoprotein; PS, phosphatidyl serine; RES, reticuloendothelial system; SD, Sprague-Dawley; STS, sodium tanshinone IIA sulfonate; Tan IIA, tanshinone IIA; Tan IIA-DMSO, tanshinone IIA DMSO solution; Tan IIA-LE, lipid emulsion of tanshinone IIA; TEM, transmission electron microscopy.

* Corresponding author. Tel.: +86 028 85501070; fax: +86 028 85502775.
E-mail address: xinba789@yahoo.com.cn (S.-j. Mao).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.12.049

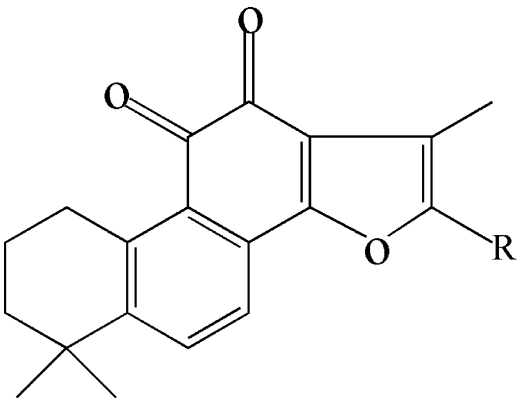

Fig. 1. Chemical structure of tanshinone IIA (R = H) and sodium tanshinone IIA sulfonate (R = SO_3Na).

a great contribution to the development of intravenous administration of Tan IIA. It should be noted that, however, none of studies have verified that STS injection also possesses anti-tumor activities in clinical practice, that is to say, the intrinsic anti-tumor activities of Tan IIA may be eliminated in the form of STS most likely due to the structure modification, while the Tan IIA-like curative effects on cardiovascular disorders remain. Given the reduced intensity on pharmacological actions of STS and the fact that there is no commercially available pharmaceutical preparation of Tan IIA at present, exploring a novel and suitable dosage form for Tan IIA with enhanced bioavailability is not only of vital importance to clinical uses of Tan IIA, but also of great benefit to the cancer treatment in future.

Up to today, several pharmaceutical strategies have been proposed such as solid lipid nanoparticles (Liu et al., 2005), solid dispersion (Hao et al., 2006), solid inclusion complex (Fan et al., 2005), pulsatile release pellet (Liang et al., 2010) and microemulsion (Li et al., 2007). But the utility of these approaches is often restricted owing to the issues involving poor stability, complexity associated with manufacturing and unqualified bioavailability, which make them unsuitable for large-scale production and for clinical application (Date and Nagarsenker, 2008). In comparison with aforementioned approaches, intravenous lipid emulsion appears to be a promising drug delivery system because of (1) the properties of intravenous administration including 100% bioavailability, quick onset and low individual difference and (2) the ability of lipid emulsion to solubilize the lipophilic drugs, to stabilize the drugs from hydrolysis and/or oxidation, to prolong the pharmacological effects, as well as the advantages including possibility of sustained release, biological compatibility, ease of mass production and low cost (Piemi et al., 1999; Tamilvanan, 2004). Furthermore, drug incorporated into lipid emulsion can enhance the activity and bioavailability of the incorporated drug, simultaneously avoid direct contact of the drug with the body fluids and tissues, leading to reduced toxicity and irritation (Lovell et al., 1994). These appealing properties give intravenous lipid emulsion an edge over other dosage forms, and suggest it to be an appropriate drug carrier for the intravenous administration of Tan IIA.

The purpose of the study was to develop a novel and safe intravenous lipid emulsion of Tan IIA (Tan IIA-LE) for liver cancer therapy with feasibility in clinical uses. This research as the first step was performed to prepare the Tan IIA-LE based on systematical investigations on the formulation and process design, and to evaluate it in terms of stability, safety and anti-tumor activity against human hepatoma cell lines *in vitro*. To our best knowledge, there has been no available information on anti-human hepatoma cell lines activity of tanshinone IIA preparation *in vitro*.

2. Materials and methods

2.1. Materials and animals

Tanshinone IIA (Tan IIA, with a purity proven by HPLC as 99.2%) was purchased from Natural Field Bio-technique Co., Ltd. (Shanxi, China). Tanshinone IIA reference substance was purchased from National Institutes for Food and Drug Control (Beijing, China). Sodium tanshinone IIA sulfonate injection was obtained from Shanghai No. 1 Biochemical and Pharmaceutical Co., Ltd. (Shanghai, China). Soybean lecithin was purchased from Lipoid KG (Ludwigshafen, Germany). Soybean oil and medium-chain triglyceride (MCT) were obtained from Tieling Beiya Pharmaceutical Oil Co., Ltd. (Liaoning, China). Poloxamer 188 (Pluronic® F68) was obtained from BASF Corp. (Ludwigshafen, Germany). Glycerol was purchased from Shantou Zhiguang Guhan Amino Acid Co., Ltd. (Guangdong, China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenylterazolium bromide (MTT) was obtained from Biosharp Inc. (USA), dimethyl sulphoxide (DMSO) was from Bodi Chemistry Co., Ltd. (Tianjin, China). All chemicals and reagents used were of analytical or chromatographic grade. Ultrapure water was used throughout the whole study.

Sprague-Dawley (SD) rats, Kunming mice and New Zealand rabbits were kindly provided by Laboratory Animal Center of Sichuan University (Sichuan, China). All the animals were housed under an air-conditioned environment at 22–24 °C with 60% of relative humidity and a 12-h light/dark cycle, and allowed to diet and water *ad libitum*. Animals were fasted for 12 h prior to studies. The experiments involving animals were approved by the University Ethics Committee for the Use of Laboratory Animals and were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Cell lines and culture

The human HepG2, SMMC 7721 and BEL 7404 hepatoma cell lines were gifts from State Key Laboratory of Biotherapy of Sichuan University (Sichuan, China). Cells were cultured in RPMI-1640 medium (Gibco Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Lanzhou National Hyclone Bio-Engineering Co., Ltd. Gansu, China), 100 U/mL penicillinum and 100 μg/mL streptomycin in a humidified incubator (BC-J160S, Shanghai Boxun Industry and Commerce Co., Ltd. Shanghai, China) with 5% CO_2 at 37 °C. All cells were passaged every two days. Cells in logarithmic growth phase were used for the further assay.

2.3. Investigation of Tan IIA-LE formulation

2.3.1. Solubility study of Tan IIA

Excessive amount of Tan IIA powder was put into three erlenmeyer flasks, which were pre-treated with 10 g of water, soybean oil and soybean oil–MCT mixture (1:1, w/w), respectively, followed by agitating at 25 °C for 24 h (air bath agitator, Harbin Dongming Medical Equipment Factory, Harbin, China). After centrifugating these mixtures at 10,000 rpm for 10 min (TGL-16G centrifuge, Shanghai Anting Scientific Instrument Factory, Shanghai, China), the supernatant of each sample was withdrawn, and filtrated through a 0.45 μm millipore filter membrane. The resultant samples were diluted to 1:200 with dehydrated alcohol immediately before HPLC analysis to determine the concentration of Tan IIA.

2.3.2. Experimental design

According to the results obtained from preliminary experiments, the formulation variables including oil phase content (%, w/v), emulsifier content (%, w/v) and co-emulsifier content (%, w/v)

Table 1
Independent variables and their values in central composite design.

Variable	Symbol	Coded variable levels				
		Lowest $-\beta$	Low -1	Center 0	High $+1$	Highest $+\beta$
Oil phase content (%, w/v)	X_1	15.0	19.05	25.0	30.95	35.0
Emulsifier content (%, w/v)	X_2	0.6	0.84	1.2	1.56	1.8
Co-emulsifier content (%, w/v)	X_3	0.1	0.18	0.3	0.42	0.5

$\beta = 2^{k/4} = 1.682$, k is the number of independent variables.

were the three main factors that affected the particle size of the Tan IIA-LE. Hence in the present study, a central composite design-response surface methodology (CCD-RSM) was applied to arrange the experiments to investigate the influence of these three independent variables on the particle size of the Tan IIA-LE (the response variable, Y), aiming to find out the optimal formulation of the Tan IIA-LE. The value range of each independent variable was defined as oil phase content (X_1) of 15–35%, emulsifier content (X_2) of 0.6–1.8% and co-emulsifier content (X_3) of 0.1–0.5%. Coded and actual levels of variables are listed in Table 1. A total number of 20 experiments consisting of 2^k factorial points, $2k$ axial points and 6 center points (k is the number of independent variables) were carried out. The details including the variables, their levels and the results are summarized in Table 2.

2.4. Optimization of homogenization process

Considering that homogenization process can affect the quality of an emulsion during manufacture, the optimal operative condition involving homogenization pressure and number of cycles was investigated in this research, using the mean particle size as the indicator for evaluating the efficiency of the process. The pressure was set from 50 to 150 MPa and the cycle number was for 1–5. The temperature was maintained at 40 °C during the whole homogenization process.

2.5. Preparation of Tan IIA-LE

The lipid emulsion of Tan IIA (Tan IIA-LE) was prepared as follows: the oil phase consisting of soybean oil, MCT, soybean lecithin and Tan IIA was heated to 80 °C under stirring until uniformly dispersed. F68, together with the glycerol was dissolved in ultrapure water and maintained at 80 °C to obtain a uniform aqueous phase. The oil phase was then added to the aqueous phase slowly under a high-speed shear mixing (JJ-2, Jintan Yitong Electronics Co., Ltd. Jiangsu, China) at 10,000 rpm. This mixing was carried out for 5 min to get the coarse emulsion. The final emulsion was obtained by passing the coarse emulsion through a high pressure homogenizer (Avestin Inc. Ottawa, Canada) under a pressure of 100 MPa for 3 cycles. The temperature of the entire homogenization process was maintained at 40 °C. Ultimately, batches of fine emulsions were sealed in 2 mL ampuls after purging with nitrogen gas, and autoclaved at 121 °C for 12 min.

