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Improving antiangiogenesis and anti-tumor activity of curcumin by biodegradable polymeric micelles

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ABSTRACT

For developing aqueous formulation and improving anti-tumor activity of curcumin (Cur), we prepared Cur encapsulated MPEG-PCL micelles by solid dispersion method without using any surfactants or toxic organic solvent. Cur micelles could be lyophilized into powder form without any cryoprotector or excipient, and the re-dissolved Cur micelles are homogenous and stable. Molecular modeling study suggested that Cur tended to interact with PCL serving as a core embraced by PEG as a shell. After Cur was encapsulated into polymeric micelles, cytotoxicity and cellular uptake were both increased. Cur micelles had a stronger inhibitory effect on proliferation, migration, invasion, and tube formation of HUVECs than free Cur. Besides, Cur micelles showed a sustained *in vitro* release behavior and slow extravasation from blood vessels in transgenic zebrafish model. Embryonic angiogenesis and tumor-induced angiogenesis were both dramatically inhibited by Cur micelles in transgenic zebrafish model. Furthermore, Cur micelles were more effective in inhibiting tumor growth and prolonged survival in both subcutaneous and pulmonary metastatic LL/2 tumor models. In pharmacokinetic and tissue distribution studies, Cur micelles showed higher concentration and longer retention time in plasma and tumors. Our findings suggested that Cur micelles may have promising applications in pulmonary carcinoma therapy.

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1. Introduction

Cancer is one of the human severe diseases and causes increasing morbidity and mortality every year in the world. As a leading cancer type, pulmonary carcinoma is account for 14% of new cancer cases and is associated with more than 25% of the total cancer-induced deaths in United States [1]. Tumor growth and systemic metastasis are highly dependent on angiogenesis [2]. Angiogenesis promotes tumor growth by supplying nutrients and oxygen and removing waste products, meanwhile, facilitating tumor invasion and metastasis [3]. Tumor less than 1 mm^3 could receive necessary nutrients and oxygen by diffusion, however, tumor is unable to grow above 1 mm^3 without neovascularization [4]. Angiogenesis is essential for tumor growth and metastasis, therefore antiangiogenesis has been proposed as a promising therapeutic strategy for clinical therapy of tumors [5]. Antiangiogenic therapy aims to prevent the formation of new vessels around tumors and to frustrate the existing abnormal capillary network that supports the tumor.

Medicines derived from natural plants have played a critical role in the health care of many cultures throughout the human history [6]. Curcumin (Cur), known as diferuloylmethane or 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a natural low molecular weight hydrophobic polyphenolic phytoconstituent. Cur is the active principle of the perennial herb *Curcuma longa* L.(known as turmeric), which was first isolated in 1815 and then synthesized by Lampe in 1910 [7]. Cur has been used as traditional medicine in Southeast Asia countries, which showed multiple pharmacological activities, including anti-inflammatory, anti-oxidant, anti-bacteria, anti-virus, and hyperlipidemic activities [8]. Previous contributions have reported that Cur showed inhibitory effect on a variety of cancers by antiangiogenesis and induction of tumor cell apoptosis, including lung, breast, colorectal, pancreatic, and prostate carcinoma [9–12]. Besides, Cur was proved to be a safe agent for *in vivo* application, since no signs of toxicity were found in animals or humans treated with Cur [13]. However, the clinical application of Cur is restricted due to its poor solubility in aqueous

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solution [14], poor oral bioavailability, and extensive fist pass metabolism [15]. Thus, an aqueous formulation with controlled release property is desired for clinical application of Cur [16].

