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Solid lipid nanoparticles of anticancer drugs against MCF-7 cell line and a murine breast cancer model

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To develop some promising anticancer drug loaded solid lipid nanoparticles (SLN) for further clinical application, SLN carrying mitoxantrone (MTO), paclitaxel (PCT), methotrexate (MTX) were prepared and their cytotoxic effects on the human breast cancer cell line, MCF-7 were investigated. The 50 % inhibitory concentration (IC_{50}) values were interpolated from growth curves obtained by MTT assay. Moreover, the inhibition effects of the drugs incorporated in SLN on a murine breast cancer model induced by MCF-7 cells were further examined. *In vitro* cytotoxicity of MTO loaded SLN (IC_{50}/72h = 1.25 ± 0.19 μM vs 2.13 ± 0.37 μM) and MTX loaded SLN (IC_{50}/72h = 93.80 ± 6.54 nM vs 153.16 ± 11.54 nM) was higher than that of free drug formulations. *In vitro* cytotoxicity of PCT-loaded SLN and free drug formulation IC_{50}/72 h were similar. Then, the MCF-7 breast cancer model in mice was established. In mice treated with SLN injections for a month, tumor was significantly inhibited. Mean tumor size of mice treated with SLN was significantly smaller than that with free drug ($P < 0.05$). Additionally, the percent inhibition of mice treated with SLN was obviously lower than that with free drug ($P < 0.05$). Therefore, the conclusion can be drawn that anticancer drugs carried by SLN, including mitoxantrone, methotrexate and paclitaxel, may be more effective than free anticancer drugs for breast cancer treatment.

1. Introduction

Anticancer drugs are conventionally administered by intravenous bolus or infusion, typically in the form of free drug solutions. However, several obstacles are frequently encountered with anticancer compounds, such as normal tissue toxicity, poor specificity and stability and a high incidence of drug resistant tumor cells. Particulate drug carrier systems offer great promise to improve the therapeutic effectiveness and safety profile of anticancer compounds. Particulate drug carrier systems include solid lipid nanoparticles (SLN), liposomes, polymeric microspheres and macromolecule conjugates; they offer numerous advantages, e.g. improved efficacy and reduced toxicity, compared to conventional dosage forms.

SLN have been proposed as alternative drug carriers (Ravi Kumar 2000). SLN are obtained using biocompatible components and are washed by diafiltration, leaving no toxic residues from the preparation process. SLN are in the colloidal size range and can be loaded with both hydrophilic and lipophilic drugs, depending on the preparation method (Cavalli et al. 2003). The composition of the warm microemulsions from which SLN are prepared is flexible, and can be varied to suit the type of drug and administration route (Gasco 2001).

Mitoxantrone (MTO) is often used to treat breast cancer. Heart toxicity and myelosupression happened in individual leucopenia patients, and there is a local toxicity at regular doses (Zingler et al. 2005). Subcutaneous injection (s.c.) induced local toxicity is more serious than intravenous injection or intraperitoneal injection (i.p.) (Oussoren et al. 1998).

The diterpenoid derivative paclitaxel has broad antineoplastic activity, including against colorectal cancer cells, and a unique mechanism of action promoting the polymerization and stabilization of tubulin to microtubules (Singla 2002; Spencer and Faulds 1994). One of the major clinical problems of using paclitaxel is its very low solubility in water, due to its extremely hydrophobic nature. In order to enhance paclitaxel's solubility, a mixture of 50:50 Cremophor EL (CrEL, a polyoxyethylated castor oil) and ethanol is used in the current clinical formulation. This determines the administration of a significant amount of CrEL, with serious side effects for 25–30 % of treated patients (Spencer and Faulds 1994).

Methotrexate is used to treat certain types of cancer of the breast, skin, head and neck, or lung. It is also used to treat severe psoriasis and rheumatoid arthritis. Central nervous system reactions to methotrexate have been reported, especially when given via the intrathecal route which includes myelopathies and leucoencephalopathies. It has a variety of cutaneous side effects, particularly when administered in high doses.

