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Increase of therapeutic activity of doxorubicin by long circulating liposomes in combination with curcumin

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Received April 23, 2011, accepted May 16, 2011

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Pharmazie 66: 871–874 (2011) *doi: 10.1691/ph.2011.1057*

In this study, doxorubicin (DOX)-loaded long circulating liposomes combined with curcumin (CUR) (DOX-CUR-LCLs) were successfully prepared as a novel formulation for cancer treatment. The particle size and distribution, zeta potential, drug loading capacity, and entrapment efficiency (EE) of the preparation were characterized. The *in vitro* anti-tumor activities of DOX-CUR-LCLs and DOX-LCLs against A549 cells were then evaluated and compared with that of free DOX. Cytotoxicity evaluation showed that DOX-CUR-LCLs had a significantly higher antitumor activity than other DOX preparations. These results suggest that novel DOX-CUR-LCLs, combination of DOX and CUR administered in long-circulating liposomes, could improve antitumor activity.

1. Introduction

Doxorubicin hydrochloride (DOX) is a widely used anthraquinone anticancer agent the treatment of breast cancer. Howerer, long-term clinical use is limited due to side effects including cardiotoxicity which may result in congestive heart failure and myelosuppression (Dresdale et al. 1983; Speth et al. 1988; Steinherz et al. 1991; Sridhar et al. 1992) Aiming to reduce the toxicity and improve the target ability of DOX, various technologies have been investigated. One of them is to encapsulate the DOX into appropriate drug carriers such as liposomes (Torchilin et al. 2005). Liposomes can enhance the permeability of the tumor vascular system, leading to an accumulation in tumor tissue and a decrease of doxorubicin concentration in the heart. A study has revealed that commercially available doxorubicin-loaded long-circulating liposomes (Doxil R, Alza Pharmaceuticals), have better therapeutic efficacy and less cardiotoxicity than free DOX (Gabizon et al. 2003).

In recent years, several approaches have been made to further improve the anticancer efficiency of Dox using folate-targeted liposomes as carriers (Goren et al. 2000) or doxorubicin-loaded long circulating liposomes modified with a peptide, growth factor antagonist (Moreira et al. 2001) and anti-HER2 monoclonal antibody fragments (Park et al. 2001).

Curcumin, a hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa* has a wide spectrum of biological and pharmacological activities. It has been used for thousands of years in the Orient as a healing agent for a variety of illnesses. Curcumin has been shown to exhibit anticancer, anti-inflammatory, anti-oxidant, anti-arthritic, and anti-amyloid properties (Aggarwal et al. 2003, 2009). Recently, research has shown that curcumin is a potent anti-inflammatory agent with strong potential against a variety of cancers. Curcumin has been shown to suppress transformation, proliferation, and metastasis of tumors (Ruby et al. 2003, 2009).

Table: Physicochemical characterization of DOX-CUR-LCLs and DOX-LCLs (n = 3)

Formulation	Particle size (nm)	Zeta potential (mV)	Entrapment efficiency(%)
DOX-CLs	121.3 ± 4.5	-27.5 ± 0.9	92.5 ± 0.47
DOX-LCLs	117.4 ± 4.6	-24.2 ± 1.9	92.9 ± 0.58
DOX-CUR-LCLs-1	118.4 ± 5.1	-25.3 ± 2.6	92.8 ± 0.65
DOX-CUR-LCLs-2	117.8 ± 2.4	-24.9 ± 2.1	92.2 ± 0.55
DOX-CUR-LCLs-3	120.8 ± 5.1	-26.3 ± 0.6	92.9 ± 0.62
DOX-CUR-LCLs-5	121.7 ± 2.3	-25.5 ± 1.6	92.5 ± 0.44

DOX-CUR-LCLs-1,2,3,5 means CUR concentration in DOX-CUR-LCLs was 1, 2, 3 and 5 μg/mL respectively.

In this study, long-circulating liposomes loaded with DOX with different amounts of curcumin were prepared by the remote-loading method. The physicochemical characteristics, the cytotoxicity against cancer cells of liposomes were investigated. The aim of the present work was also to investigate the influence of the addition amount of curcumin on the cytotoxicity and antitumor efficacy of DOX liposomes.

2. Investigations, results and discussion

2.1. Characterization of DOX-CUR-LCLs and DOX-LCLs

The physicochemical properties of DOX-CUR-LCLs and DOX-LCLs are shown in the Table. TEM shows that the particles had round and uniform shapes. The mean diameters of DOX-CUR-LCLs were similar to that of DOX-LCLs. The EE and zeta potential did not show any significant difference for DOX-CUR-LCLs and DOX-LCLs, indicating that the physicochemical properties of the drug has not been significantly affected by incorporation of curcumin.