2.6. Characterization of Tan IIA-LE

The particle size and ζ-potential of the Tan IIA-LE were measured by a Zetasizer Nano ZS90 analyzer (Malvern Instruments Co., Worcestershire, UK). Before assessment, Tan IIA-LE samples were diluted 1:5000 with ultrapure water immediately at 25 °C. The determination of pH value was accomplished with a pH-meter (Leici®, Shanghai Precision Science Instrument Ltd. Shanghai, China) with a glass electrode at 25 °C. The amount of Tan IIA in Tan IIA-LE was determined by means of HPLC analysis. For this purpose, 0.5 mL of the Tan IIA-LE samples (0.5 mg/mL) was collected and diluted with dehydrate alcohol to 25 mL, shaked well to obtain the test sample. The reference sample (10 μg/mL) was prepared by dissolving accurately weighed amount of Tan IIA reference substance in methylene chloride, followed by a dilution

Table 2
Central composite design for the study of three independent variables with experimental results.

Experiment Number	Coded values of independent variables			Actual values of independent variables			Values of response variable
	X_1	X_2	X_3	Oil phase content (%, w/v)	Emulsifier content (%, w/v)	Co-emulsifier content (%, w/v)	Particle size (nm)
1	−1	−1	−1	19.05	0.84	0.18	293.9
2	1	−1	−1	30.95	0.84	0.18	377.7
3	−1	1	−1	19.05	1.56	0.18	241.5
4	1	1	−1	30.95	1.56	0.18	286.4
5	−1	−1	1	19.05	0.84	0.42	247.0
6	1	−1	1	30.95	0.84	0.42	307.2
7	−1	1	1	19.05	1.56	0.42	212.1
8	1	1	1	30.95	1.56	0.42	259.9
9	0	0	0	25	1.2	0.3	264.3
10	0	0	0	25	1.2	0.3	262.2
11	0	0	0	25	1.2	0.3	267.5
12	0	0	0	25	1.2	0.3	261.6
13	0	0	0	25	1.2	0.3	264.9
14	0	0	0	25	1.2	0.3	265.7
15	−1.682	0	0	15	1.2	0.3	217.0
16	1.682	0	0	35	1.2	0.3	322.5
17	0	−1.682	0	25	0.6	0.3	321.6
18	0	1.682	0	25	1.8	0.3	237.5
19	0	0	−1.682	25	1.2	0.1	307.3
20	0	0	1.682	25	1.2	0.5	243.2

of 1:1000 with methanol. All the samples were prepared freshly before HPLC analysis. Drug content was calculated as follows (Eq. (1)):

$$\text{Drug content}\,(\%) = \text{actual amount of Tan IIA/labelled amount of Tan IIA} \times 100 \quad (1)$$

2.7. Stability study of Tan IIA-LE

2.7.1. Centrifugal accelerated testing

To investigate the physical stability of the Tan IIA-LE under the condition of accelerated test, 5 mL of the prepared emulsion samples was collected and centrifugated at 10,000 × *g* for 15 min. Visual observation was made to examine whether a phenomenon of creaming occurs or not during the whole testing.

2.7.2. Stress testing

In this study, a stress testing was performed to estimate the physicochemical stability of the Tan IIA-LE when exposed to elevated temperature and light. Briefly, two batches of Tan IIA-LE samples were prepared according to Section 2.5, placed at 60 °C and room temperature with 4500 ± 500 lx, respectively, for 10 days. During the storage, a collection of parameters including physical appearance, mean particle size, ζ-potential, pH value and drug content was monitored to evaluate the thermal- and light-stability of this emulsion product. The results obtained in this section can also provide a foundation for selecting a proper experimental condition for the following studies, as well as for choosing an appropriate storage condition for Tan IIA-LE.

2.7.3. Long-term stability

The Tan IIA-LE was prepared as described in Section 2.5, stored at 25 ± 2 °C away from light over 12 months. At the time intervals of 0, 3, 6, 9 and 12 months, emulsion samples were collected and investigated from aspects of physical appearance, mean particle size, ζ-potential, pH value and drug content to evaluate the physicochemical stability of the Tan IIA-LE during storage.

2.8. HPLC analysis

An HPLC system consisting of a Diamonsil-C_{18} column (150 mm × 4.6 mm, 5 μm, Dikma Technology Co., Ltd. Beijing, China), a Shimadzu LC-10AT pump and a Shimadzu SPD-10A UV–vis detector (Shimadzu, Japan) was employed for the determination of Tan IIA *in vitro*. The mobile phase was composed of methanol–water (80:20, v/v) at a flow rate of 1.0 mL/min. The wavelength was fixed at 270 nm and an injection volume of 20 μL was used. The column temperature was maintained at 30 °C.

2.9. Safety study of Tan IIA-LE

2.9.1. Rabbit ear vein irritation study

Three New Zealand rabbits weighing 2.0–2.5 kg were used in this study for the assessment of venous irritation caused by the Tan IIA-LE. Each rabbit received Tan IIA-LE (0.5 mg/mL) at a daily dose of 4 mg/kg through the left ear marginal vein, while an equivalent volume (8 mL/kg) of 0.9% normal saline was injected into their right ear marginal veins as control. This injection was performed for three consecutive days. Irritation signs such as edema and erythema at the injection sites were observed and recorded daily. After last dosing for 24 h, all the animals were subjected to a sacrifice, then their ears were removed and preserved in 10% formalin solution to prepare histological sections for histopathological examination.

2.9.2. Acute toxicity study

Sixty Kunming mice (weighed 18–22 g) were equally designated into three groups (each group has 10 males and 10 females), the first two groups were given Tan IIA-LE and Tan IIA-free lipid emulsion *via* tail veins at a dosage level of 15 mg/kg, respectively, while the third group received an equivalent volume (30 mL/kg) of 0.9% normal saline as control. The injection repeated three times within 24 h. Following a period of 14 days after the last administration, routine clinical observations were made daily, and the body weights of mice were measured and recorded in detail.

2.10. Anti-hepatoma activity study in vitro

2.10.1. Cell viability assay

In this study, MTT assay was employed to quantify the cell viability of three human hepatoma cell lines (HepG2, SMMC 7721 and BEL 7404) after treatment with Tan IIA-LE to confirm its *in vitro* anti-tumor activity against human hepatoma cell lines. Cells in logarithmic growth phase were harvested, adjusted to a density of 2×10^5 cells/mL and plated in 96-well microplates (Costar Corning, Rochester, NY) in 100 μL/well. A range of concentrations (1, 2, 4 and 8 μg/mL) of tanshinoneIIA DMSO solution (Tan IIA-DMSO, the Tan IIA-DMSO stock solution was prepared by dissolving a known amount of Tan IIA in DMSO to a final concentration of 1.7×10^3 μg/mL. The final concentration of DMSO was 0.03% (v/v), the results of our preliminary experiments suggested that DMSO would not cause changes of cellular morphology at a concentration ≤0.1%) and Tan IIA-LE, 10 μg/mL of STS solution were added into designated wells to be served as experimental groups, while the cells treated with Tan IIA-free culture medium and Tan IIA-free lipid emulsion were taken as the negative control group (NC) and blank vehicle control group, respectively. Each group included four parallel wells. After incubation for 24, 48 and 72 h, 20 μL of 5 mg/mL MTT solution was added into each well and cultivated for another 4 h. The supernatant was then removed and replaced with 100 μL of DMSO to dissolve the formazan crystal under shaking with a microplate thermostatic oscillator (MB100-4P, Hangzhou Allsheng Instruments Co., Ltd. Zhejiang, China). The absorbance value (*A* value) of each well was measured with a microplate reader (Bio-Rad, Richmond, CA, USA) at 570 nm. Cell viability was calculated according to the following formula (Eq. (2)):

$$\text{Cell viability}\,(\%) = \frac{A_t}{A_{nc}} \times 100 \quad (2)$$

where A_t is the *A* value of the treatment group (including experimental groups and blank vehicle control group) and A_{nc} represents the *A* value of the negative control group.

2.10.2. Observation of cellular morphology

After incubation for 48 h, cells which were treated with 10 μg/mL of STS solution, 8 μg/mL of Tan IIA (Tan IIA-LE and Tan IIA-DMSO) were observed under an inverted phase contrast microscope (CFM-500, Changfang Optical Instrument Co., Ltd. Shanghai, China). Cells in NC group and blank vehicle control group were also observed as comparison.

2.11. Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA) using the statistical package for social science (SPSS, Version 17.0). All the descriptive parameters were expressed as

Table 3
Comparison of the predicted and observed mean particle size value of the Tan IIA-LE prepared according to the optimized formulation.

Response variable	Predicted values	Observed values	Bias (%)[a]
Mean particle size (nm)	240.48	252.5	4.76

[a] Bias was calculated as (observed value − predicted value)/observed value × 100%.

mean ± standard deviation, and a value $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Investigation of Tan IIA-LE formulation

3.1.1. Investigation of oil phase composition

Oil phase as an essential component in lipid emulsions, significantly affects the physicochemical properties and the stability of the emulsions (Jumaa and Müller, 1998). Long-chain triglycerides (LCTs) derived from vegetable sources are always the primary choice as the oil phase in the parenteral lipid emulsions for their biocompatibility and long-term acceptability (Trotta et al., 2002), and they have already been incorporated in most of FDA-approved lipid emulsion products. Besides, medium-chain triglycerides (MCTs) have been reported to be 100 times more soluble in water than LCTs and to have an enhanced solubilizing capability (Floyd, 1999). A lipid emulsion mixture containing MCT and LCT was found to be less toxic than the pure LCT-based lipid emulsions, and to be more stable (Driscoll et al., 2002, 2000; Smymiotis et al., 2001), thus some commercial lipid emulsions have adopted a mixture of LCT and MCT as their oil phase. Considering that the solubility of the drug usually drives the oil selection to achieve the maximal drug loading in the emulsion, the solubility of Tan IIA in water, soybean oil and a soybean oil–MCT mixture (1:1, w/w) has been determined. Fig. 2 shows that Tan IIA was almost insoluble in water, but was soluble in soybean oil and the soybean oil–MCT mixture. The solubilizing capacity followed the order of soybean oil–MCT mixture (1:1, w/w) > soybean oil > water. Therefore, the soybean oil–MCT mixture (1:1, w/w) was selected as the oil phase for Tan IIA-LE.