Nanotechnology is a fast developing field and attracts increasing attention in drug delivery and cancer therapy, which provides an important route to develop aqueous formulations of hydrophobic drugs [17–21]. Micelles prepared from synthetic biodegradable polymers are widely applied as drug delivery system (DDS) due to their core–shell geometry [22,23]. Polymeric micelles are nanoscale assemblies of amphiphilic polymers, in which hydrophobic domain packed together in aggregates (core) serves as a potent nanocontainer of hydrophobic drugs and hydrophilic segments (shell) serves as a stabilizing interface of the particles [19]. Encapsulation of hydrophobic compounds in to polymeric micelles can render the compound dispersible in aqueous solutions to form a stable and homogenous solution for intravenous applications [24]. Besides, nanoscale of micelles and presenting of hydrophilic stabilizing interface can prolong their circulation time *in vivo* and enhance the cellular uptake. More importantly, polymeric micelles can passively target to tumors by the by the enhanced permeability and retention (EPR) effect, therefore improving their antitumor effects [25].

In this work, Cur loaded polymeric micelles (Cur micelles) were prepared and characterized, and antiangiogenesis and anti-tumor activity of Cur micelles were investigated *in vitro* and *in vivo*. Molecular modeling study was employed to investigate the structure and interactions of Cur micelles. Cytotoxicity, apoptosis, cellular uptake, and *in vitro* antiangiogenesis activity of Cur micelles were studied in detail. In addition, i*n vitro* drug release behavior was tested, and *in vivo* drug extravasation study was performed in transgenic zebrafish model. Inhibitory effect of Cur micelles on embryonic angiogenesis and tumor-induced angiogenesis were also evaluated in transgenic zebrafish model. Subsequently, pulmonary metastatic and subcutaneous LL/2 mouse models were established and used to evaluated anti-tumor activity of Cur micelles. Besides, pharmacokinetic and tissue distribution studies of Cur micelles were conducted. Our findings indicated that Cur micelles showed improved antiangiogenesis and anti-tumor activity both *in vitro* and *in vivo*, and may have potential applications in pulmonary carcinoma therapy.

2. Materials and methods

2.1. Materials, cell lines, and animals

Poly(ethylene glycol) methyl ether (MPEG, Mn = 2000, Fluka, USA), ε-caprolactone (ε-CL, Alfa Aesar, USA), stannous octoate ($Sn(Oct)_2$, Sigma, USA), curcumin (Sigma, USA), 4′, 6-diamidino-2-phenylindole 2hci (DAPI, Sigma, USA), alginate sodium (Sigma, USA), Matrigel (BD Biosciences, USA), methanol (HPLC grade, Fisher Scientific, UK), 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (methyl thiazolyl tetrazolium, MTT, Sigma, USA), fluorescein isothiocyanate-dextran (FITC-dextran, Sigma, USA), CM –Dil (Invitrogen, USA), propidium iodide (PI, Sigma, USA), tricaine (Sigma, USA), and 1-phenyl-2-thiourea (PTU, Sigma, USA) were used without further purification. All the materials used in this article were analytic reagent (AR) grade and used as received.

L929 cells, LL/2 cells, and B16-F10 cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD). LL/2 cells and B16-F10 cells grew in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplement with 10% fetal bovine serum (FBS, Gibco, USA), and L929 cells grew in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Gibco, USA) supplement with 10% FBS. Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by a standard procedure [26], and grew in EBM-2 medium with Single Quots (Lonza, USA) containing VEGF and other growth factors. HUVECs at passages 2 to 4 were used for all experiments. All above cells were maintained at 37 °C in a humidified incubator containing 5% CO_2.

C57BL/6 mice (18 ± 2 g) were used for *in vivo* anti-tumor tests and pharmacokinetic studies. The animals were purchased from the Laboratory Animal Center of Sichuan University, which were sex-separately housed at controlled temperature of 20–22 °C, relative humidity of 50–60% and 12 h light–dark cycles. Animals were provided with standard laboratory chow and tap water *ad libitum*. All the animals would be in quarantine for a week before treatment. All animal procedures were performed following the protocol approved by the Institutional Animal Care and Treatment Committee of Sichuan University (Chengdu, P.R. China). All mice were treated humanely throughout the experimental period.

