Research Reports

Poly (ethylene glycol) modified solid lipid nanoparticles as carriers of doxorubicin for the treatment of hepatocarcinoma cells

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Abstract Doxorubicin encapsulated solid lipid nanoparticles (DOX–SLNs) have already been invested for the chemotherapy of solid tumor, but after i.v. administration, DOX–SLNs are easily removed from circulation by the reticuloendothelial system (RES). In order to avoid clearance by RES, we prepared Poly (ethylene glycol) modified solid lipid nanoparticles (PEG–modified–SLNs) as carriers of doxorubicin, their correlative parameters and anti–tumor efficacy against hepatocarcinoma cells *in vitro* and *in vivo* were detected. The diameter of nanoparticles, determined by transmission electron microphotography, was 120±48 nm, and the loading efficiency was 68.6%. The release kinetics of DOX from PEG–modified–SLNs *in vivo* showed apparently controlled drug release efficiency. *In vitro* cytotoxicity assessed by MTT test was found to be concentration–dependent, and the effect of inhibition of hepatocarcinoma cell growth *in vivo* suggested that DOX–loaded–PEG–modified–SLNs could effectively inhibit the growth of hepatocarcinoma cells, which indicated favorable dosage–efficacy relationship. Therefore PEG–modified–SLNs might act as a promising carrier for controlled drug delivery.

Keywords: Poly (ethylene glycol); solid lipid nanoparticle; stearic acid; Doxorubicin; chemotherapy; hepatocarcinoma

1 Introduction

Doxorubicin is an anthracycline antibiotic that is of great importance in the treatment of leukemia and solid tumors in humans. But prolonged use of doxorubicin can cause severe heart damage, even years after you have stopped taking doxorubicin, which is the most serious side effect of this drug. Therefore, with the aim of achieving better therapeutic efficacy and limiting side effects of the drug, many colloidal carriers of doxorubicin have been studied as delivery systems, such as liposomes [1,2], micelles [3,4] and solid lipid nanoparticles[4,5]. Colloidal carriers, after i.v. administration, are removed from circulation by cells of the reticuloendothelial system (RES). Sterically stabilized liposomes and nanoparticles have been prepared in previous studies by modifying their surface with hydrophilic polymers[6–8] in order to increase the blood circulation time of the particulate. Poly (ethylene glycol) derivatives (PEG derivatives), in particular, have been widely employed to obtain the steric stabilization of nanoparticles, thus reducing their uptakes by the RES cells.

In the present study, DSPE–PEG2000 was used to modify the solid lipid nanoparticles´ surface to prepare a kind of sterically stabilized SLN for carrying Doxorubicin. The aim was to evaluate differences in pharmacokinetics of two kinds of Doxorubicin at an equivalent DOX dose (10 mg/kg) of commercial Doxorubicin solution and Doxorubicin incorporated in PEG modified SLN, in a conscious animal model. Another animal model loading transplanted tumor, was prepared to detect the difference of Doxorubicin without or within PEG modified SLN in antitumor cytotoxicity.

2 Materials and methods

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2.1 Materials

Doxorubicin was supplied by Wanle Corporation (Shenzhen, China). Stearic acid, Pluronic F68 and 75% DSPE -PEG2000 were purchased from Sigma (USA). Ethionine and lecithin was purchased from Fisher (USA). The other chemicals were of analytical reagent grade. Hepatocarcinoma cell was a kind gift from CAS (Shanghai, China). RPMI 1640 was purchased from Gibco (USA), and MTT also from Sigma (USA).

2.2 Preparation of DOX-PEG modified SLNs

The DOX-PEG modified SLN were prepared by the method of "emulsion evaporation -solidification at low temperature", which was described as follow. 10 mg of Doxorubicin and 100 mg of Stearic acid were dissolved in 10 ml of Acetone in a conical flask, and dispersed by ultrasound. Then lecithin at the prescription dosage was added into the mixture, and melted together at 70℃, which made the organic phase. A mount of Pluronic F68 and DSPE-PEG2000 were dissolved in 30 ml distilled water, which formed the inorganic phase. Then the organic phase was added dropwise to the inorganic phase at a magnetic stirring (1,000 rpm, 70℃). After 4 h of continual stirring, the organic solution was evaporated completely, and 5 ml semifinished solution was remained. Then the semifinished solution was mixed quickly with 10 ml cold inorganic solution (0~2℃) as described above, and stirred at 1 000 rpm for 2 h, then the final product, that is DOX-loaded-PEG-modified-SLNs, was obtained in the equilibrium solution.