The main aim of the present study was to evaluate the cytotoxic effect of these drugs incorporated in SLN, *versus* free drugs, on the human breast cancer cell line, MCF-7. Moreover, we further examined the inhibition effects of the drugs incorporated in SLN on a murine breast cancer model induced by MCF-7 cells. SLN were more effective than the free drugs against MCF-7 cell line and in the murine breast cancer model. The available data will be supplied to develop the promising anticancer drug loaded SLN for further clinical application.

2. Investigations and results

2.1. Particle size and zeta potential of SLN

The average diameter of obtained MTO-SLN was about 108 nm with the polydispersity index (PDI) of 0.219. The zeta potential was about −26 mV. The drug content was 4.07 ± 0.24 % and encapsulation yield 81.76 ± 3.69 %. The average diameters of PCT-SLN and MTX-SLN were 192 and 224 nm, with the PDI of 0.245 and 0.278, respectively. The drug content of PCT-SLN was 3.11 ± 0.38 % with an encapsulation efficiency of 71.8 ± 4.61 %. The drug content of MTX-SLN was 4.68 ± 0.41 % with an encapsulation efficiency of 67.3 ± 5.20 %.

2.2. Cytotoxicity of SLN

The results showed that the amount of MTO required to achieve the IC_{50} value was lower with loaded SLN than with free drug and the cytotoxicity was time-dependent (Table 1). Fig. 1 shows the percentage survival of MCF-7 cells after exposure to MTO solution, either as MTO-SLN or in unloaded SLN for 72 h. The IC50 of free MTO and MTO-SLN were 2.13 ± 0.37 μM and 1.25 ± 0.19 μM at 72 h exposure, respectively. PCT-SLN and free PCT showed the strong decrease of cell growth after exposure. Both of them shared a similar dose dependent inhibitory activity (Fig. 2) and the IC_{50} in MCF-7 cells at different exposure time were reported in Table 1. As shown in Fig. 3, Both of MTX and MTX-SLN showed obvious cytotoxicity to MCF-7 cells. In particular, MCF-7 cells were more sensitive to MTX-SLN than to free MTX, 50 % inhibition of cell growth being achieved with 93.8 nM of MTX-SLN at 72 h exposure, while the IC_{50} of free MTX was 153.16 nM (Table 1). No cytotoxicity of the unloaded SLN being observed on MCF-7 cell line at corresponding SLN concentration used, as cell viability remained constant.

2.3. Establishment of the MCF-7 breast cancer model

Human MCF-7 breast cancer cells were inoculated in 6∼8 weeks old BALB/c-nu nude mice under the breast. Two weeks after inoculation, the first measurement of the tumor volume was carried out. After four weeks, the mean tumor volume in mice was 386.0 ± 201.7 mm. However, there was considerable variation in the size of the MCF-7 tumor of mice, which was difficult to use in the later evaluation of tumor inhibition. Therefore, the method to form primary cancer was given up, and the procedures using cancer phyma cubes to form secondary breast cancer was adopted.

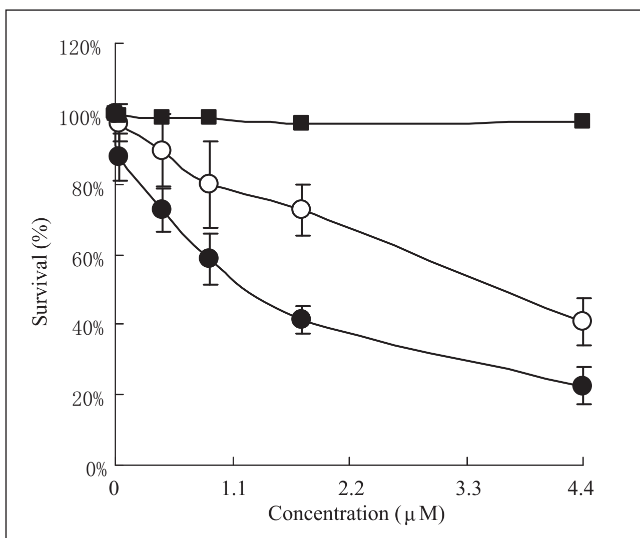

Fig. 1: Cytotoxicity of MTO(○), MTO-SLN (●) and unloaded SLN (■) in MCF-7 cells after 72 h exposure. The data are expressed as mean ± SD (n = 6)