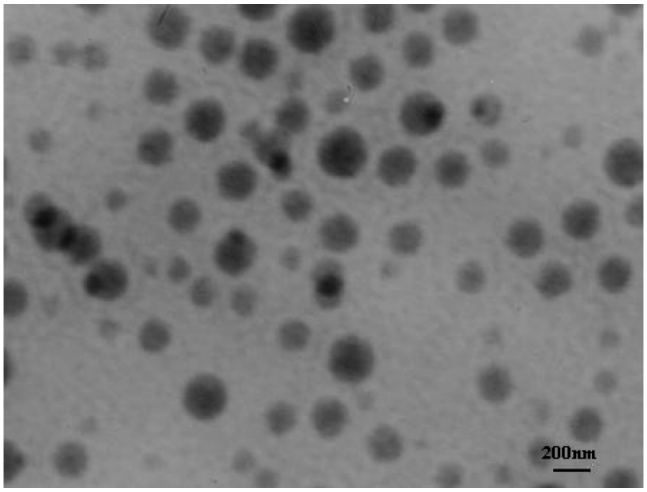


Fig. 1: Transmission electron microscope images of morphology of DOX-CUR-LCLs

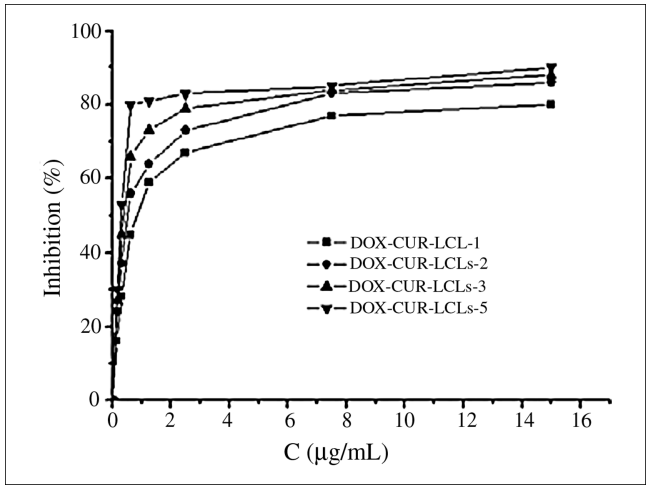


Fig. 3: The inhibitory rate (IR %) of DOX-CUR-LCLs with different CUR concentration to A549 cancer cell lines. DOX-CUR-LCLs-1,2,3,5 means CUR concentration in DOX-CUR-LCLs was 1, 2, 3 and 5 μg/mL respectively

2.2. In vitro cytotoxicity assay

In vitro anti-cancer effect of doxorubicin preparations was determined by a MTT cytotoxicity assay. Figs. 1 and 2 shows the cellular viabilities after the cells were incubated for 3 days with free DOX, DOX-LCLs, DOX-Ls and DOX-CUR-LCLs at different drug concentrations.

There was no significant difference between the anticancer effects of free DOX and DOX-LCLs at the concentrations tested. It was found that the inhibitory rate (IR) was increased when CUR was incorporated into DOX-LCLs, and the IR was further increased with the increase of CUR concentration in DOX-CUR-LCLs (at CUR concentrations of 1 to 5 μg/mL), these results suggested that addition of CUR increased the antitumor activity of DOX. The IR of DOX-LCLs was similar to that of DOX solution ($P < 0.05$).

In contrast, MTT assay results indicated that there was no apparent toxicity to A549 cells at the concentrations tested during the treatment with blank LCLs and Ls formulations (data not shown). In summary, the *in vitro* cytotoxicity assay demonstrated that curcumin could increase the cytotoxicity of DOX in A549 cells.

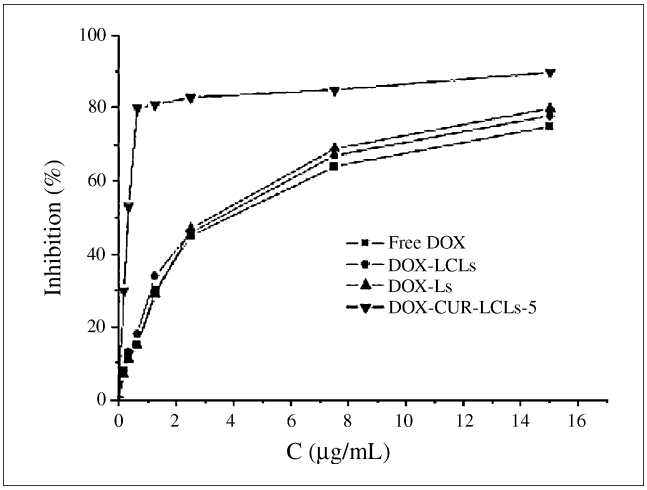


Fig. 2: The inhibitory rate (IR %) of DOX preparations to A549 cancer cell lines DOX-CUR-LCLs-5 (CUR concentrations was 5 μg/mL)