2.3 Photon correlation spectroscopy

The average diameters and polydispersity indices of SLNs were determined by photon correlation spectroscopy (PCS) using a Submicron Particle Size Analyzer (Santa Barbara, California, USA) at a temperature of 25℃. The wavelength of the laser light was 632.5 nm.

2.4 Transmission electron microscopy

Transmission electron microscopy (TEM) analysis was performed using a CM10 Philips instrument (Eindhoven, Netherlands). TEM samples were diluted 1:25 with ultrapure water and stained with a 2% solution of osmium tetraoxide before analysis.

2.5 Determination of DOX-loading content

The amount of Doxorubicin incorporated in the DOX-loaded-PEG-modified-SLNs was determined by HPLC using a LC-6A pump unit control, a C-R5A Chromatopac integrator and an SPD-2A UV detector (Shimadzu Corporation, Kyoto, Japan) set at 227 nm. A reversephase Waters Sephadex G50-ODSC18 column (4.6 mm × 250 mm) was used. The mobile phase consisted of acetonitrile-water (70/30, v/v), pH adjusted to 2.8 with phosphoric acid. The flow rate was 1.0 ml/min.

2.6 *In vivo* release kinetics of DOX from PEG-modified-SLNs

A number of Kunming rats at a weight of 20±2 g, was set up into 3 groups at random, and each group contained 50 rats. The same dose of doxorubicin in the two different formulations (DOX, 10 mg/ kg in each rat for each drug prescription) was injected through the tail vein. Each time, six rats for each drug formulation were killed at 15, 30, 60 min, and 2, 4, 8 and 14 h after drug administration. Blood samples were collected from the supraorbital vein. Plasma extracts were prepared by mixing plasma with four volumes of methanol. Precipitated proteins were removed by centrifugation at 10,000 g for 5 min and 100 μl of the clear supernatants were injected for HPLC analysis.

2.7 In vitro cytotoxicity study against SMMC-7721 Hepatocarcinoma cell

Human SMMC-7721 Hepatocarcinoma cells were seeded onto 24-well plates with a seeding density of 600 cells per well. Cells were maintained in Roswell Park Memorial Institute media (RPMI-1640, Sigma), and incubated for 24 h at 37℃ in a humidified atmosphere with 5% CO_2. Then the cells were incubated in the free DOX or DOX-loaded-PEG-modified-SLNs in a series of different drug concentration, and incubated for another 36 h at 37℃ in a humidified atmosphere with 5% CO_2. After cells were harvested, the effect of inhibition of hepato-carcinoma cell growth in vitro was measured by MTT assay. The cytotoxicity of DOX and DOX-loaded-PEG-modified-SLNs in aqueous solution was analyzed and compared.

2.8 *In vivo* anti-tumor effect against Hepatocarcin-oma in tumor-bearing murine modals

Kunming rats (8-10 weeks old) breed in Center of experimental animal Sun Yat-sen University (Guangzhou, China) were used as the tumor-bearing murine models. At

day 0, 100 μl of 1 ×10^6 hepatocarcinoma cells were inoculated subcutaneously at the oxter of each rat´s right forelimb, and drug injection via tail vein was started at day 13 when the tumor diameter reached approximately 8 mm. The amount of injection is 10 mg/kg as doxorubicin concentration. Rats were divided into three different groups, and each group contained 12 rats. Each of the first group, as control, was injected with 700 μl of 0.9% saline solution for 7 days. The second group was injected with 700 μl of free doxorubicin (10 mg/kg body weight) and the third group was injected with 700 μl of DOX-loaded-PEG modified SLNs (10 mg of equivalent DOX/kg) in the same way. At day 20, all the rats were executed, and body weight of each rat was monitored. Then the transplanted tumors were anatomized and the weight of each solid tumor was also monitored.