3.1.2. Investigation of emulsifier composition

Emulsifier is another absolutely necessary and key component in lipid emulsions, as it can substantially improve the stability of emulsion droplets by forming a mechanical barrier and/or by producing an electrical barrier (Levy and Benita, 1989; Tamilvanan, 2004; Trotta et al., 2002). Natural lecithins obtained from animals or vegetables are the most commonly used emulsifiers in the parenteral emulsion formulations. The excellent biocompatibility, and the affinity to cellular membrane which leads to an increased absorption of several drugs (Paolino et al., 2002), have greatly

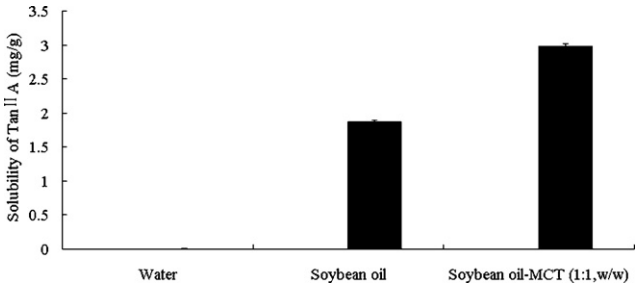

Fig. 2. Solubility of Tan IIA in water, soybean oil and soybean oil–MCT mixture (1:1, w/w) ($n = 3$).

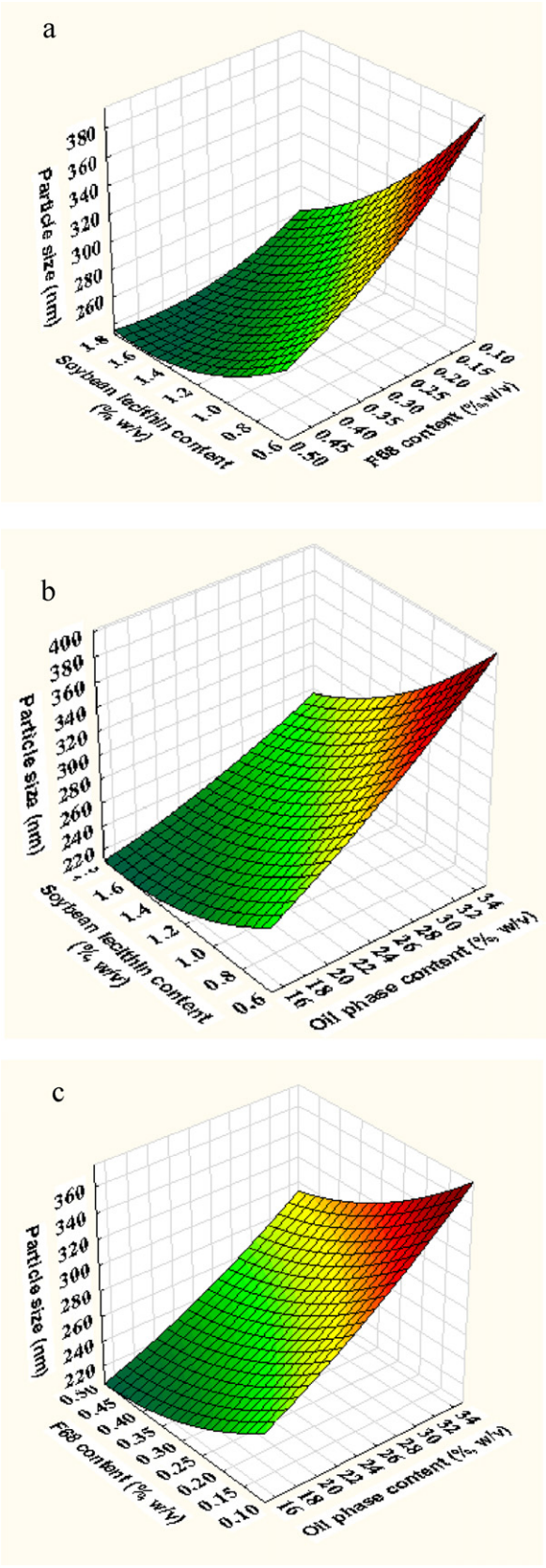

Fig. 3. The response surface plots showing the effects of (a) soybean lecithin content and F68 content; (b) oil phase (soybean oil–MCT of 1:1) content and soybean lecithin content and (c) oil phase (soybean oil–MCT of 1:1) content and F68 content on the particle size of Tan IIA-LE.

Table 4
Mean particle size (nm) of the Tan IIA-LE prepared under different operative conditions ($n = 3$).

Number of cycles	Homogenization pressure (MPa)			
	50	80	100	150
1	350.3 ± 18.2	322.7 ± 15.5	273.3 ± 16.9	272.6 ± 15.8
3	326.5 ± 10.3	283.4 ± 14.7	252.5 ± 10.8	251.7 ± 16.6
5	287.2 ± 11.1	272.0 ± 12.3	254.1 ± 10.7	251.9 ± 13.9

contributed to the extensive utilization of lecithins in parenteral emulsions. Lecithins as a kind of natural mixtures contain non-ionic and anionic lecithins, among which the acidic lipids including phosphatidyl serine (PS), phosphatidyl glycerol (PG) and phosphatidic acid (PA) play an important role in stabilizing emulsions by means of increasing the surface charge (Chansiri et al., 1999; Rubino, 1990). Therefore, as far as emulsifying capacity concerned, the soybean lecithin seems to be superior to the egg lecithin, since the soybean lecithin possesses a higher content of anionic lecithins than egg lecithin. The results of our preliminary experiment also demonstrated that the soybean lecithin-stabilized emulsion exhibited a better stability than the emulsion stabilized with only egg lecithin after thermal sterilization (Data were not shown). Consequently, the soybean lecithin was selected as the main emulsifier in the present study.

Most of the time, lecithin alone is not sufficient to yield a desired emulsion due to its high hydrophobicity, necessitating an addition of another emulsifier to adjust the HLB of lecithin to inhibit its tendency to form lamellar liquid crystalline phase (Date and Nagarsenker, 2008). Poloxamer 188 (Pluronic® F68) should be an excellent choice, not only because of its ability to stabilize the newly created interface immediately (Shi et al., 2009), but also due to its high cloud point which can make the emulsifier mixture containing F68 more resistant to breakdown during the autoclaving process (Jumaa et al., 1999). Furthermore, the combination of F68 with lecithin as an emulsifying complex was suggested to form a close-packed mixed film at the oil–water interface, leading to a much more stable emulsion than those obtained with only one emulsifier (Weingarten et al., 1991). As a result, F68 was chosen as the co-emulsifier in the Tan IIA-LE formulation.

3.1.3. Optimization of Tan IIA-LE formulation

The droplet size of parenteral emulsions is not only of great relevance to its safety of application since particles larger than 5 μm was suggested to pose a risk of lung embolism (Laval-Jeantet et al., 1982), also a relatively small particle size indicates a good physical stability owing to the prevention of creaming by Brownian movement (Trotta et al., 2002). Based on the results of preliminary experiments, the particle size of Tan IIA-LE was found to be significantly influenced by the contents of oil phase, emulsifier and co-emulsifier, thus in the present study, the effects of these three variables on the particle size were investigated by applying CCD-RSM, aiming to find out the optimal formulation of Tan IIA-LE with a desirable mean particle size. Table 2 summarizes all the experiments conducted along with the results. According to the data, the mathematical relationship between the response variable (Y) and the independent variables (X_1, X_2 and X_3) can be approximated using a second order polynomial equation as shown below (Eq. (3)):

$$Y = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + b_3 \cdot X_3 + b_{11} \cdot X_1^2 + b_{22} \cdot X_2^2 + b_{33} \cdot X_3^2 + b_{12} \cdot X_1 \cdot X_2 + b_{13} \cdot X_1 \cdot X_3 + b_{23} \cdot X_2 \cdot X_3 \quad (3)$$

where Y is the response variable and X_1, X_2, X_3 represent the independent variables. b_0 is the average of the results of the replicated center point (constant term); b_1, b_2 and b_3 are the main half-effects of the independent variables X_1, X_2 and X_3 (linear effects), respectively; b_{11}, b_{22} and b_{33} are the quadratic effects; b_{12}, b_{13} and b_{23} are two factor interaction half-effects.

Analysis of multiple non-linear regression and estimation of coefficients were performed by applying Statistica 10.0 statistical package (StatSoft, Inc., USA). The polynomial equation was generated as follows (Eq. (4)):

$$Y = 363.605 + 6.325X_1 - 165.079X_2 - 481.582X_3 + 0.069X_1^2 + 46.468X_2^2 + 310.71X_3^2 - 2.994X_1X_2 - 3.624X_1X_3 + 177.951X_2X_3 \quad (4)$$

The R^2 value for this model is 0.9936, signifying the suitability of this polynomial regression model.