2.3. Intravenous safety assessment

2.3.1. Hemolysis test

In the study, complete hemolysis was observed in tube of the positive control at 15 min, and no erythrocyte survived at the bottom of the tube indicating complete hemolysis. While the erythrocyte precipitated at the bottom of the other six tubes (negative control and tested DOX-CUR-LCLs) during the 3 h observation. These experimental results demonstrated that both 0.9% saline injection and DOX-CUR-LCLs at different concentrations did not cause hemolysis or erythrocyte agglutination at 37 °C.

2.3.2. Irritation assessment

After a 3-day administration, there was no obvious visible damage such as erythema and edema at the injection site (Fig. 3a, b). The histopathologic examination of the rabbit ear border vein showed that there was no angiectasia, thrombus or vascular congestion in blood vessel at the site of injection. Furthermore, there were no pathological changes such as hemorrhage, edema, necrosis and inflammatory cell infiltrate in the vessel wall and surrounding tissues. The histopathologic examination results indicated that no intravenous irritation took place after i.v. administration of DOX-CUR-LCLs.

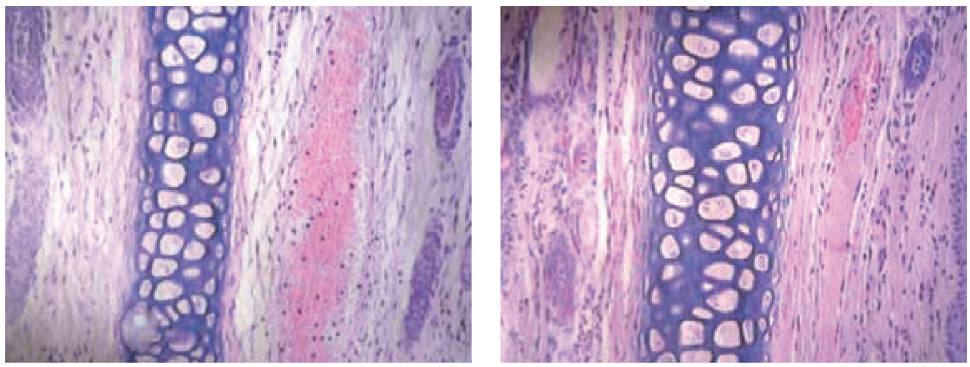
3. Experimental

3.1. Materials

Doxorubicin hydrochloride (DOX) was obtained from Zhejiang Haizheng Pharmaceutical Co. Ltd. (Zhejiang province, China). Polyethylene glycol derivative of distearoylphosphatidyl ethanolamine (PEG-DSPE) was supplied by NOF Co. Ltd. (Tokyo, Japan), Egg phosphatidylcholine (EPC) was provided by TaiWei Pharmaceutical Co. (Shanghai, China). RPMI 1640 medium was purchased from Solarbio Co. (Bejing, China). Human lung cancer cell line A549 cells was kindly supplied by the Department of Pharmacology, Hebei University. MTT was purchased from Amresco Co., Solon, USA. All other solvents were of analytical or chromatographic grade.

3.2. Preparation of liposomes

Liposomes were prepared according to the following procedures. Lipid mixture (Egg PC:Chol:PEG-DSPE = 65:30:5 molar ratio) was dried by rotary evaporator at 50 °C. 250 mM citrate buffer solution (pH 4.0) was added to the thin film of lipids and the mixture was hydrated at 50 °C. The obtained suspension was passed through a high pressure homogenizer for 6 homogenization cycles and then was extruded 3 times through polycarbonate membranes of 100 nm pore size. Then the pH of the liposome suspension was raised to pH 7.8 with NaOH solution. DOX was encapsulated into lipo-



(a) (b)

Fig. 4: The histopathologic examination pictures of the rabbit ear border vein after i.v. administration of DOX-CUR-LCLs (a) and 0.9% saline injection (b)

somes using the remote-loading method. DOX was dissolved at 7 mg/mL in PBS (pH 7.8), and then, immediately mixed with liposomal suspension and incubated at 35 °C for 30 min. DOX containing-liposomes were used within 1 week after preparation. There was no change of liposome size and no leakage of the encapsulated drug at 4 °C during storage.

