European Journal of Pharmaceutics and Biopharmaceutics 112 (2017) 209–223

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Co-assembly of doxorubicin and curcumin targeted micelles for synergistic delivery and improving anti-tumor efficacy

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ARTICLE INFO

Article history:
Received 2 July 2016
Revised 7 November 2016
Accepted in revised form 22 November 2016
Available online 29 November 2016

Keywords:
Doxorubicin
Multifunctional micelles
Curcumin
Synergistic anti-tumor
Active targeting

ABSTRACT

Chemotherapeutic drugs have a series of limitations in anti-tumor treatment, mainly including multidrug resistance (MDR) and serious adverse reactions. Co-delivery system with two or more synergistic therapeutic drugs is an effective strategy to settle these limitations. In this study, active tumor-targeted co-delivery micelles (DOX + Cur)-PMs, with two synergistic drugs of a therapeutic drug of doxorubicin (DOX) and a chemosensitizer of curcumin (Cur) co-encapsulated into hyaluronic acid-vitamin E succinate (HA-VES) graft copolymer, were prepared and delivered simultaneously into tumor cells for improving therapeutic effects of DOX. (DOX + Cur)-PMs had uniform particle size, high encapsulation efficacy, sustained release profile and good colloidal stability. *In vitro* cytotoxicity study, (DOX + Cur)-PMs exerted the strongest cytotoxicity and highest cell apoptosis-inducing activities against DOX-resistant MCF-7/Adr cells. Moreover, (DOX + Cur)-PMs more efficiently internalized into cancer cells and enhanced the cellular uptake of DOX via energy-dependent and caveolae-mediated endocytosis, and significantly reversed MDR effects via CD44 targeting delivery and the synergic effect of released Cur. More importantly, *in vivo* results illustrated that (DOX + Cur)-PMs not only displayed better tumor accumulation and tumor targeting, and more efficiently inhibited the growth of tumor in 4T1 tumor-bearing mice, but also induced significantly less pathological damage to the cardiac tissue in comparison with free DOX, even DOX-PMs and DOX-PMs + Cur. In summary, this targeted combinational micellar delivery system with DOX and Cur could be a promising vehicle in tumor therapy.

1. Introduction

Chemotherapeutics plays an important role in clinical cancer treatment [1,2]. In recent years, multidrug resistance (MDR) and high toxicity has been recognized as a major obstacle of chemotherapeutics in anti-tumor treatment [3–5]. Doxorubicin (DOX), the most active single-agent drug, is widely used for the treatment of a variety of malignancies, such as breast, ovarian, prostate, brain, lung, and leukemia due to its broad spectrum of antitumor activity. However, the clinical use of DOX has been severely limited by its critical cardiotoxicity, narrow therapeutic window, and the development of MDR.

Nowadays, much effort has been focused on the co-delivery system with different drugs to simultaneously delivery drugs into tumor cells and achieve a synergistic therapeutic effect in cancer treatment. Curcumin (Cur), the polyphenol derived from the perennial herb *Curcuma longa*, exhibits various of pharmacological activities, including anti-oxidant, anti-toxic, anti-inflammatory, anti-microbial, and anticancer activities [6–11]. Additionally, Cur is also known to inhibit cancer cell proliferation by blocking human epidermal growth factor receptor-2 (HER2 activity, nuclear factor kappa B (NF-*k*B) activation and B-cell lymphoma-2 (bcl-2) and regulating of various cell signaling and genetic pathways, including MAPK and PI3K/PKB signaling pathways [12,13]. Furthermore, Cur is also an ideal chemosensitizer to inhibit P-glycoprotein (P-gp) over expression and reverse MDR in cancer cells [14,15]. Meanwhile, Cur has a potential of myocardial protective effect and no systemic side effects to human organs even at high doses [16,17]. Recently, several studies have demonstrated that Cur had synergistic effects on DOX [18–24]. However, Cur is limited by poor water solubility and low stability. Excitingly, co-delivery DOX and Cur into nano-drug delivery system is an efficient strategy to exert the synergistic effects.

Co-delivery system mainly including liposomes, nanoparticles, and polymeric micelles. However, self-assembled polymeric micelles have been investigated widely for their potential functions

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http://dx.doi.org/10.1016/j.ejpb.2016.11.033
0939-6411/

in cancer therapy. Polymeric micelles have a core-shell structure, where hydrophobic drugs can be solubilized and stabilized in the hydrophobic core of micelles, and hydrophilic shell can prolonged the circulation time and improve the steric stabilization by reducing opsonization in blood circulation [25,26]. Furthermore, polymeric micelles could selectively and effectively accumulate in tumor through enhanced permeability and retention (EPR) effect, thereby enhancing the therapeutic effects of chemotherapeutic drugs [26]. So co-delivery micellar system had attracted more attentions. Moreover, co-delivery system with chemotherapy and MDR sensitizer has been developed as an effective strategy to overcome MDR and reduce side effects of chemotherapeutics in cancer therapy.

More interestingly, in our previous study, we prepared PEGylated Cur and DOX passive targeted co-delivery micelles, which significantly improved the anticancer effects of DOX [18]. Nevertheless, passive targeting micelles had a low targeted efficiency. Moreover, active-targeted polymeric micelles could be internalized by specific receptor mediated endocytosis in tumor cells, achieve a higher chemotherapy efficacy and be conducive to reduce the side effects in normal tissues. However, active-targeted co-delivery micelles of DOX and Cur, has not been reported till now.