In order to obtain a better understanding on the particle size as a function of contents of oil phase, emulsifier and co-emulsifier, three dimensional (3D) response surface plots were made by keeping one independent variable at its center level to show the effects of the other two variables on the response variable. Fig. 3a–c shows the effects of soybean lecithin content and F68 content, soybean oil–MCT mixture (1:1, w/w) content and soybean lecithin content, soybean oil–MCT mixture (1:1, w/w) content and F68 content on the particle size of the Tan IIA-LE, respectively, when kept the third variable at its center level. As can be seen, an increased oil phase often led to a larger particle size, while reduced droplet size can be achieved by increasing the soybean lecithin content and/or the F68 content. The minimum particle size would be obtained with minimum level of oil phase, maximum level of emulsifier and maximum level of co-emulsifier. Nevertheless, as for a lipophilic drug

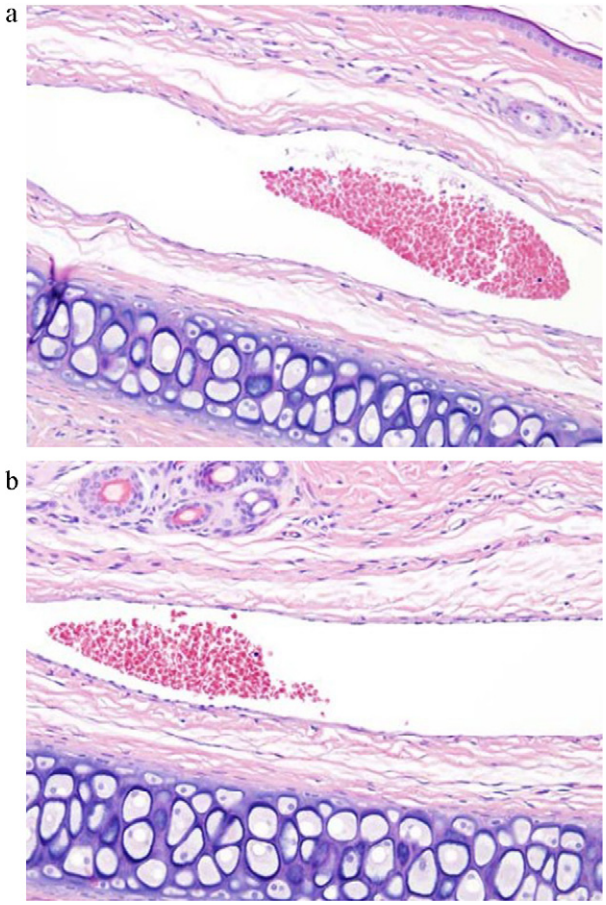

Fig. 4. The histophathological examination of rabbit ear veins. (a) Administered by 4 mg/kg of Tan IIA-LE and (b) administered by 0.9% normal saline.

Table 5
Characterization of Tan IIA-LE stored under 60 °C and room temperature with 4500 ± 500 lx for 10 days (n = 3).

Storage condition	Time point (day)	Physical appearance	Mean particle size (nm)	ζ-Potential (mV)	pH value	Drug content (%)[a]
60 °C	0	Orange-yellow appearance	252.5 ± 10.8	−32.3	6.53	100.4
	5	Orange-yellow appearance	260.3 ± 11.5	−31.9	6.15	98.63
	10	Orange-yellow appearance	314.7 ± 14.1	−33.5	5.87	95.34
Room temperature with 4500 ± 500 lx	0	Orange-yellow appearance	252.5 ± 10.8	−32.3	6.53	100.4
	5	Milky appearance	258.9 ± 12.6	−32.8	6.41	73.23
	10	Milky appearance	267.0 ± 13.1	−33.6	6.13	51.42

[a] Drug content (%) = actual amount of Tan IIA/labelled amount of Tan IIA × 100.

formulated in an oil-in-water parenteral emulsion, a relatively small oil phase content usually results in a low drug amount, which would be unfavorable for clinical application. An additional amount of emulsifiers can decrease the particle size distribution to some extent, but this would bring a higher viscosity of the parenteral emulsion, causing a pain when administered intravenously (Jumaa and Müller, 1998). Bearing these considerations in mind and based on Fig. 3a–c, the optimal levels of the three independent variables for the Tan IIA-LE with a desirable particle size were achieved at the soybean oil–MCT mixture of 20.0% (w/v), soybean lecithin of 1.2% (w/v) and F68 of 0.3% (w/v). Table 3 shows a comparison between the predicted and the observed value of mean particle size of Tan IIA-LE prepared according to the optimized formulation, a low percentage bias of 4.76% suggested that this optimized formulation was reliable and reasonable.

Since the solubility of Tan IIA in soybean oil–MCT mixture (1:1, w/w) was determined to be 2.98 mg/g, the labelled amount of this Tan IIA-LE was designed to be 0.5 mg/mL. In addition, 2.2% (w/v) of glycerol was formulated to adjust the osmosis. Therefore, the final optimized formulation of Tan IIA-LE developed in our study consisted of 0.05% (w/v) Tan IIA, 20% (w/v) soybean oil–MCT mixture (1:1, w/w), 1.2% (w/v) soybean lecithin, 0.3% (w/v) F68 and 2.2% (w/v) glycerol.

3.2. Optimization of homogenization process

To find out the optimal homogenization process, a series of Tan IIA-LE samples were prepared under different operative conditions with regard to homogenization pressure and number of cycles, while other conditions were the same as described in Section 2.5. The homogenization was performed under 50–150 MPa for 1–5 cycles, and the mean particle size corresponding to each operative condition is recorded in Table 4.

As shown in Table 4, increasing the homogenization pressure from 50 to 100 MPa produced a stepwise decrease in the particle size, but further increasing pressure from 100 to 150 MPa almost had no influence on the particle size irrespective of the number of cycles. This observation agreed with the earlier findings which found that no linear relationship between the decrease in size and increase in pressure existed (Keck and Müller, 2006). As far as the cycle numbers concerned, our results showed that reducing particle size on increasing the number of cycles only could be obtained at the pressures of 50 and 80 MPa. For relatively high pressures of 100 and 150 MPa, a slight increase in particle size was observed after 3 cycles, which may be because that the particle size had reached its maximum dispersity at the given power density (Cheong et al., 2008). The smallest mean particle size of 251.7 nm was obtained by passing the emulsion product through the homogenizer for 3 cycles at 150 MPa. This value was not significantly smaller than the value of 252.5 nm, which was produced at the pressure of 100 MPa for 3 cycles. The higher pressure, however, often needs more energy and is a disadvantage for industrial production. Thus the operative condition for the preparation process was set to 100 MPa for 3 cycles.

Table 7
Body weight (g) of mice before and after administration of corresponding solutions.

Time	Body weight (g)		
	Tan IIA-LE	Tan IIA-free lipid emulsion	Normal saline
Before administration	20.2 ± 1.15	19.5 ± 0.87	20.6 ± 0.82
14 days after administration	26.7 ± 2.84	25.5 ± 2.15	28.2 ± 1.98

3.3. Characterization of Tan IIA-LE

The Tan IIA-LE prepared in our study exhibited an uniformly orange-yellow appearance without visible oil droplets on the surface after thermal sterilization. The mean particle size of Tan IIA-LE was 252.5 ± 10.8 nm, with a PDI value of 0.058. The ζ-potential was −32.3 mV, and the pH value was measured to be 6.53. The drug content of Tan IIA in Tan IIA-LE was determined to be 100.4% (the actual amount of Tan IIA was calculated to be 0.502 mg/mL).

3.4. Stability study of Tan IIA-LE

3.4.1. Centrifugal accelerated testing

Centrifugal accelerated testing is usually conducted to estimate the physical stability of a lipid emulsion in a relatively short period by means of accelerating the creaming process, which serves as an important indicator for evaluating the stability of the lipid emulsion. The Tan IIA-LE was found to be physically stable under a centrifugation at 10,000 × g for 15 min, with no creaming or phase separation occurred during and after the whole study.

3.4.2. Stress testing

To investigate the thermal- and light-stability of the Tan IIA-LE , a stress testing was carried out by exposing the Tan IIA-LE

Table 6
Characterization of Tan IIA-LE stored at 25 ± 2 °C away from light during 12 months (n = 3).

Time (month)	Physical appearance	Particle size (nm)	pH value	ζ-Potential (mV)	Drug content (%)[a]
0	Orange-yellow appearance	252.5 ± 10.8	6.53	−32.3	100.4
3	Orange-yellow appearance	255.2 ± 9.8	6.50	−31.8	99.86
6	Orange-yellow appearance	261.4 ± 14.1	6.51	−32.1	98.71
9	Orange-yellow appearance	260.3 ± 13.3	6.47	−32.4	97.60
12	Orange-yellow appearance	265.7 ± 15.2	6.45	−32.6	97.14

[a] Drug content (%) = actual amount of Tan IIA/labelled amount of Tan IIA × 100.

Table 8
Cell viability (%) of HepG2, SMMC 7721 and BEL 7404 in different groups for 24, 48 and 72 h ($n = 3$).