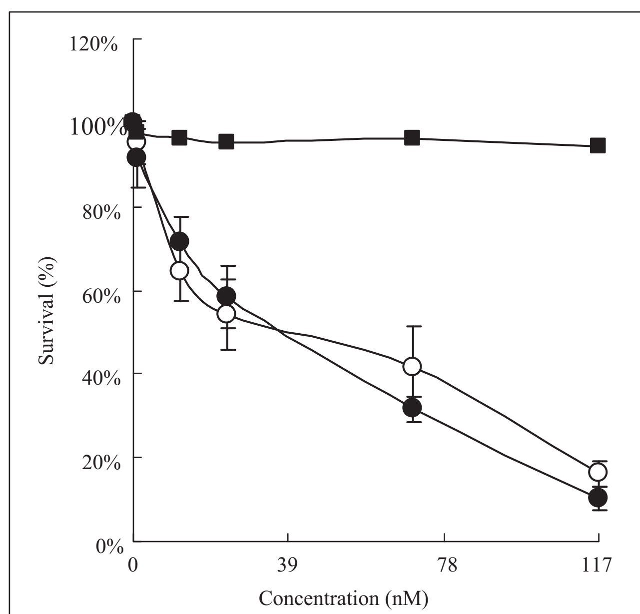

Fig. 2: Cytotoxicity of PCT(○), PCT-SLN (●) and unloaded SLN (■) in MCF-7 cells after 72 h exposure. The data are expressed as mean ± SD (n = 6)

2.4. Inhibition tests of SLN on human MCF-7 breast cancer

The tumor size and percent inhibition of the mice treated with SLN were compared in Table 2. Cytotoxicity assay indicated that the inhibition of SLN and free anticancer drugs to MCF-7 cells was dose-dependent. The excessive dose of anticancer drugs can cause the serious side-effect to kill the mice. In order to save the number of expensive nude mice, 0.05∼1.0 mg/kg dose level for

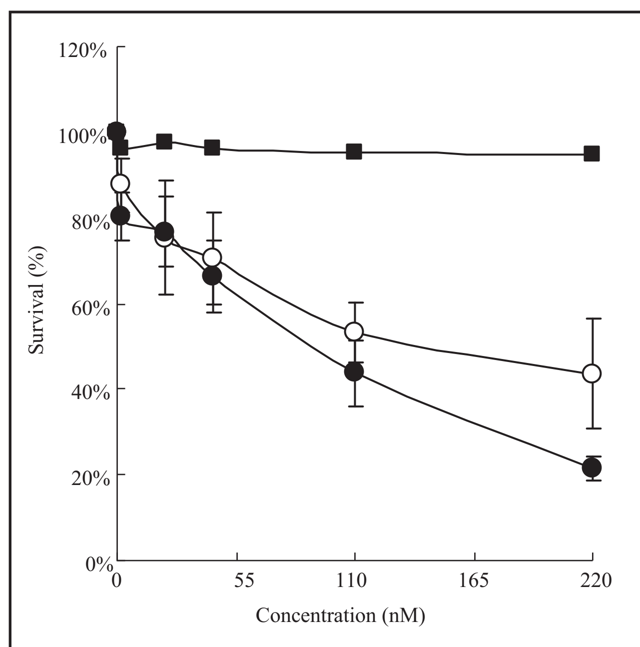

Fig. 3: Cytotoxicity of MTX(○), MTX-SLN (●) and unloaded SLN (■) in MCF-7 cells after 72 h exposure. The data are expressed as mean ± SD (n = 6)