3 Results

3.1 Size analysis

The average diameters and the polydispersity indices of SLNs shown by PCS was 120 ± 48 nm. TEM analysis confirmed the spherical shape and colloidal sizes of the SLNs (As shown in Fig. 1).

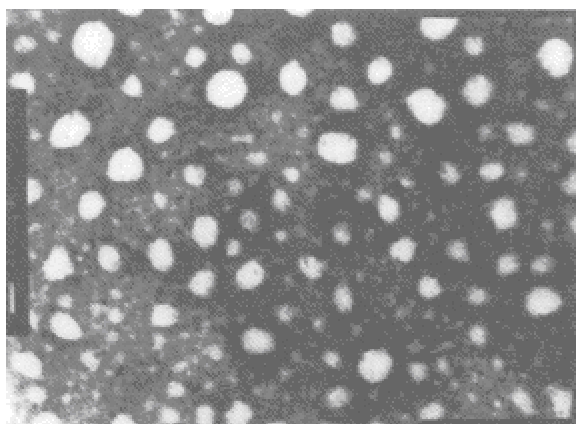

Fig. 1 Transmission electron microscoph of DOX-loaded- PEG modified SLNs

3.2 Percentage of DOX incorporated

Three groups of DOX-loaded-PEG modified SLNs were made and determined by HPLC analysis respectively. The amount of DOX incorporated in the SLNs was 0.32±0.07 g/L, and the loading efficiency (%) was 68.6%.

3.3 Release kinetics of DOX from PEG modified SLNs

At the beginning 30 min, both the concentration of DOX in the free DOX and DOX-loaded-PEG modified SLNs were decreased sharply, indicating that DOX was metabolized rapidly. After 4 h, the serum drug concentrations in the free DOX group reduced to 0.16 mg/L, while the DOX-loaded-PEG modified SLNs group 0.78 mg/L, even after 14 h, the concentrations of DOX in PEG modified SLNs group remained 0.24 mg/L, much higher than the free DOX group at 4 h, which indicated apparently controlled drug release (As Tab. 1 shown).

Tab. 1 The concentrations of DOX in serum for each formulation

T (h)	DOX (mg/L)	DOX- SLNS-PEG (mg/L)
0	35.94±6.21	32.21±5.78
0.5	3.39±0.51	1.96±0.38
1	2.03±0.35	1.36±0.24
2	0.63±0.12	0.85±0.18
4	0.16±0.06	0.78±0.11
8	-	0.68±0.10
14	-	0.24±0.02

3.4 *In vitro* and *in vivo* anti-tumor effect against hepatocarcinoma cells

The in vitro result from MTT assay show that the inhibition of DOX-SLNs group was not much more excelled but a little weaker than the free DOX group, while compared with control group, it showed statistically significant difference. (As Tab. 2 shown)

Tab. 2 The inhibition of DOX-loaded-PEG modified SLNs on growth of hepatocarcinoma cell in vitro

Groups	Num	OD	Inhibition Rate (%)
Control group	4	0.522±0.242	-
DOX group	4	0.224±0.098	60.1
DOX-SLNs	4	0.242±0.126*	59.6

* Compared with control group, p<0.01 comparatively

In vivo, the average weight of solid tumor in DOX-SLNs group was 0.59±0.39 g, apparently smaller than that

of free DOX group (P<0.01), which was 2.19±0.78g. And the inhibition rate of DOX-SLNs group (86%) was also higher than that of free DOX group (50%).