Groups	Concentration (μg/mL)	Time (h)		
		24	48	72
HepG2				
NC		100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Blank vehicle		100.00 ± 1.08	100.24 ± 0.55	99.98 ± 1.12
Tan IIA-DMSO	1	87.30 ± 2.85**	82.20 ± 3.67**	65.21 ± 4.36**
	2	83.50 ± 2.27**	75.33 ± 3.42**	51.61 ± 3.07**
	4	74.90 ± 1.39**	61.60 ± 3.17**	40.61 ± 3.60**
	8	65.57 ± 2.91**	44.43 ± 4.06**	30.43 ± 4.00**
Tan IIA-LE	1	92.25 ± 2.85**	89.46 ± 2.39**	68.80 ± 3.11**
	2	91.34 ± 2.23**	87.03 ± 2.78**	53.19 ± 3.88**
	4	87.74 ± 3.54**	83.16 ± 2.20**	46.17 ± 3.56**
	8	77.91 ± 3.57**	61.59 ± 3.14**	38.90 ± 2.89**
STS solution	10	99.61 ± 0.65	100.19 ± 1.35	100.49 ± 2.30
SMMC 7721				
NC		100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Blank vehicle		100.08 ± 1.10	100.77 ± 1.66	99.85 ± 1.30
DMSO	1	89.95 ± 1.06**	85.24 ± 4.64**	64.44 ± 4.23**
	2	80.89 ± 4.81**	73.75 ± 3.06**	27.34 ± 2.48**
	4	72.84 ± 2.56**	52.43 ± 3.12**	24.84 ± 2.01**
	8	68.56 ± 2.21**	43.48 ± 1.05**	23.29 ± 1.14**
LE	1	93.15 ± 2.78**	88.09 ± 2.29**	65.87 ± 2.82**
	2	90.33 ± 2.43**	80.75 ± 2.98**	33.24 ± 3.02**
	4	86.68 ± 1.29**	69.07 ± 1.99**	29.24 ± 2.24**
	5	80.88 ± 2.95**	54.27 ± 2.15**	26.82 ± 2.07**
STS solution	10	100.33 ± 2.40	100.23 ± 2.15	99.97 ± 1.77
BEL 7404				
NC		100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Blank vehicle		100.24 ± 0.89	100.18 ± 1.49	100.69 ± 2.35
Tan IIA-DMSO	1	91.05 ± 2.65**	85.30 ± 6.29**	69.41 ± 2.64**
	2	77.36 ± 4.32**	71.48 ± 4.64**	52.09 ± 3.11**
	4	66.28 ± 4.69**	58.91 ± 2.41**	33.30 ± 3.28**
	8	63.14 ± 4.75**	54.94 ± 2.60**	28.12 ± 5.84**
Tan IIA-LE	1	95.19 ± 2.68*	90.56 ± 3.25**	73.13 ± 3.20**
	2	88.34 ± 2.58**	81.78 ± 2.09**	56.50 ± 4.00**
	4	79.10 ± 3.94**	72.49 ± 3.53**	41.91 ± 2.81**
	8	71.89 ± 2.18**	65.14 ± 4.16**	31.17 ± 3.43**
STS solution	10	100.23 ± 2.61	99.95 ± 2.01	100.11 ± 2.51

* $p < 0.05$ compared with the NC group.
** $p < 0.01$ compared with the NC group.

samples to elevated temperature and light, respectively, for 10 days. Based on Table 5, after exposure to 60 °C for 10 days, the Tan IIA-LE still maintained a good physical appearance, while the particle size became 60 nm larger than that on day 0. An increase in ζ-potential, accompanied by a decrease in pH was observed, but these changes were slight and may be attributed to the formation of free fatty acid (FFA) produced by hydrolysis of lecithin and/or oil phase. Furthermore, a decrease of drug content from 100.4% to 95.34% indicated that an elevated temperature may not significantly affect the chemical stability of the Tan IIA-LE. When the emulsion was kept under 4500 ± 500 lx, it underwent a complete change in physical appearance, implying that the light may induce degradation and/or isomerization of the Tan IIA to generate other substances. Besides, a significant decrease by up to 48.78% in drug content after 10 days also hinted at obvious light-sensitivity of the Tan IIA. These results agreed with the study published previously, which suggested that Tan IIA was physicochemically unstable under light (Liu and Xia, 2010). However, the information concerning the specific mechanism of unstability of Tan IIA, along with the degradation and/or isomerization products from Tan IIA under light hardly exists up to date. Therefore, further investigations to clarify the light-sensitivity of Tan IIA are needed and will be certainly helpful for the development of Tan IIA preparation. In short, the suitable storage condition for this intravenous lipid emulsion of Tan IIA should be at room temperature and away from light.

3.4.3. Long-term stability study

Since lipid emulsion system is intrinsically unstable, it often suffers from various instability processes such as creaming, flocculation and coalescence during storage. Thus, investigating the long-term stability of a lipid emulsion product under a practical condition is essential. In this research, the physical and chemical stability of the Tan IIA-LE at 25 ± 2 °C in dark was evaluated for 12 months by monitoring a collection of parameters, and the results are listed in Table 6. It can be seen that the Tan IIA-LE kept an orange-yellow appearance with no creaming, coalescence or visible oil droplets observed during the storage, while its mean particle size slightly increased. Other parameters including pH value, ζ-potential and drug content did not significantly change either upon duration, indicating its satisfactory physicochemical stability. Hence, this Tan IIA-LE product appeared to be physicochemically stable during 12-month storage in dark, and the investigation of long-term stability is still under way.

3.5. Safety study of Tan IIA-LE

3.5.1. Rabbit ear vein irritation study

During and after administration of Tan IIA-LE and 0.9% normal saline, no damages including edema and erythema were observed at the injection sites. Histopathological examination demonstrated that no degeneration, necrosis or hyperkeratosis occurred in the

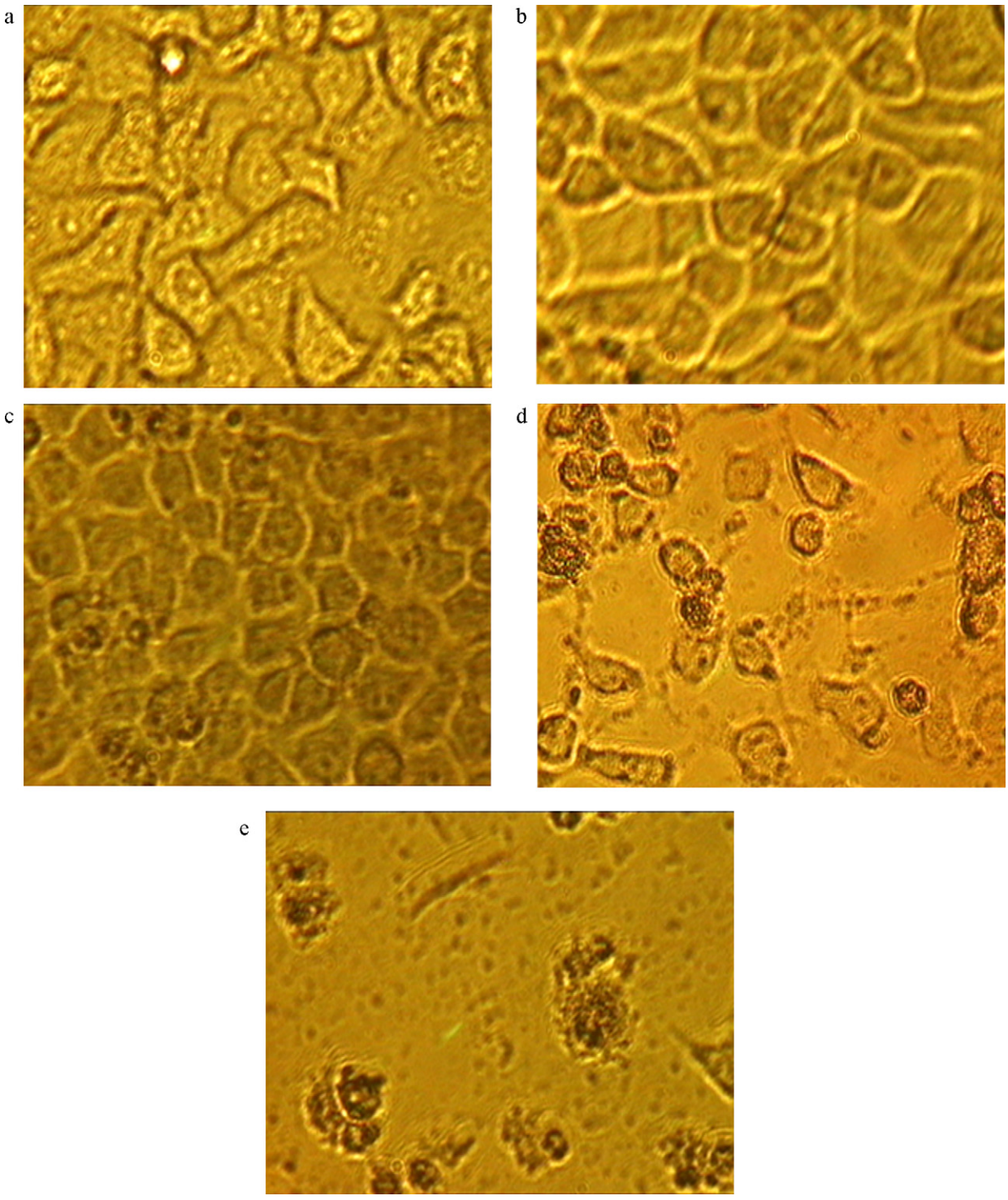

Fig. 5. Observation of HepG2 cell morphology after incubation for 48 h. (a) Treated with Tan IIA-free culture medium, (b) treated with Tan IIA-free lipid emulsion, (c) treated with 10 μg/mL of STS solution, (d) treated with 8 μg/mL of Tan IIA-LE and (e) treated with 8 μg/mL of Tan IIA-DMSO. The observation was carried out under an inverted phase contrast microscope (magnification 250×).

dermal tissues at and away from the injection sites, also there was no thrombus, angiectasia or vascular congestion in blood vessels for both the two administration sites (Fig. 4). These similar results obtained from the rabbit ears administered with 4 mg/kg of Tan IIA-LE and 0.9% normal saline indicated that the Tan IIA-LE would not cause venous irritation after i.v. administration at a dose of 4 mg/kg.