Table 1: IC_{50} values evaluated after 24, 48 and 72 h exposure of cells to mitoxantrone (MTO and MTO-SLN), paclitaxel (PCT and PCT-SLN) and methotrexate (MTX and MTX-SLN). Data are expressed as mean ± SD (n = 6)

Samples	IC_{50}		
	24 h	48 h	72 h
MTO-SLN	2.58 ± 0.44 μM	1.64 ± 0.28 μM	1.25 ± 0.19 μM*
free MTO	5.13 ± 0.78 μM	3.51 ± 0.57 μM	2.13 ± 0.37 μM
PCT-SLN	98.70 ± 8.53nM	62.31 ± 6.12 nM	46.70 ± 5.47 nM
free PCT	92.64 ± 7.48 nM	67.34 ± 6.74 nM	52.48 ± 4.81 nM
MTX-SLN	154.76 ± 10.59 nM	98.48 ± 6.87 nM	93.80 ± 6.54 nM*
free MTX	267.84 ± 17.86 nM	195.16 ± 13.65 nM	153.16 ± 11.54 nM

* $P < 0.05$ (drug loaded SLN vs free drug within the group for 72 h)

SLN and the same dosage level for free anticancer drugs were chosen to perform the inhibition tests.
In mice treated with MTO-SLN injections (1.0 mg/kg) for a month, tumor was significantly inhibited. Mean tumor size of mice was 68.58 ± 34.27 mm^3 and the inhibition rate was 78.53 ± 16.65 %. In the MTO group, the effects of treatment with the same dose of free MTO were observed. Mean tumor size of mice treated with the same dose of free MTO was significantly larger than that after treatment with MTO-SLN ($P < 0.05$). Additionally, the inhibition rate was only 52.37±18.50 %, which was obviously lower than that of MTO-SLN ($P < 0.05$) (Table 2). The same trend could also be observed in PCT group and MTX group. A greater reduction in tumor growth occurred in the PCT group of animals treated with PCT-SLN. About 85 % inhibition rate indicated the highest antitumor effect in all experimental groups, whereas 62.84 ± 23.27 % inhibition effect was observed in mice treated with a commercial formulation of PCT. In the MTX group, MTX-SLN was effective against tumors of mice given inoculations of MCF-7 cell cubes. However, free MTX seems to be less effective against tumors (percent inhibition of 43.62 ± 25.77 %) of mice at the same dose level.

3. Discussion

In most cases, tumor chemotherapy is limited by the low therapeutic index of the anticancer drugs in clinical use. A significant pharmacokinetic problem is to determine the reason for low anticancer activity and/or severe side effects. Especially, the drugs are not specific against tumor cells, thus they accumulate not only in tumors but also in healthy tissues. It is clear that new approaches that can deliver drugs more specifically and produce less toxicity are required (Wong et al. 2007). Some studies have shown that nanoparticle-bound anti-tumor agents prolong drug retention in tumors, reduce tumor growth and increase survival of tumor-bearing animals (Lee et al. 2007; Lu et al. 2006; Kohler et al. 2005). In order to evaluate the activity of anticancer drug loaded SLN on human breast cancer cells proliferation, the inhibitory effects of SLN, including mitoxantrone SLN as well as of paclitaxel and methotrexate loaded SLN, on MCF-7 cell growth were determined.
Various PCT preparations have been developed as possible alternatives to Cremophor EL-based formulations or for new applications. In our research, due to the poor solubility of PCT, it was necessary to screen the excipient formula carefully in SLN preparation. According to Lee's method, trimyristin has the proper fatty acid chain length to be miscible with stabilizers and to solubilize PCT. Drug immobilization inside a solid lipid could increase the preparation stability. By means of this method, we have successfully prepared a PCT-SLN with the average diameter of 192 nm and a PDI of 0.245. The drug content of PCT-SLN was 3.11 ± 0.38 % with encapsulation efficiency of 71.8 ± 4.61 %.
In the study, we first showed that cytotoxicity of MTO-SLN and MTO to MCF-7 cell is time-dependent and dose-dependent. Moreover, MCF-7 cells were more sensitive to MTO-SLN than to MTO, indicating that SLN may be an alternative approach to delivering MTO to human breast cancer cells. Heart toxicity and myelosupression of MTO are the most serious side-effects and the local toxicity happens at a regular dose. In addition, local toxicity induced by subcutaneous injection of MTO is more serious than after i.v. injection or i.p. injection (Oussoren et al. 1998; Zingler et al. 2005). Taking this into account, it is necessary to decrease the dose of MTO to save the number of model mice. Having given the dose 1.0 mg/kg MTO to model mice, MTO-SLN showed better therapeutic effect than free MTO provided at the same dose level.
Improved efficacy was also achieved with MTX-SLN, the amount of MTX required to achieve 50% MCF-7 cell growth inhibition at 72 h exposure was significantly lower with MTX-SLN than with a commercial formulation. The results indicated that MTX-SLN increase the drug's cytotoxicity against the human breast carcer cell line. The results were consistent with recent research (Battaglia et al. 2011). They reported that methotrexate-loaded SLN showed an increased cytotoxicity towards MCF-7 and Mat B-III (human and murine breast tumor cell lines) cell lines compared with free drug. After intravenous administration of SLN to breast tumor model rats, a major drug

