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Highly efficient and lowly toxic docetaxel nanoemulsions for intravenous injection to animals

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Hypersensitivity many occur with commercial docetaxel injections containing Tween 80 and ethanol. An alternative formulation of docetaxel, an oil-in-water nanoemulsion was prepared using the high-pressure homogenization method. It was composed of medium-chain triglyceride, oleic acid, egg lecithin, and poloxamer. These ingredients are known as safe agents for intravenous (i.v.) injection. The nanoemulsion had a small size of 169 nm, and a high surface charge with the zeta potential of -33.9 mV. It maintained well stable even under high centrifugation. Acute toxicity of i.v. injection, erythrocyte hemolysis experiment, and rabbit ear vein irritation test showed no toxicity for the docetaxel nanoemulsion. The docetaxel nanoemulsion led to a larger apparent distribution volume and area under curve than the docetaxel injection after i.v. administration to rats. The histopathological test of tumor further demonstrated the highly anticancer efficiency of the docetaxel nanoemulsion. Thus, the nanoemulsion is a promising delivery system for docetaxel with highly anticancer efficiency and low toxicity.

1. Introduction

Nanoemulsions are transparent or translucent oil-in-water (O/W) or water-in-oil (W/O) droplets with a mean droplet diameter in the range between 100 and 500 nm. Unlike the thermodynamically stable microemulsions, nanoemulsions are kinetically stable with great stability in suspensions due to their small droplet size (Tadros et al. 2004). The O/W nanoemulsions have been introduced as an effective delivery vehicle for nonpolar drugs whose i.v. administration was hampered greatly by their low solubility in water (Wim et al. 2006). The i.v. emulsions of diazepam, propofol, fat soluble vitamins, and amphotericin B, have already been marketed. The unique characteristic of nanoemulsions is that the preparation procedure is relatively simple, favoring large-scale production.

Docetaxel as a potent anticancer agent has widely clinical applications for treatment of advanced breast cancer, non-small-cell and small-cell lung cancers, ovarian cancer, head and neck cancer, and gastric cancer, melanoma, soft tissue sarcomas, and so on (Cortes and Pazdur 1995; Earhart 1999). A major problem of docetaxel is its low aqueous solubility and the design of suitable formulations has been a difficult step. The commercial product of docetaxel, Taxotere®, contains Tween 80 as the solubilizer and ethanol as the auxiliary solvent, which is packaged as the condensed injection solution and the diluting solvent, respectively. Before application, the solvent is first added into the condensed injection solution and mixed uniformly, then diluted with 0.9% NaCl solution or 5% glucose solution. The whole dilution procedure is complicated, inconvenient for clinical use and prone to cause secondary contamination. Moreover, the patients have to receive anti-hypersensitivity pretreatment in advance. In addition, it is prone to cause hemolysis though Tween 80 is less toxic (Tije 2003). Therefore, the development of a safe intravenous (i.v.) formulation devoid of Tween 80 is an important issue for investigation (Engels 2007). Many alternative docetaxel formulations, such as liposomes (Immordino 2003), polymeric nanoparticles (Hwang 2008), solid lipid nanoparticles (Xu 2009), and polymeric micelles (Liu 2008), have been investigated. However, the preparation procedures of the docetaxel formulations mentioned above were complicated and not suitable for large-scale preparation.

It is well known that nanoscale particles could target to tumor tissues through the enhanced penetration and retention (EPR) effect if long circulation is achieved after avoiding macrophage's phagocytosis (Moghimi et al. 2001). The tumor targeting effect of paclitaxel nanoemulsions has been demonstrated (Constantinides et al. 2008). A docetaxel nanoemulsion was prepared in this study. Its toxicity and anticancer efficiency for i.v. injection to tumor-bearing mice were explored.

2. Investigations, results and discussion

2.1. Characteristics of docetaxel nanoemulsions

The mean size of docetaxel nanoemulsions was 169 nm, and the zeta potential was -33.9 mV. The high surface charge is regarded as the key factor to keep particles stable due to the strong electrostatic repulsion when the absolute zeta potential is more than 30 mV (Jin et al. 2009). The stability of docetaxel nanoemulsions was also demonstrated according to the stability constant Ks that was low to 36.02%. Therefore, the stability of docetaxel nanoemulsions was good, and maintained for a long time.