3.5.2. Acute toxicity study

Clinical observations showed that both Tan IIA-LE and Tan IIA-free lipid emulsion caused decreased activity in some mice following administration immediately, but this symptom disappeared within about 30 min. No other clinical signs of toxicity or death were observed during the injection and observation period. Based on Table 7, Tan IIA-LE and Tan IIA-free lipid emulsion appeared to

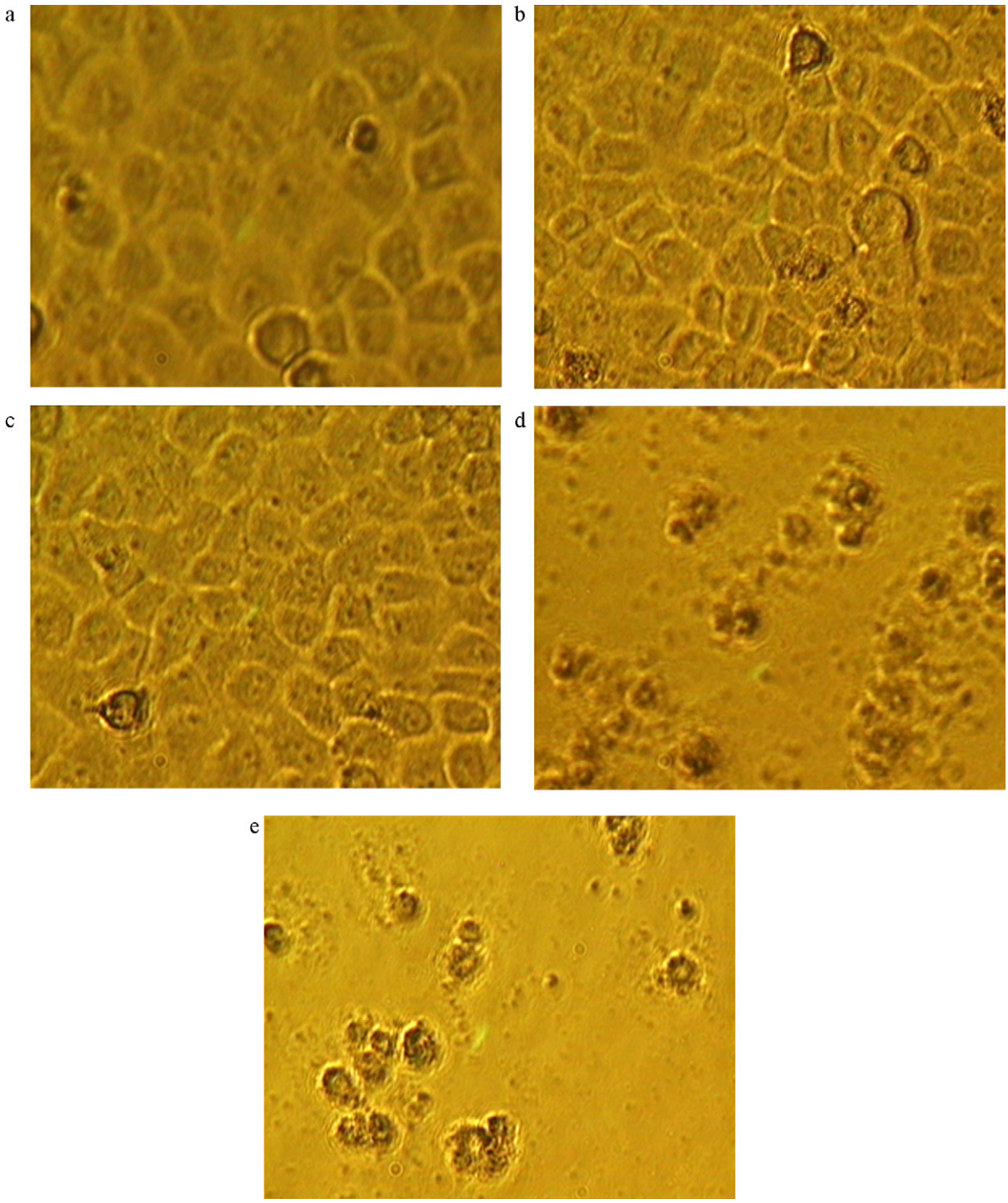

Fig. 6. Observation of SMMC 7721 cell morphology after incubation for 48 h. (a) Treated with Tan IIA-free culture medium, (b) treated with Tan IIA-free lipid emulsion, (c) treated with 10 μg/mL of STS solution, (d) treated with 8 μg/mL of Tan IIA-LE and (e) treated with 8 μg/mL of Tan IIA-DMSO. The observation was carried out under an inverted phase contrast microscope (magnification 250×).

have similar effects on the body weights of mice, since comparative body weights of animals in these two groups were obtained after administration for 14 days. In comparison with the control group (normal saline), both Tan IIA-LE and Tan IIA-free lipid emulsion groups showed reduced body weights, but the differences were not significant ($p > 0.05$). Therefore, it can be deduced that neither the drug carrier (drug-free emulsion) nor the Tan IIA has obvious acute toxicity to the mice.

3.6. Anti-hepatoma activity study in vitro

3.6.1. Cell viability assay

The anti-tumor activity of Tan IIA against a variety of cancer cell lines has been confirmed in a number of preclinical studies, and a nanoparticle formulation containing Tan IIA was also found to be cytotoxic to mice H_{22} hepatocarcinoma cell line (Li et al., 2008). In order to investigate the cytotoxicity of the lipid emulsion of Tan IIA,

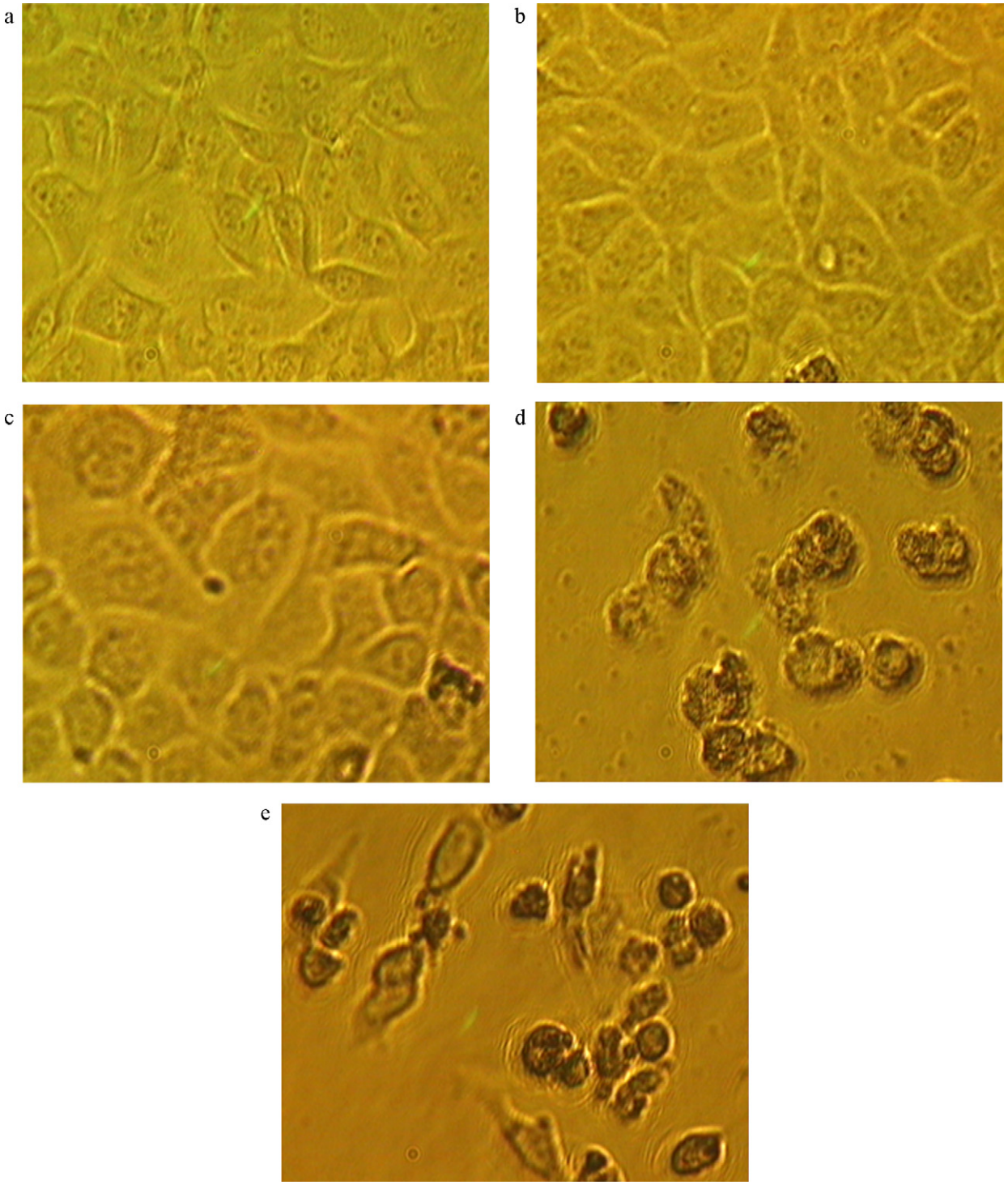

Fig. 7. Observation of BEL 7404 cell morphology after incubation for 48 h. (a) Treated with Tan IIA-free culture medium, (b) treated with Tan IIA-free lipid emulsion, (c) treated with 10 μg/mL of STS solution, (d) treated with 8 μg/mL of Tan IIA-LE and (e) treated with 8 μg/mL of Tan IIA-DMSO. The observation was carried out under an inverted phase contrast microscope (magnification 250×).

MTT assay was conducted in this study to assess the effects of Tan IIA-LE on cell viability. The anti-tumor activity of STS, which is the derivate of Tan IIA was also evaluated.