Table 2: Size and percent inhibition of the breast cancer in different groups. Data are expressed as mean ± SD (n = 6)

groups	subgroups	Dose (mg/kg)	Tumor size (mm^3)	Inhibition (%)
Blank group	blank	–	374.86 ± 136.84	–
MTO group	MTO-SLN	1.0	68.58 ± 34.27	78.53 ± 16.65*
	free MTO	1.0	116.69 ± 51.94	52.37 ± 18.50
PCT group	PCT-SLN	0.05	45.15 ± 33.42	86.40 ± 21.84*
	free PCT	0.05	89.61 ± 36.67	62.84 ± 23.27
MTX group	MTX-SLN	0.1	84.63 ± 38.35	66.80 ± 29.94*
	free MTX	0.1	146.29 ± 58.87	49.62 ± 25.77

* $P < 0.05$ (drug loaded SLN vs free drug within the group)

accumulation within neoplastic tissue can be observed. However, the authors did not supply any pharmacodynamic data *in vivo*. Therefore, pharmacodynamics *in vivo* were further studied. Pharmacodynamic results showed that MTX-SLN were more efficient than the free drug to treat tumor-bearing mice at the same dosage level. The possible reason may be related to the fast internalization of MTX-SLN followed by the drug's release from SLN inside the cells, enhancing their pharmacodynamic action. Taken together, these results suggested that MTX-SLN can be more effective than MTX for breast cancer treatment.

At each time of exposure the same dose-dependent inhibition of MCF-7 cell growth was observed with the PCT-SLN and free PCT (commercial formulation) (Table 1). The obtained data suggested that, in the case of the commercial formulation, a significant inhibitory effect is due to the diluent CrEL, whereas for PCT-SLN the cytotoxicity only is due to the amount of PCT released (since unloaded SLN showed no toxicity). Serpe et al. (2004) also observed a similar phenomenon on HT-29 colorectal cancer cell line after treatment with PCT-SLN and commercial PCT formulation. However, in our further study, PCT-SLN exhibited an obvious inhibition effect on tumor bearing mice, whereas a weak inhibition was observed in the free PCT group (Table 2). Hence, the results still indicated that PCT carried by SLN showed an enhanced pharmacodynamic action.