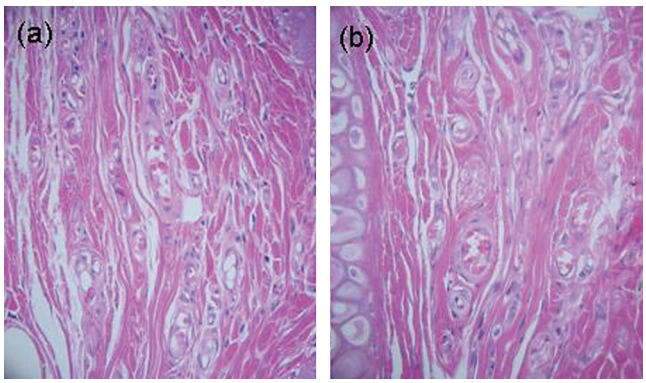

Fig. 1: Histopathological slices of rabbit ear-rim auricular veins after injection of saline (a), and docetaxel nanoemulsions (b)

2.2. *Formulation analysis of docetaxel nanoemulsions*

The components of docetaxel nanoemulsions were selected based on stability and biocompatibility. MCT is a family of triglycerides, containing predominantly, caprylic and capric fatty acids with less amounts of caproic and lauric fatty acids (Traul et al. 2000). Docetaxel is soluble in MCT especially at high temperature, which was demonstrated by us. More importantly, MCT is essentially non-toxic in acute toxicity tests conducted in several species of animals. MCT is widely used for parenteral nutrition (Traul et al. 2000). No adverse effect is found after injecting MCT to human subjects, and hemostatic safety is shown (Pradier et al. 2008). The lecithin used is the model of Lipoid E80 that contains 80% phosphatidylcholine, 8% phosphatidylethanolamine, 3.6% non-polar lipids and 2% sphingomyelin. This lecithin is commonly used in lipid formulations, and the injection safety is good (Li et al. 2010). Poloxamers can prevent particles from aggregating through inserting the PPO chains into the lipid core and extending the PEO chains to the aqueous medium (Santander-Ortega et al. 2006). The injection safety of poloxamer 188 has been ensured (Ballas et al. 2004). Glycerin is commonly used as an osmotic modifier.

The unique advantage of docetaxel nanoemulsions is the absence of the surfactant Tween 80 and the organic solvent ethanol, compared with the commercial docetaxel injection. Tween 80 is known to produce hypersensitivity. In addition, the docetaxel nanoemulsion can directly i.v. injected or infused while the docetaxel injection has to be diluted with glucose or saline solution before i.v. infusion.

2.3. *Toxicity of docetaxel nanoemulsions*

The volume percentages of docetaxel nanoemulsions in the erythrocyte suspensions were from 2% to 10%. No hemolysis was found within 4 h for all experimental samples, whereas the positive control showed complete hemolysis within 15 min. Therefore, the docetaxel nanoemulsion is suitable for i.v. injection. No adverse effect was found in mice after i.v. administration of a high dose of docetaxel nanoemulsions. All the mice showed a healthy status, and stayed alive for 48 h. Thus, the docetaxel nanoemulsion did not show acute injection toxicity.

The result of rabbit ear vein irritation test could indicate the topical irritation of docetaxel nanoemulsions. No congestion, dropsy, hemorrhage and putrescence were observed in the injection sites and the surroundings after i.v. administration of nanoemulsions and saline. The histopathological slices also showed that no difference existed between the nanoemulsion and saline (Fig. 1). Therefore, no topical irritation happened after i.v. injection of docetaxel nanoemulsions.

It is well known that the i.v. infusion of commercial docetaxel injection produces hypersensitivity reactions that are mainly attributed to the intrinsic toxic effects of Tween 80. The oxidation products present in Tween 80 can cause histamine release. Moreover, the injection can increase the membrane or vessel wall permeability, and change the erythrocyte morphology, leading to possible cardiovascular side effects. The side-effects are decreased by pre-medication with corticosteroids and antihistamines (Engels 2007). Therefore, docetaxel nanoemulsions are safer than docetaxel injection.