Table 8 displays the cell viability of different human hepatoma cell lines (HepG2, SMMC 7721 and BEL 7404) after treatment with Tan IIA-free culture medium (negative control group, NC), Tan IIA-free lipid emulsion (blank vehicle control group), 10 μg/mL of STS solution and different concentrations (1, 2, 4 and 8 μg/mL) of Tan IIA-LE and Tan IIA-DMSO for 24, 48 and 72 h. For each cell line, it was clearly demonstrated that both Tan IIA-LE and Tan IIA-DMSO can significantly inhibit cell proliferation compared with the negative control group ($p < 0.05$), and their inhibitory effects appeared to be in dose- and time-dependent manner. Since the Tan IIA-free lipid emulsion did not exhibit any adverse impact on cell viability, it can be deduced that the apparent proliferation inhibitory effect displayed by Tan IIA-LE was almost attributed to the inherent anti-tumor activity of the drug itself rather than the drug carrier. Besides, observations were noted that when compared with

the Tan IIA-DMSO group, the cell viability in Tan IIA-LE group at the equivalent concentration was obviously greater at 24 and 48 h, while the incubation time prolonged to 72 h, the cell viability between these two groups was close at each corresponding concentration, irrespective of the cell line type. This phenomenon may be explained as follows: for Tan IIA dissolved in DMSO, it contacted cells directly that enable it exert cytotoxicity immediately, resulting in a pronounced cell proliferation inhibitory effects at initial treatment time. As for Tan IIA-LE, Tan IIA was incorporated in the interior phase of the lipid emulsion, thus it will take time for the release of Tan IIA from lipid emulsion to inhibit cell growth effectively. After incubation for a long enough time (*e.g.* 72 h), the incorporated drug may release completely from the interior phase, showing a comparative cytotoxicity on cell proliferation as the Tan IIA-DMSO did. This explanation was also supported by the observation that, taking HepG2 cells as an example, the cell viability after treatment with Tan IIA-LE at 1 μg/mL for 48 h was similar to that after treatment with the equivalent dose of Tan IIA-DMSO for 24 h (89.46% and 87.30%, respectively), substantiating the sustained release property of lipid emulsion as a drug carrier. In addition, our observation regarding the cytotoxicity of STS solution was in agreement with the findings published elsewhere (Chan et al., 2011), which reported that STS had no influence on cell viability, irrespective of treatment time, indicating that the intrinsic anti-tumor activity of Tan IIA was eliminated in the form of STS, which may be attributed to the structure modification. It has been well documented that the anti-tumor activity of Tan IIA is mainly based on its furano-o-quinone moiety, which exerts the cytotoxicity either by producing reactive free radicals in the close vicinity of the bases to cause DNA damage (Wu et al., 1991) or by binding to DNA groove to cause structure damage to duplex DNA (Zhang et al., 2008, 2009). These mechanisms were all dependent on the lipophilicity of Tan IIA that facilitates the entry of the drug into cells to play the anti-tumor activity. STS as the derivate of Tan IIA after sulfonation, its high hydrophilicity makes it impossible for STS to transport across the cell membrane to cause damage. This may be part of the reasons why STS exhibited no cytotoxicity.

3.6.2. Observation of cellular morphology

Figs. 5–7 show the cellular morphology of HepG2, SMMC 7721 and BEL 7404 cells after 48 h incubation with Tan IIA-free culture medium (negative control group, NC), Tan IIA-free lipid emulsion (blank vehicle control group), 10 μg/mL STS solution, 8 μg/mL of Tan IIA-LE and 8 μg/mL of Tan IIA-DMSO, respectively. It can be seen that for these three cell lines, the cells in drug-free groups (NC and blank vehicle control group) proliferated adhesively with a relatively normal gross morphology, indicating that the drug carrier did not cause damages to cellular morphology. Likewise, the observation that cells grew well in the STS group also verified that STS had no adverse influence on the cell proliferation, in accordance with the findings in MTT assay. In both the Tan IIA-LE and Tan IIA-DMSO groups, the number of cells decreased apparently in comparison with that in negative control group, reflecting a significant inhibited proliferation. Furthermore, the phenomena including detachment of cells from the wall, morphological changes of becoming round and cell breakage was found in these two groups, verifying that both the Tan IIA-LE and Tan IIA-DMSO exerted cytotoxicity against these cell lines regardless of cell line types, while the Tan IIA-DMSO exhibited a somewhat greater degree than the Tan IIA-LE.

Based on the results obtained from cell viability assay and cellular morphology observation, it can be concluded that the Tan IIA-LE developed in our study possessed significant cytotoxic effects on human hepatoma cancer cell lines (HepG2, BEL 7404 and SMMC 7721), and the drug carrier of lipid emulsion guarantees a sustained release of the entrapped drug.

Lipid emulsions as drug carrier with passive targeting effects have been reported to preferentially accumulate in reticuloendothelial system (RES) including the liver, and to be readily uptaken by tumor cells (Igarashi et al., 1996). Therefore, the Tan IIA incorporated in the lipid emulsion can be expected to target to the liver to exert the anti-tumor activity with enhanced efficacy and reduced systematical toxicity. Further investigation of anti-tumor activity against human hepatoma *in vivo* of the Tan IIA-LE is now being carried out in our laboratory.

4. Conclusion

In conclusion, this research initially provided a novel intravenous lipid emulsion of tanshinone IIA (Tan IIA-LE) based on systematical investigations with respect to formulation and preparation process. The results of stability study demonstrated that the Tan IIA-LE was sensitive to light but remained physicochemically stable during 12-month storage in the dark, indicating its excellent long-term stability when kept away from light. This Tan IIA-LE not only displayed a satisfactory safety in animal models tests, but also exerted an obvious anti-tumor activity against human hepatoma cell lines (HepG2, SMMC 7721 and BEL 7404) *in vitro*. Taking all these findings into consideration, the Tan IIA-LE developed in this paper offers a potential for clinical application and will be beneficial for liver cancer therapy in future. To our best knowledge, this is the first research to confirm the anti-tumor activity of lipid emulsion of tanshinone IIA against human hepatoma cell lines *in vitro*.

Acknowledgements

This work was supported by the grants from National Natural Science Foundation of China (No. 30772668), Doctoral Fund of Ministry of Education of China (No. 20070610050), Sichuan Province Science and Technology Support Program (No. 2011SZ0009) and National Scientific and Technological Major Special Project of China – "Key New Drugs Creation and Manufacturing" (No. 2009ZX09310-002 and No. 2009ZX09503-020).

References

Cao, E.H., Liu, X.Q., Wang, J.J., Xu, N.F., 1996. Effect of natural antioxidant tanshinone II-A on DNA damage by lipid peroxidation in liver cells. Free Radic. Biol. Med. 20, 801–806.

Chan, S.E., Lai, H.W., Su, C.C., Kuo, S.J., Chien, S.Y., Lin, H.Y., Chen, D.R., 2011. Effect of supplementation of Tanshinone IIA and sodium tanshinone IIA sulfonate on the anticancer effect of epirubicin: an *in vitro* study. Evid. Based Complement. Altern. Med., doi:10.1155/2011/841564.

Chang, P.N., Mao, J.C., Huang, S.H., Ning, L., Wang, Z.J., On, T., Duan, W., Zhu, Y.Z., 2006. Analysis of cardioprotective effects using purified *Salvia miltiorrhiza* extract on isolated rat hearts. J. Pharmacol. Sci. 101, 245–249.

Chansiri, G., Lyons, R.T., Patel, M.V., Hem, S.L., 1999. Effect of surface charge on the stability of oil/water emulsions during steam sterilization. J. Pharm. Sci. 88, 454–458.

Cheng, T.O., 2007. Cardiovascular effects of Danshen. Int. J. Cardiol. 121, 9–22.

Cheng, C.Y., Su, C.C., 2010. Tanshinone IIA may inhibit the growth of small lung cancer H146 cells by up-regulating the Bax/Bcl-2 ratio and decreasing mitochondrial membrane potential. Mol. Med. Rep. 3, 645–650.

Cheong, J.N., Tan, C.P., Man, Y.B.C., Misran, M., 2008. α-Tocopherol nanodispersions: preparation, characterization and stability evaluation. J. Food Eng. 89, 204–209.

Date, A.A., Nagarsenker, M.S., 2008. Parenteral microemulsions: an overview. Int. J. Pharm. 355, 19–30.

Dong, X., Dong, J., Zhang, R., Fan, L., Liu, L., Wu, G., 2009a. Anti-inflammatory effects of tanshinone IIA on radiation-induced microglia BV-2 cells inflammatory response. Cancer Biother. Radiopharm. 24, 681–687.

Dong, K., Xu, W., Yang, J., Qiao, H., Wu, L., 2009b. Neuroprotective effects of Tanshinone IIA on permanent focal cerebral ischemia in mice. Phytother. Res. 23, 608–613.

Driscoll, D.F., Bacon, M.N., Bistrian, B.R., 2000. Physicochemical stability of two different types of intravenous lipid emulsion as total nutrient admixtures. J. Parenter. Enteral Nutr. 24, 15–22.

Driscoll, D.F., Nehne, J., Peterss, H., Franke, R., Bistrian, B.R., Niemann, W., 2002. The influence of medium-chain triglycerides on the stability of all-in-one formulations. Int. J. Pharm. 240, 1–10.