In this paper, we demonstrated that SLN formulations of mitoxantrone, methotrexate and paclitaxel have been shown to be superior to the corresponding free drug solutions in terms of anticancer activity and drug pharmacodynamics. The reasons can be summarized as follows: (i) SLN can lead to increased tumor drug concentrations via the EPR effect. In addition, it was reported that subcutaneous administration can lead to a multiplefold increase in the tumor drug concentrations 24 h post injection. The slower and progressive penetration of the SLN from the subcutaneous injection site into the tumor may result in more favorable patterns of drug distribution (Zara et al. 1999). (ii) SLN can carry drug into the cancer cells by endocytosis to gain this additional anticancer activity (Lo 2000; Tang et al. 2003). (iii) the terminal half-lives of the drugs were significantly increased when administered via SLN.

4. Experimental

4.1. Materials

Mitoxantrone was kindly granted from Sichuan Shenghe Pharmacy and methotrexate was purchased from Jiangsu Hengrui Pharmaceutical Co., Ltd. Paclitaxel was supplied by Jiangsu Hongdoushan Pharmaceutical Co., Ltd. MCF-7, human breast cancer cell line, was obtained from ATCC. MEM medium, fetal bovine serum (FCS) and antibiotics for cell culture were purchased from Sigma. The MTT assay kit was from Boehringer Mannheim. Other chemicals were of laboratory grade purity and used as obtained.

BALB/c-nu nude mice (6∼8 weeks old) were provided by the Experimental Animal Center of Chinese Academy of Science (Shanghai, China). All *in vivo* protocols were approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animals, Chinese Academy of Sciences.

4.2. Preparation of solid lipid nanoparticles

According to Lu's method (Lu et al. 2006) with some modification, mitoxantrone loaded SLN (MTO-SLN) were prepared. In brief, 0.1 g Compritol®888 and 0.3 g soy phosphatidylcholine were dissolved in 5 ml chloroform, and then the solution was evaporated to form a thin layer of uniform film at the bottom of the bottle. Residue of the organic solvent was expelled under vacuum. 10 ml aqueous solution containing 0.05 g Tween 80 and 0.02 g mitoxantrone was added and then the mixture further dispersed ultrasonically for 2 min.

Paclitaxel loaded SLN (PCT-SLN) were prepared by homogenization at elevated temperature. 1.0 g trimyristin, 0.3 g soy phosphatidylcholine, 0.03 g sodium taurocholate and 0.06 g PCT were dispensed into a 100 mL pear-shaped bottle. Then, the mixture was sonicated for 1 h at 70 °C in a bath type sonicator to dissolve the PCT. 10 mL preheated (70 °C) water was added into the bottle and the mixtures were sonicated until hot, milky and homogeneous crude emulsions were obtained. These emulsions were then further homogenized for 5 cycles at 60–70 °C and 100 MPa using a high pressure homogenizer. The resulting fine hot emulsion was immersed in a water bath at room temperature (Lee et al. 2007).

To obtain methotrexate loaded SLN (MTX-SLN), tripalmitin and soy phosphatidylcholine, also used as a lipid matrix, were melted at 70 °C. 0.1 g MTX was added to the melted mixture containing tripalmitin (1.0 g) and soy phosphatidylcholine (0.3 g) to form the dispersion. Then, the dispersion was added carefully drop wise into ice cold water (2–3 °C) containing 0.03 g sodium taurocholate with continuous stirring to form nanosuspension.

Unloaded SLN without any drug for each formulation were also prepared. All drug-loaded SLN and unloaded SLN were then sterilized by autoclaving (15 min at 121 °C). The SLN aqueous dispersions were stable for more than 12 months stored at 4 °C. The amount of drug incorporated was determined as follows: the dispersion was separated by a G-50 Sephadex column (1 cm i.d. × 20 cm) with a on-line ultraviolet equipment. The sample was used with purified water as the elution fluid at an elution rate of 0.5 mL/min. This portion containing nanoparticles was collected and extracted with ethanol. The amount of drug in the nanoparticles was thus determined by UV or HPLC method. Finally, the encapsulation yield and drug content were calculated according to the following equations:

$$\text{Encapsulation yield} = \frac{W_{\text{drug in SLN}}}{W_{\text{total drug}}} \times 100\% \quad (1)$$

$$\text{Drug content} = \frac{W_{\text{drug in SLN}}}{W_{\text{total}}} \times 100\% \quad (2)$$

W_{total}: the total amount of the drug and the excipients.