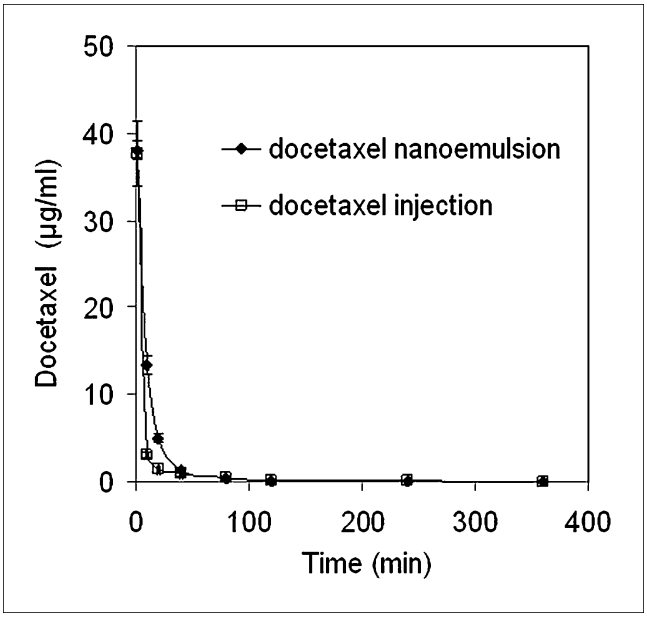

Fig. 2: Pharmacokinetic profiles of docetaxel nanoemulsions and injections after i.v. administration to rats, $n = 5$

2.4. *Pharmacokinetics of docetaxel nanoemulsions and docetaxel injections*

The docetaxel nanoemulsion and injection showed similar pharmacokinetic profiles with the two-compartment model after bolus i.v. injection to rats (Fig. 2). They showed a rapid distribution and a slow elimination. Comparing the pharmacokinetic parameters of the two formulations, the docetaxel nanoemulsion had a smaller clearance rate (Cl), a larger apparent volume of distribution (V(c)), and a larger area under curve (AUC) (Table 1). The large V(c)) should indicate much tissue distribution of the nanoemulsion. Generally, nanoparticles are rapidly eliminated from circulation due to macrophage recognition and uptake (Jin et al. 2006). However, the inserted poloxamer 188 in the docetaxel nanoemulsion could lead to a slow clearance due to the long-circulating effect, like other long-circulating nanoparticles (Moghimi et al. 2001). This

Table 1: Pharmacokinetic parameters of the docetaxel nanoemulsion and the docetaxel injection after i.v. administration to rats

Parameter	Docetaxel nanoemulsion	Docetaxel injection
AUC ($\mu g \cdot ml^{-1} \cdot min$)	$435.36 \pm 37.96^*$	267.65 ± 28.47
$t_{1/2\alpha}$ (min)	7.60 ± 1.11	6.26 ± 0.11
$t_{1/2\beta}$ (min)	101.22 ± 2.47	99.87 ± 8.0
$V_{(c)}$ (ml)	$0.109 \pm 0.039^*$	0.088 ± 0.004
Cl (ml/min)	$0.011 \pm 0.002^*$	0.016 ± 0.001

$^*P < 0.01$. The data were the mean ± SD ($n = 5$). AUC, area under curve; $t_{1/2\alpha}$, half life-time of phase I, i.e. distribution phase; $t_{1/2\beta}$, half life-time of phase II, i.e. elimination phase; $V_{(c)}$, apparent volume of distribution; *Cl*, clearance rate from plasma.

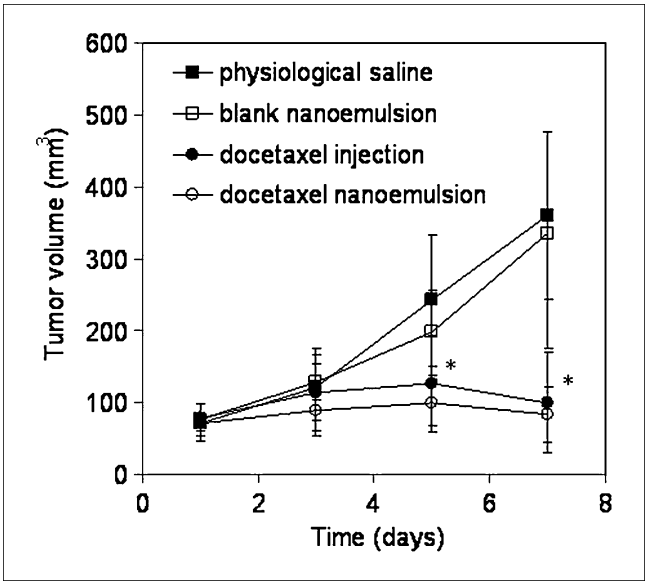

Fig. 3: Curves of tumor growth after i.v. administration of different agents to mice, $n = 8$. $^*P < 0.01$, the docetaxel nanoemulsion and injection groups *vs.* the blank nanoemulsion group