Fan, Y., Li, J.F., Dong, C., 2005. Preparation and study on the inclusion complexes of two tanshinone compounds with β-cyclodextrin. Spectrochim. Acta A: Mol. Biomol. Spectrosc. 61, 135–140.
Fan, G.W., Gao, X.M., Wang, H., Zhu, Y., Zhang, J., Hu, L.M., Su, Y.F., Kang, L.Y., Zhang, B.L., 2009. The anti-inflammatory activities of Tanshinone IIA, an active component of TCM, are mediated by estrogen receptor activation and inhibition of iNOS. J. Steroid Biochem. Mol. Biol. 113, 275–280.
Fang, J., Xu, S.W., Wang, P., Tang, F.T., Zhou, S.G., Gao, J., Chen, J.W., Huang, H.Q., Liu, P.Q., 2010. Tanshinone II-A attenuates cardiac fibrosis and modulates collagen metabolism in rats with renovascular hypertension. Phytomedicine 18, 58–64.
Floyd, A.G., 1999. Top ten considerations in the development of parenteral emulsions. Pharm. Sci. Technol. Today 4, 134–143.
Gong, Y., Li, Y., Lu, Y., Li, L., Abdolmaleky, H., Blackburn, G.L., Zhou, J.R., 2011. Bioactive tanshinones in *Salvia Miltiorrhiza* inhibit the growth of prostate cancer cells *in vitro* and in mice. Int. J. Cancer 129, 1042–1052.
Hao, H., Wang, G., Cui, N., Li, J., Xie, L., Ding, Z., 2006. Pharmacokinetics, absorption and tissue distribution of tanshinone IIA solid dispersion. Planta Med. 72, 1311–1317.
Igarashi, R., Takenaga, M., Matsuda, T., 1996. Distribution of lipid microsphere preparations. Adv. Drug Deliv. Rev. 20, 147–154.
Jiao, J.W., Wen, F., 2011. Tanshinone IIA acts via p38 MAPK to induce apoptosis and the down-regulation of ERCC1 and lung-resistance protein in cisplatin-resistant ovarian cancer cells. Oncol. Rep. 25, 781–788.
Jumaa, M., Müller, B.W., 1998. The effect of oil components and homogenization conditions on the physicochemical properties and stability of parenteral fat emulsions. Int. J. Pharm. 163, 81–89.
Jumaa, M., Kleinebudde, P., Müller, B.W., 1999. Physicochemical properties and hemolytic effect of different lipid emulsion formulations using a mixture of emulsifiers. Pharm. Acta Helv. 73, 293–301.
Keck, C.M., Müller, R.H., 2006. Drug nanocrystals of poorly soluble drugs produced by high pressure homogenization. Eur. J. Pharm. Biopharm. 62, 3–16.
Laval-Jeantet, A.M., Laval-Jeantet, M., Bergot, C., 1982. Effect of particle size on the tissue distribution of iodized emulsified fat following intravenous administration. Invest. Radiol. 17, 617–620.
Levy, M.Y., Benita, S., 1989. Design and characterization of a submicronized o/w emulsion of diazepam for parenteral use. Int. J. Pharm. 54, 103–112.
Li, H., Zhang, Z., Ma, L., Chen, X., 2007. Preparation of tanshinone microemulsion and its absorption in rat intestine in situ. China J. Chin. Mater. Med. 32, 1024–1027.
Li, Q., Wang, Y., Fan, Z., Feng, N., Gao, H., Nan, Y., Ni, L., Bao, W., Sui, H., 2008. Effect of tanshinone IIA and its nanoparticles on apoptosis and expression of p38 MAPK and TGFβ$_1$ signaling proteins of hepatoma cells in mice. Tumor 28, 8–12.
Liang, X., Zheng, Y., Fan, T., 2010. Preparation and in vitro evaluation of tanshinone IIA pulsatile release pellets. Beijing Da Xue Xue Bao 18, 559–564.
Liu, J., Zhu, J., Du, Z., Qin, B., 2005. Preparation and pharmacokinetic evaluation of Tanshinone IIA solid lipid nanoparticles. Drug Dev. Ind. Pharm. 31, 551–556.
Liu, M., Xia, X., 2010. Study on the chemical stability of Tanshinone IIA. Chin. Med. Mater. 33, 606–609.
Liu, T., Jin, H., Sun, Q.R., Xu, J.H., Hu, H.T., 2010. The neuroprotective effects of tanshinone IIA on β-amyloid-induced toxicity in rat cortical neurons. Neuropharmacology 59, 595–604.
Lovell, M.W., Johnson, H.W., Hui, H.W., Cannon, J.B., Gupta, P.K., Hsu, C.C., 1994. Less-painful emulsion formulation for intravenous administration of clarithromycin. Int. J. Pharm. 109, 45–57.
Lu, Q., Zhang, P., Zhang, X., Chen, J., 2009. Experimental study of the anti-cancer mechanism of tanshinone IIA against human breast cancer. Int. J. Mol. Med. 24, 773–780.
Mao, S.J., Hou, S.X., Liang, Z., Bi, Y.Q., Wu, Y., Li, H., Jin, H., 2006. Ion-pair reversed-phase HPLC: assay validation of sodium tanshinone IIA sulfonate in mouse plasma. J. Chromatogr. B 831, 163–168.
Paolino, D., Ventura, C.A., Nisticò, S., Puglisi, G., Fresta, M., 2002. Lecithin microemulsions for the topical administration of ketoprofen: percutaneous adsorption through human skin and in vivo human skin tolerability. Int. J. Pharm. 244, 21–31.
Piemi, M.P.Y., Korner, D., Benita, S., Marty, J.P., 1999. Positively and negatively charged submicron emulsions for enhanced topical delivery of antifungal drugs. J. Control. Release 58, 177–187.
Rubino, J.T., 1990. The influence of charged lipids on the flocculation and coalescence of oil-in-water emulsions. I: kinetic assessment of emulsion stability. J. Parenter. Sci. Technol. 44, 210–215.
Shan, Y.F., Shen, X., Xie, Y.K., Chen, J.C., Shi, H.Q., Yu, Z.P., Song, Q.T., Zhou, M.T., Zhang, Q.Y., 2009. Inhibitory effects of tanshinone II-A on invasion and metastasis of human colon carcinoma cells. Acta Pharmacol. Sin. 30, 1537–1542.
Shi, S., Chen, H., Cui, Y., Tang, X., 2009. Formulation, stability and degradation kinetics of intravenous cinnarizine lipid emulsion. Int. J. Phram. 373, 147–155.
Smymiotis, V.E., Kostopanagiotou, G.G., Arkadopoulos, N.F., Theodoraki, K.A., Kotsis, T.E., Lambrou, A.Th., Vassiliou, J.G., 2001. Long-chain versus medium-chain lipids in acute pancreatitis complicated by acute respiratory distress syndrome: effects on pulmonary hemodynamics and gad exchange. Clin. Nutr. 20, 139–143.
Sun, D., Shen, M., Li, J., Li, W., Zhang, Y., Zhao, L., Zhang, Z., Yuan, Y., Wang, H., Cao, F., 2011. Cardioprotective effects of tanshinone IIA pretreatment via kinin B2 receptor-Akt-GSK-3β dependent pathway in experimental diabetic cardiomyopathy. Cardiovasc. Diabetol. 10, 4.
Tamilvanan, S., 2004. Oil-in-water lipid emulsions: implications for parenteral and ocular delivering systems. Prog. Lipid Res. 43, 489–533.
Trotta, M., Pattarino, F., Ignoni, T., 2002. Stability of drug-carrier emulsions containing phosphatidylcholine mixtures. Eur. J. Pharm. Biopharm. 53, 203–208.
Weingarten, C., Magalhaes, N.S.S., Baszkin, A., Benita, S., Seiller, M., 1991. Interaction of a non-ionic ABA copolymer surfactant with phospholipid monolayers: possible relevance to emulsion stabilization. Int. J. Pharm. 75, 171–179.
Won, S.H., Lee, H.J., Jeong, S.J., Lee, H.J., Lee, E.O., Jung, D.B., Shin, J.M., Kwon, T.R., Yun, S.M., Lee, M.H., Choi, S.H., Lü, J., Kim, S.H., 2010. Tanshinone IIA induces mitochondria dependent apoptosis in prostate cancer cells in association with an inhibition of phosphoinositide 3-kinase/AKT pathway. Biol. Pharm. Bull. 33, 1828–1834.
Wu, W.L., Chang, W.L., Chen, C.F., 1991. Cytotoxic activities of tanshinones against human carcinoma cell lines. Am. J. Chin. Med. 19, 207–216.
Yu, X.Y., Lin, S.G., Zhou, Z.W., Chen, X., Liang, J., Liu, P.Q., Duan, W., Chowbay, B., Wen, J.Y., Li, C.G., Zhou, S.F., 2007. Role of P-Glycoprotein in the intestinal absorption of Tanshinone IIA, a major active ingredient in the root of *Salvia miltiorrhiza* Bunge. Curr. Drug Metab. 8, 325–340.
Zhang, Z., Zhang, J., Jin, L., Song, T., Wu, G., Gao, J., 2008. TanshinoneIIA interacts with DNA by minor groove-binding. Biol. Pharm. Bull. 31, 2342–2345.
Zhang, Z., Gao, J., Wang, Y., Song, T., Zhang, J., Wu, G., Zhang, T., Du, G., 2009. TanshinoneIIA triggers p53 responses and apoptosis by RNA polymerase II upon DNA minor groove binding. Biochem. Pharmacol. 78, 1316–1322.
Zhang, R., Sun, J., Ma, L., Wu, X., Pan, G., Hao, H., Zhou, F., A, J., Liu, C., Ai, H., Shang, L., Gao, H., Peng, Y., Wan, P., Wu, H., Wang, G., 2011. Induction of cytochromes P450 1A1 and 1A2 by tanshinones in human HepG2 hepatoma cell line. Toxicol. Appl. Pharmacol. 252, 18–27.
Zhou, L., Zuo, Z., Chow, M.S., 2005. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. J. Clin. Pharmacol. 45, 1345–1359.