4 Discussion

Stearic acid is one of the useful types of saturated fatty acids that come from many animal and some plant foods like chocolate, which act as one of the important energy sources. Unlike most saturated fats, stearic acid does not seem to increase cholesterol levels in the blood, because liver enzymes convert it to an unsaturated fat during digestion, which show great compatibility with our organism. So it was widely used as carrier in the drug delivery, such as solid lipid nanoparticles. Many different synthetic techniques were used to prepare various solid lipid nanoparticles, such as emulsification-coacervation method [9], microemulsion method [10], solvent diffusion method [11] *et al*. In present study, we prepared the DOX-loaded-PEG modified-SLNs by the method "emulsion evaporation-solidification at low temperature", which was a bit easier to operate. Compared to SLNs prepared by microemulsion method, the average diameters of PEG modified SLNs we prepared were much smaller, which was 120 nm, meanwhile their diameter was about 160 nm [12]. It suggested that the diameter may be affected with different synthetic techniques. Meanwhile the diameter could also be influenced by the categories and quantities of DSPE-PEG2000 used in the experiment, which was similar with Sadzuka´s conclusion reported in his study [13]. By contraries, the factor affecting the diameter excluded the velocity of magnetic stirring and the quantities of Acetone or distilled water in use.

The inclusion of phospholipid with grafted poly (ethylene glycol) (PEG) side chains into the membrane surface prolongs liposomes circulation time in the blood stream and increases the potential applicability of liposomes for drug delivery [6,13,14]. And Sadzuka even demonstrated that the thickness of the PEG aqueous layer of PEG-modified doxorubicin liposomes has connection with improvement of circulation in blood and the involvement of anti-tumor activity [13].

Distearoylphosphatidylethanolamine-polyethylenegly-col 2000 (DSPE-PEG2000) is commonly used as a major component to help to increase liposome stability. It is well established that adding certain amounts of DSPE-PEG2000 into liposomes is one of the effective methods to eliminate the binding of plasma macromolecules including potential opsonizing factors such as immunoglobulins and complement proteins to liposomes and thus improve circulation life time as well as pharmacokinetics and antitumor therapeutic efficacy of liposomes [15-17]. In the present study, DSPE-PEG2000 was used to increase the stability. DSPE, as the hydrophobic blocks, were inserted into the core of solid lipid nanoparticles, where the Stearic acid was located, containing an amount of Doxorubicin. Meanwhile the hydrophilic PEG blocks were assembled at the surface of SLN, to form an aqueous layer, with the liquid absorbed at the surface. The aqueous layer will protect the SLN from being detected and removed by the recticuloendothelial system (RES), and thus results in a prolongation of blood circulation times of the particulate. With the EPR effect (enhanced permeability and retention), which is the basis for the selective targeting of drugs to solid tumor [18], the macromolecular anticancer agents with a prolonged blood circulation times have more opportunities to permeate the tumor blood vessels and sustain at the site of tumor, and thus increase the antitumor therapeutic efficacy.

In the clinical application of doxorubicin for che-motherapy, it is well known that the larger the dosage of doxorubicin was, the more severe side-effects the patient present, and at the interphase of administration the drug concentration in serum will decrease sharply, because of the quick clearance by liver and kidney (as Fig. 1 shown). Since the drug concentration at the tumor site was limited, thus the anticancer efficacy was depressed. In order to overcome these limits, Chiannikulchai prepared a kind of doxorubicin-nanoparticles [19], which could significantly reduce the side-effect of severe heart damage, and their anticancer efficacy was 10 times higher than that of free DOX injection. In the present study, we also show enhanced anticancer efficacy, though *in vitro* result from MTT assay indicated that the inhibition of DOX-SLNs group was not much more excelled but a little weaker than that of the free DOX group (as Tab. 2 shown). Maybe its causes lies in that, the time limit of anticancer effect on

tumors in vitro are much shorter, which was only 6–12 h, comparatively, the time limit in vivo was 8–10 d. within that limited time, the advantage of controlled drug release of the nano–carriers was not present, meanwhile the continuous anticancer effect of doxorubicin on Hepato–carcinoma cells was not embodied yet. But the information from anti–tumor effect on transplanted tumor–bearing models showed that the anti–tumor efficacy of DOX–loaded–PEG–modified–SLNs was better than free DOX group, furthermore it indicated a favorable effect–dosage relationship, suggesting that PEG modified SLNs will act as a promising controlled drug delivery carrier in the future, with its benefit of prolonged blood circulation time and enhanced anti–tumor efficacy.

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