could provide the opportunity of tumor targeting of docetaxel nanoemulsions based on the EPR effect.

2.5. High anticancer efficiency of docetaxel nanoemulsions

Both docetaxel nanoemulsions and docetaxel injections showed the inhibition of tumor growth after the second administration, while the saline group and the blank nanoemulsion group showed rapid tumor growth (Fig. 3). With drug nanoemulsions and injections a significant inhibition happened after the third administration. In the whole treatment field, the tumor volume of the docetaxel nanoemulsion group was lower than that of the docetaxel injection group. On the seventh day, the tumor volume of the docetaxel nanoemulsion group was even close to the original volume.

The tumor weight data also showed the high anticancer effect of docetaxel nanoemulsions (Table 2). The tumor inhibitory rate of docetaxel nanoemulsions was 78.11%, a little higher than that of docetaxel injections (73.43%). Both the formulations showed a marked tumor inhibition compared with the saline and blank nanoemulsion groups. However, the docetaxel nanoemulsion showed higher safety than the injection when the equal dose was used according to the above safety experiment. The high anticancer efficiency makes the docetaxel nanoemulsion to become a potential docetaxel delivery system for clinical application.

2.6. Histopathological analysis

The therapethic effect of docetaxel formulations can be further evaluated by the histopathological analysis of tumor tissues.

Table 2: Tumor inhibition of the docetaxel nanoemulsion and the docetaxel injection after i.v. administration to mice

Group	Average tumor weight (g)	Tumor inhibitory rate (%)
Saline	1.069 ± 0.950	–
Blank nanoemulsion	0.829 ± 0.270	–
Docetaxel injection	$0.284 \pm 0.182^*$	73.43
Docetaxel nanoemulsion	$0.234 \pm 0.203^*$	78.11

The data were the mean ± SD ($n = 8$). $^*P < 0.01$, the docetaxel nanoemulsion and injection groups *vs.* the blank nanoemulsion group