4.3. Measurement of particle size and zeta potential

The mean particle size and zeta potential of the SLN were determined using a Zetasizer nano (Malvern, UK). The size distribution of the SLN population was also measured. SLN water dispersions were diluted with ultrapure water before analysis. Each value reported is the average of six measurements.

4.4. Cell culture and cytotoxicity

MCF7 cells were grown as a monolayer culture in MEM medium supplemented with 10 % heat-inactivated FCS, 2 mM L-glutamine and penicillin/streptomycin (100 units/ml), at 37 °C in 5 % CO_2 humidified atmosphere, and passaged weekly. At the beginning of the experiments, cells in exponential growth phase were removed from the flasks with 0.05 % trypsin-0.02 % EDTA solution. Cells were seeded in 24 wells/plate (5×10^4 cells/well) in MEM medium with 10 % FCS. The cells were allowed to attach for 72 h, and seeding medium was removed and replaced by experimental medium.

Cells were maintained for 3 days in medium supplemented with increasing concentrations of mitoxantrone (free or loaded SLN), paclitaxel (free or loaded SLN) and methotrexate (free or loaded SLN). The concentrations of MOT-SLN and mitoxantrone varied from 0.044 to 4.4 μM and the concentrations of paclitaxel and methotrexate (free or loaded SLN) varied from 1.17 to 117 nM and from 2.20 to 220 nM, respectively.

4.5. Establishment of the MCF-7 breast cancer model

BALB/c-nu nude mice (6∼8 weeks old) were inoculated with human MCF-7 breast cancer cells under the breast at a dose of 0.2 mL of 1×10^7/mL per mouse. When the tumor grew to a certain volume, the mice were sacrificed by the removal of the eye and the tumor was taken and cut into cubes with 2 mm edge length. Then, the obtained the tumor cube was implanted into BALB/c-nu nude mice under the breast to form secondary breast cancer. Growth rates were determined by measuring the tumors with calipers every week. Tumor volumes were calculated according to Eq. (3) (Yue et al. 1994):

$$V_{\text{tumor}} = \frac{4}{3\pi {r_1}^2 r_2} \quad (3)$$

4.6. Inhibition tests of SLN on human MCF-7 breast cancer

A week later when the tumor grew normally, sixty mice were randomly divided into three groups: MTO group, PCT group and MTX group. The group was further classified into subgroups: drug loaded SLN and free drug. Each subgroup contained five mice.

The subgroups of MTO group received subcutaneous (s.c.) injection close to the tumor with a dose of 1.0 mg/kg MTO solution and MTO-SLN (containing

1.0 mg/kg MTO), respectively; the mice in PCT group were received with 0.05 mg/kg PCT solution and PCT-SLN (containing 0.05 mg/kg PCT) by s.c. injection, respectively; in the MTX group the mice received 0.1 mg/kg MTX solution and MTX-SLN (containing 0.1 mg/kg MTX), respectively. Another five model mice as the blank group were injected with physiological saline. The administration continued for successive seven times with an s.c. injection every 4 d. Four days after the last administration, the mice were sacrificed and dissected. The tumor was weighed and tumor volume was determined according to the equation mentioned above. Finally, the tumor inhibition rate was calculated based on the tumor weight of the drug group versus the weight of the blank group (Eq. 4).

$$\text{percent inhibition} = \left(1 - \frac{W_{\text{drug group}}}{W_{\text{blank group}}}\right) \times 100\% \quad (4)$$

$W_{\text{drug group}}$: the tumor weight of mice in the drug group;
$W_{\text{blank group}}$: the tumor weight of mice in the blank group

4.7. Statistical analyses

Data were expressed as means ± SD and were compared by one-way ANOVA analysis followed by Dunnett's multiple comparison tests. A p-value < 0.05 was considered statistically significant in all cases.

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