This study was to develop an active-targeted co-delivery system of DOX and Cur with hyaluronic acid-vitamin E succinate (HA-VES) graft polymer, which could selectively target onto tumors, bind to the HA receptor CD44, efficiently internalize into tumor cells, simultaneously delivered into tumor cells and exerted synergistic effects. Co-delivery micelles of DOX and Cur ((DOX + Cur)-PMs) were prepared and characterized. The cellular uptake, efflux, cytotoxicity, apoptosis, endocytosis mechanism, and the efficacy on reversal MDR of different formulations were evaluated in MCF-7 and MCF-7/Adr cells *in vitro*. Furthermore, pharmacokinetic, biodistribution, antitumor effects and systemic toxicity of different formulations were also carried out on rats and 4T1-tumor bearing mice, respectively.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng United Technology Co., Ltd. (Beijing, China). Curcumin (Cur) was obtained from Sigma Aldrich (St. Louis, MO, USA). Hyaluronic acid-vitamin E succinate was synthesized by our group. Vitamin E succinate was obtained from Wuhan Yuancheng Gongchuang Technology CO. Ltd (Hubei, China). Sodium hyaluronate (average molecular weight: 7 kDa) was purchased from Frida biological engineering Co. Ltd (Shandong, China). 1-ethy l-3-(3-dimethylaminopropyl) carbodiimide (EDCI), *N*-Hydroxysuccinimide (NHS) and 1-hydroxybenzotriazole (HoBt) were both provided by Zhejiang Pukang Pharm Co. Ltd (Zhejiang, China). Apoptosis Assay kit and Annexin V-FIT Kit was provided by Beyotime Institute of Biotechnology (Shanghai, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) of analytical reagent grade were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). CK, CKMB, LDH and AST were obtained from Beijing Leadman Biochemistry Co. Ltd (Beijing, China). RMPI 1640 medium and fetal bovine serum was provided by Gibco (BRL, MD, USA). All solvents used were of HPLC grade.

2.2. Preparation and characterization of (DOX+Cur)-PMs

(DOX + Cur)-PMs were prepared by probe ultrasonication method. Firstly, 5 mg DOX·HCl was reacted with two molar excess of triethylamine in methanol to obtain free doxorubicin base (DOX). Then, 20 mg HA-VES copolymer was dissolved in 10 mL distilled water and 1 mg DOX and 1 mg Cur were dissolved in 0.5 mL acetone. Then the mixture of drug solutions was slowly injected into HA-VES aqueous solution with stirring and stirred for another 24 h at room temperature. The mixture was ultrasonicated for 20 min under an ice bath at 200 W (active every 2 s with an interval of 3 s, Vernon Hills Co. Ltd, USA). The final micelles were centrifuged at 3500 rpm for 30 min and filtered through 0.45 μm microfiltration membrane to remove free drugs. The DOX-PMs and blank micelles were prepared in the same way with only DOX or no drugs. And the DOX-PMs + Cur were prepared via physical mixing DOX-PMs and Cur-Sol.

The size distribution and zeta potential of (DOX + Cur)-PMs were determined by dynamic light scattering (DLS) using a Zeta sizer Nano ZS90 (Malvern Instruments, Malvern, UK). All measurements were performed in triplicate. The morphology of (DOX + Cur)-PMs was observed by transmission electron microscopy (TEM, Tecnai 20,200 kV, FEI). Diluted micelles were placed on a copper grid, negative stained with 1% uranyl acetate and dried at room temperature before observation by TEM. The colloidal stability of (DOX + Cur)-PMs was evaluated by measuring the size and zeta potential after incubation with 1%, 5%, and 10% fetal bovine serum (FBS) at 37 °C for 24 h.

The amount of DOX or Cur in (DOX + Cur)-PMs was determined by an HPLC system (Agilent 1260) on Eclipse XDB-C_{18} column (4.6 × 250 mm, 5 μm, Agilent) with the mobile phase of methanol-3 mmol/L monopotassium phosphate-acetic acid (58:42:0.5, v/v/v) at an analysis wavelength of 258 nm. The micellar solution and freeze-dried micelles were diluted with methanol and sonicated for 10 min before analyzed by HPLC. The encapsulation efficiency and drug loading was calculated using the following equation:

$$\text{Encapsulation efficiency (EE)}\,\% = \frac{\text{the amount of loaded doxorubicin or curcumin}}{\text{total amount of doxorubicin or curcumin used for micelles}} \times 100$$

$$\text{Drug loading (DL)}\,\% = \frac{\text{the amount of loaded doxorubicin or curcumin}}{\text{total amount of freeze-dried micelles}} \times 100$$

2.3. In vitro release

In vitro release of DOX or Cur from (DOX + Cur)-PMs were carried out by a dialysis method. Specifically, a total of 3 mL of (DOX + Cur)-PMs were sealed in dialysis bags (MW 14,000), then the dialysis bags were immersed in 50 mL of phosphate buffer (pH 7.4, 6.5 and 5.5) or acetate buffer (pH 4.5) containing Tween 80 (0.5%, v/v) at 37 °C with gentle shaking. At predetermined time points, the released medium was withdrawn and replaced with equivalent volume of fresh medium. The amount of released DOX and Cur was analyzed by HPLC as described above. The *in vitro* release studies were carried out in triplicate.

2.4. Cell culture and animals

MCF-7/Adr (DOX-resistant) [27], MCF-7 (DOX-sensitive and relative high CD44 receptor expression) [28] and HepG2 (relative low CD44 receptor expression) [29] were obtained from Nanjing Kaiji Biotech. Ltd. Co. (Jiangsu, China). MCF-7 cells were cultured in RPMI-1640 medium, MCF-7/Adr cells were incubated in RPMI-1640 medium with the addition of DOX (1 μg/mL), and HepG2 cells were cultured in DMEM medium with 5% CO_2 at 37 °C. Drug resistance of MCF-7/Adr cells was maintained by addition of DOX (1 μg/