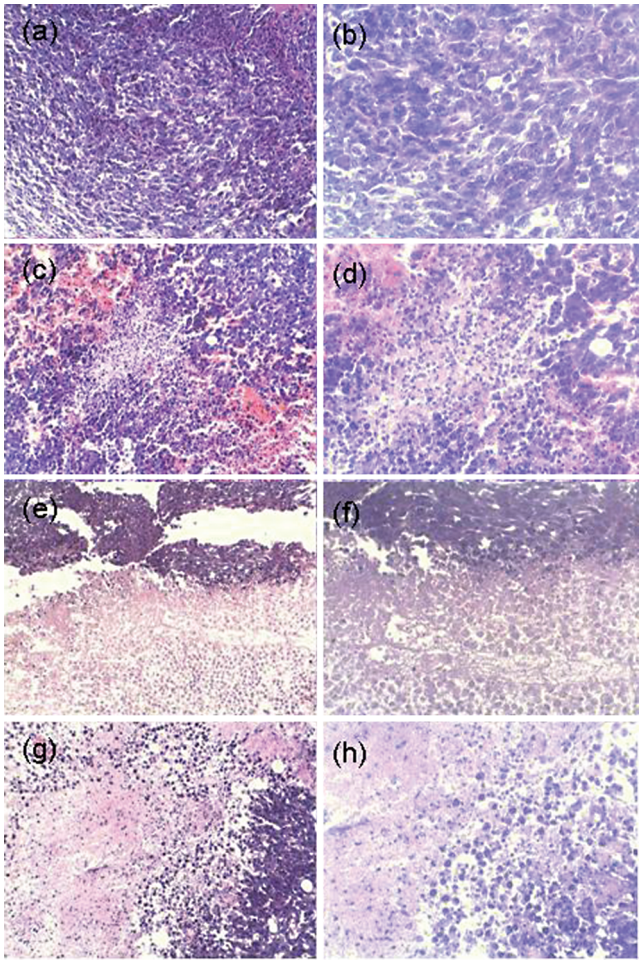

Fig. 4: Microscopic images of histopathological slices of tumor tissues after i.v. administration of different agents to mice. Images (a) and (b) are from the saline group; (c) and (d), the blank nanoemulsion group; (e) and (f), the docetaxel injection; (g) and (h), the docetaxel nanoemulsion group. Image (a) is 100-fold magnification; Images (c), (e) and (g) are 200-fold magnification; Images (b), (d), (f) and (h) are 400-fold magnification

Microscopic images of histopathological slices were shown in Fig. 4. The docetaxel treatment groups showed marked difference from the controls. The saline group showed the unclear tumor border, and the tissues arranged tightly to form the cancer nests (Fig. 4a). The cancer cells showed low-level differentiation, and some were necrotic (Fig. 4b). These implied that the tumor growth was good and the carcinomatous change may be enlarged. The blank nanoemulsion group showed phenomena similar to the saline group (Fig. 4c and 4d). There were severe bleeding sites and the neutrophilic granulocyte infiltration in the necrosis region due to the rapid growth and possible inflammation of tumor. The docetaxel injection group showed a large necrotic area (Fig. 4e). The cancer nests remained, and the tumor was surrounded with the loose fibrous connective tissue (Fig. 4f). The docetaxel nanoemulsion group showed phenomena similar to the drug injection group (Fig. 4 g and 4 h). Besides the large necrotic area, many monocytes existed between tumor and necrotic region. The results showed that the docetaxel nanoemulsion had significant anticancer efficiency.

3. Experimental

3.1. Materials

Docetaxel was provided by Shanghai Jinhe Bio-Technology Co., Ltd., China. Docetaxel injection was purchased from Jiangsu Heng Rui Medicine Co., Ltd. (China). Egg lecithin, oleic acid and medium-chain triglyceride (MCT) were purchased from Lipoid GmbH (Ludwigshafen, Germany).

Poloxamer is the collective name for a large group of triblock copolymers with a hydrophobic middle block (poly(propylene oxide); PPO) and hydrophilic end blocks (poly(ethylene oxide); PEO), also written as PEO-PPO-PEO. Poloxamer 188 was supplied by Shenyang Jiqi Pharmaceutical Co., Ltd. (China). Double-distilled water was used otherwise specially indicated. Other chemicals used were of analytical grade. Solvents of chromatographic grade were used for chromatographic measurement.

3.2. Animals

Male Sprague-Dawley rats (270–330 g) and male Kunming mice (18–20 g) were received from the Laboratory Animal Center of Beijing Institute of Pharmacology and Toxicology. Food and water were supplied *ad libitum*. All studies were carried out in accordance with the Declaration of Helsinki.

3.3. Preparation of nanoemulsions

Docetaxel nanoemulsions were prepared with the high-pressure homogenization technique. Docetaxel was dissolved in the MCT containing 3% (w/v) oleic acid through bath sonication under 60 °C to obtain a 1% (w/v) docetaxel solution as the oil phase. An aqueous solution containing 2.5% (w/v) glycerin was prepared and heated to 80 °C, and then 1.3% (w/v) lecithin and 1.3% (w/v) poloxamer 188 were added and agitated to thoroughly disperse to obtain the water phase. The water phase was transferred to a tissue mixer (Model 985370, Cole Parmer Instrument Co., USA), maintained at 50 °C, and homogenized at 10000 rpm for a moment. The 50 °C oil phase was then added into the hot water phase with the volume ratio of 1:9 followed by homogenization at 10000 rpm for 5 min. The formed emulsion was immediately transferred to the high-pressure homogenizer (EmulsiFlex-C5, Avestin, Canada), and further homogenized at 8000 psi for 6 times. The formed emulsion was filtered through a 0.22 μm filter, and sealed in the vials filled with nitrogen and stored in a 4 °C refrigerator until use. The final nanoemulsion contained 0.1% (w/v) or 1 mg/ml docetaxel. The blank nanoemulsion was prepared as the above procedure without docetaxel to be the control in the pharmacodynamic study.