Conventional liposomes were constructed by the same method described above with a 2:1 ratio of Egg PC to cholesterol. The DOX-to-Egg PC ratio of 1:10 (wt/wt) was used in this study for all investigated formulations. Control liposomes were prepared without doxorubicin using the same lipid components and in the same concentrations as in conventional liposomes.

3.3. Characterization of DOX-CUR-LCLs

The particle size and zeta potential of different DOX-CUR-LCLs and DOX-LCLs were measured by photon correlation spectroscopy (PCS) using a NICOMP particle sizing system (CW380, Santa Barbara, California, USA) at 25 °C. Zeta potential measurements was operated using the same instrument at electrical field strength of 10 v/cm and at the same temperature. All the measurements were performed in triplicate.

The morphologies of DOX-CUR-LCLs and DOX-LCLs were observed using a transmission electron microscopy (TEM, JEM-1200EX, JEOL, Tokyo, Japan). After dilution with distilled water, the samples were negatively stained with 2% (w/v) phosphotungstic acid for observation.

3.4. Drug encapsulation efficiency (EE) and drug loading (DL)

In order to determine the DL and EE, the resulting preparations were first eluted through a Sephadex G-50 column to remove free DOX. The drug concentrations in preparations before and after elution were determined by HPLC method using a Shimadzu instrument consisted of a LC-20A pump and SPD-20A UV/VIS detector (Shimadzu, Kyoto, Japan) and the chromatographic column was a Kromasil C-18 (5 μm, 250 × 4.6 mm). The mobile phase was mixture of 0.01 M sodium dodecyl sulfate (containing with 0.02 M phosphoric acid):acetonitrile:methanol (50:50:6, v/v). The flow rate was 1.0 ml/min; UV detection wavelength was 254 nm. Each sample was assayed in triplicate.

The equations for the drug EE and DL are as follows:

DD (%) = (amount of the drug in preparations/amount of the total material and drug) × 100%;

EE (%) = (amount of the drug in preparations/amount of the total drug) × 100%.

3.5. In vitro cytotoxicity analysis

The cytotoxic activity was assessed by the the methylthiazoletetrazolium (MTT) assay. Cytotoxicity of DOX-CUR-LCLs was compared to free DOX, DOX-LCLs, DOX-Ls, negative control, blank LCLs and Ls. The tumor cells (human lung cancer cell line A549) were plated at a density of 1×10^4 cells/well in 100 μl RPMI-1640 medium in 96-well plates and grown for 24 h. The cells were then exposed to different concentrations of DOX-loaded formulations for 2 days, and the viability of cells was measured using the *MTT* method. Briefly, 100 μl of *MTT* solution were added to each well. The plates were incubated for 4 h at 37 °C. After the incubation, 100 μl of DMSO was added to each well for 10 min. And the absorbance was measured at 570 nm using a microplate reader. The *in vitro* anticancer cytotoxic activity was expressed as inhibitory rate (IR %) by the equation below:

IR (%) = (1−Nt/Nc) × 100%

where Nt is the absorbance of the cells cultured with doxorubicin preparations, and Nc is the absorbance of the negative control.

3.6. Safety assessment for intravenous injection

3.6.1. Hemolysis test

In this study, rabbits' erythrocytes (2%, v/v) were dispersed in a saline injection, 2.5 ml of the above erythrocytes suspension was added to 0.1, 0.2, 0.3, 0.4, and 0.5 ml of DOX-CUR-LCLs. Then, 0.9% saline injection was added every tube to get a final volume of 5 mL. A solution containing 2.5 mL 2% erythrocyte dispersion and 2.5 mL 0.9% saline injection was used as negative control. The positive control was prepared by addition of 2.5 mL distilled water into 2.5 mL 2% erythrocyte. After vortexing, the tubes were incubated at 37 °C and observed microscopically from 15 min to 3 h.

3.6.2. Vascular irritation test

The irritation test was used to evaluate the level of irritation for DOX-CUR-LCLs after injection. Rabbits weighing 2.0–3.0 kg were used for this study. Each rabbit received DOX-CUR-LCLs via the auricular vein in the right ear and 0.9% saline injection in the left one for 3 days. After injection, visual observation of the appearance of the veins was recorded, and then the rabbits were sacrificed. 24 h after the last administration, the rabbits were sacrificed and the ears were cut and fixed in 10% formaldehyde. The histopathological changes of ear veins were observed.

3.7. Data analysis

Data were expressed as mean ± S.D. Statistical evaluation of data was performed using one-way analysis of variance (ANOVA). Statistical significance was determined using the Student's t-test.

Acknowledgements: This work was supported by the National High Technology Research and Development Program of China (863 Program), No. 2007 AA021810 and also by the Talent Introduction Program of Hebei University, No. y2005064.

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