3.4. Characterization of nanoemulsions

Sizes and zeta potentials of nanoemulsions were measured using a laser light scattering technique with a particle size analyzer (Sympatec Nanofox, Germany) and a zeta potential analyzer (Malvern Zetasizer 2000, UK). The nanoemulsions were diluted with water prior to measurement. The measurement was performed at 25 °C and each sample was measured in triplicates.

3.5. Stability investigation of nanoemulsions

The stability constant (K_s) was used to evaluate the stability of nanoemulsions. It was determined by the centrifugation-spectrophotometric method (Xiong et al. 2010). Small K_s implies good stability. Docetaxel nanoemulsions were 250-fold diluted with water, and the absorbance (A_0) was measured on a Cintra 10e UV-Visible Spectrometer (Australia) at 500 nm. The nanoemulsion (1.0 ml) was centrifuged at 3000 rpm for 10 min followed by withdrawing of supernatants, and the bottom sample was 250-fold diluted and measured as above to obtain the absorbance (A). The K_s was calculated as the following equation: $K_s = |A_0 - A|/A_0 \times 100\%$, wherein $|A_0 - A|$ indicates the absolute value of the difference between the original absorbance and the sample absorbance. Signifiicantly, the small K_s indicates little precipitating or floating of nanoemulsions, i.e. good stability.

3.6. Safety investigation

3.6.1. Biocompatibility

The erythrocyte lysis assay was done according to the literature with a little modification (Jin et al. 2006). Whole blood was obtained from rabbit heart and agitated with a glass stick to remove fibrinogen. Erythrocytes were separated by centrifugation at 1000 rpm for 15 min, and washed three times with saline. The sediment cells were diluted with saline to obtain the erythrocyte suspension (2%, v/v). The 5-ml samples containing 1% erythrocytes, a series of docetaxel nanoemulsions and supplementary saline were prepared, and incubated at 37 °C. The hemolytic effect was observed with naked eyes and/or a microscope. Pure water can lead to total hemolysis as the positive control, and saline was added as the negative control. Four situations could happen: a) no hemolysis, i.e. erythrocytes sinking, transparent supernatant, and no coacervation under microscope; b) coacervation, i.e., erythrocyte agglomeration after dispersion with saline; c) partial hemolysis, i.e. red appearing in supernatant, a little erythrocytes sinking and distortion; d) complete hemolysis, i.e., wholly red appearing in supernatant, and no erythrocytes in the bottom. If the latter three situations appear in 3 h, the experimental samples could not be suitable for i.v. injection.

3.6.2. Acute toxicity

Ten mice (18–20 g) were i.v. administered with the docetaxel nanoemulsion via tail vein with the dose of 0.5 ml docetaxel emulsion per mouse, or 25 mg docetaxel/kg. The injection process was finished within 5 s. The mice were observed whether to be death within 48 h.

3.6.3. Rabbit ear vein irritation test

Three rabbits (2.0 kg) received infusion of the docetaxel nanoemulsion with the dose of 2.0 ml nanoemulsion/kg or 2.0 mg docetaxel/kg and the injection rate of 1 ml/min through the right ear veins. Saline was also i.v. injected into their left ear veins with the dose of 2.0 ml/kg. The infusion was continued for 3 days with once per day. Whether congestion, dropsy, hemorrhage and putrescence happened in the injection sites and the surroundings was visually observed after infusion. The rabbits were ethically sacrificed after 24 h from the last administration, and a piece of vascular tissue at the injection site was removed for histopathological examination (Lu et al. 2008).

3.7. High-performance liquid chromatographic determination of docetaxel

HPLC experiments were performed on a Hitachi HPLC system (Japan), consisting of L-2130 pump, L-2400 UV detector, L-2200 automatic injector, and TL-2000 chromatographic workstation. The Venusil MP-C_{18} ODS HPLC columns (150 mm × 4.6 mm, 5 μm) were purchased from Agela Technologies Inc. (China). A 20-μl loop (7725i, Rheodyne, USA) was used. Docetaxel was measured at 230 nm and room temperature with the mobile phase of acetonitrile/water (55:45, v/v). The flow rate of mobile phase was 1.0 ml/min. The retention time (t_R) of docetaxel was 6.5 min. The nanoemulsion was 100-fold diluted with the above mobile phase to destroy the emulsion followed by centrifugation at 14000 rpm for 10 min. The supernatant was measured to obtain the amount of docetaxel in the nanoemulsion.

The determination of docetaxel in plasma samples was described as follows. A 100-μl aliquot of plasma samples was thoroughly mixed with the norethisterone standard solution in methanol (27.5 μg/ml, 10 μl, as the internal standard) and supplementary methanol (10 μl). Tert-butyl methyl ether (1 ml) was added into the above sample, vortexed for 3 min, and centrifuged at 7000 rpm for 5 min. The supernatant was pipetted into a tube. The extraction procedure was repeated once for the same sample using *tert*-butyl methyl ether. The organic solvent in the tube was evaporated under 40 °C. The residue was dissolved with methanol (50 μl) followed by the HPLC measurement. The docetaxel content was calculated after comparing the HPLC peak area of docetaxel and norethisterone.

3.8. Pharmacokinetic study

Rats were randomly assigned into two groups with 5 rats per group. They were fasted and freely supplied with water for 12 h prior to the experiment. The saline dilution of the docetaxel injection (1 mg/ml) and the docetaxel nanoemulsion (1 mg/ml) were i.v. injected to rats via tail vein with the dose of 11.5 mg/kg. Blood (0.5 ml) was collected to heparinized tubes via orbit at 1, 10, 20, 40, 80, 120, 240, 360, 480 min after injection. After centrifugation, the obtained plasma was stored at −20 °C until determination. The 3P87 pharmacokinetics software (supplied by the Committee of Mathematic Pharmacology of the Chinese Society of Pharmacology) was used to analyze the data.

3.9. Tumor-bearing animal model

Lewis lung cancer cell line was provided by Beijing Institute of Basic Medical Science. The cultural medium was the DMEM medium (Sigma, USA) supplemented with 10% fetal bovine serum and penicillin–streptomycin solution (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were cultured at 37 °C in the 5% CO_2 atmosphere. Mice were bred with food pellets and free access to tap water with a normal 12 h light/dark cycle. Ambient temperature and relative humidity were maintained at 22 ± 1 °C and 45 ± 5%, respectively. The Lewis cell suspension (3.5×10^6/ml) was prepared in sterile phosphate buffered solution. Each mouse was injected subcutaneously with the cell suspension (200 μl) in the armpit of its forelimb.

3.10. Pharmacodynamic study

The size of tumor could achieve 5 mm × 5 mm after transplant for 5 days. The mice were divided into 4 groups randomly with 8 mice each group, including the saline group, the blank nanoemulsion group, the docetaxel injection group, the docetaxel nanoemulsion group. The drug concentration in the injection and nanoemulsion was 1 mg/ml as above. After transplant for 6 days, the mice of all groups were i.v. administered via tail vein with the above formulations. The doses of docetaxel groups were 25 mg/kg, and

the same volume doses were used in the other two groups. All the mice were repeatedly injected with one day interval for a total of 4 times.
Xenograft sizes were measured in two perpendicular dimensions by a caliper once a day after treatment. The first administration day was recorded as day 0. Tumor volume (V) was calculated by $V = 0.5L \times W^2$, where L was the largest superficial diameter and W the smallest superficial diameter of the xenograft. After 48 h from the last administration, the mice were sacrificed. Tumors were dissected and weighed to calculate the tumor inhibitory rate based on the following equation:

$$\text{Tumor inhibitory rate } (\%) = (W_{blank}\text{-}W_{test})/\ W_{blank} \times 100$$

where W_{blank} and W_{test} were the tumor average weight of control group (Group D) and test groups, respectively. Statistically significant differences for multiple groups were determined using a one way ANOVA with a Dunnett T3 test. All testing was done using SPSS 16.0 (SPSS Inc., Somers, New York, USA).

3.11. Histological investigation

The tumor of ethically sacrificed mice were isolated and rinsed with a saline solution, solidified with 10% neutral carbonate buffered formaldehyde, embedded in paraffin using an embedding center, and cut into slices. The slices were stained with hematoxylin and eosin, and observed on a light microscope (CK30/CK40 model, Olympus, Japan). The explored content involved tumor growth, tumor necrosis, and infiltration of inflammation cells.

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