2.2. Synthesis of the copolymer

Monomethyl poly(ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) copolymer was synthesized by ring-opening polymerization of ε-CL on MPEG using $Sn(Oct)_2$ as catalyst, which was reported in our previous contributions [22,27,28]. Briefly, MPEG and ε-CL were introduced into a dry glass ampoule under nitrogen atmosphere, and $Sn(Oct)_2$ was added into reaction vessel under mild agitation. The reaction system was kept at 130 °C for 6 h. The purified MPEG-PCL copolymer was kept in desiccators before further use. The obtained MPEG-PCL copolymer was characterized by Fourier transform infrared spectroscopy (FTIR, NICOLET 200SXV, Nicolet, USA), ^1H nuclear magnetic resonance spectroscopy (^1H-NMR, Varian 400 spectrometer, Varian, USA), and gel permeation chromatography (GPC, Agilent 110 HPLC, USA). Molecular weight of synthesized MPEG-PCL copolymer was 3800 (2000-1800), calculated by ^1H-NMR.

2.3. Preparation and characterization of Cur micelles

Cur micelles were prepared by a one-step solid dispersion method. Briefly, 100 mg of Cur and MPEG-PCL copolymer with different ratios were co-dissolved in 5 mL of dehydrated alcohol under mild stirring. Then, the solution was evaporated in rotary evaporator at 60 °C. During this process, homogenous coevaporation was obtained, and Cur was distributed in MPEG-PCL copolymer as amorphous substance. Subsequently, the coevaporation was dissolved in water at 60 °C to self-assemble into micelles with Cur encapsulated in. The Cur micelle solution was filtered through a 0.22 μm syringe filter (Millex-LG, Millipore Co., USA), and was lyophilized and stored at 4 °C before use.

The particle size distribution, polydisperse index (PDI), and zeta potential of prepared blank micelles and Cur micelles were determined by Malvern Nano-ZS 90 laser particle size analyzer at 25 °C. All results were the mean of three test runs, and all data were expressed as the mean ± standard deviation (SD).

The morphological characteristics of Cur micelles were examined by transmission electron microscope (TEM, H-6009IV, Hitachi, Japan). Cur micelles were diluted with distilled water and placed on a copper grid covered with nitrocellulose. Samples was negatively stained with phosphotungstic acid and dried at room temperature.

The concentration of Cur was determined by high performance liquid chromatography (HPLC, Waters Alliance 2695) instrument and sample was diluted before measurement. Solvent delivery system equipped with a column heater and a plus autosampler. Detection was taken on a Waters 2996 detector. Chromatographic separations were performed on a reversed phase C_{18} column (4.6 × 150 mm–5μm, Sunfire Analysis column). The column temperature was kept at 28 °C. Methanol/0.3% acetic acid (80/20, v/v) was used as eluant at a flow rate of 1 mL/min. The standard curve equation is: $H = 37138^*X\text{-}4383.7$ (H: The area of peak; X: the concentration of Cur) and the correlation coefficient is 0.9999.

Drug loading (DL) and encapsulation efficiency (EE) of Cur micelles were determined as follows. Briefly, 10 mg of lyophilized Cur micelles were dissolved in 0.1 mL of methanol. The amount of Cur in the solution was determined by HPLC. The DL and EE of Cur micelles were calculated according to Eqs. (1) and (2):

$$\mathrm{DL} = \frac{\mathrm{Drug}}{\mathrm{Polymer} + \mathrm{Drug}} \times 100\% \quad (1)$$

$$\mathrm{EE} = \frac{\mathrm{Experimental\ drug\ loading}}{\mathrm{Theoretical\ drug\ loading}} \times 100\% \quad (2)$$

Cur micelles were kept at 4 °C after preparation, and the stability of Cur micelles was evaluated qualitatively by the observation of aggregates. A homogeneously transparent solution implies stable of the Cur micelles, whereas the presence of precipitation indicates instable.

2.4. Molecular modeling study

Generally, the three-dimensional (3D) structures of macromolecules such as proteins could be determined experimentally by employing techniques such as X-ray crystallography and NMR spectroscopy. Unlike protein, diblock copolymer MPEG-PCL exhibits atactic structure. It is uneasy to determine the structure of MPEG-PCL copolymer at the level of atom experimentally. Therefore, we tried to get the 3D structure of the copolymer theoretically by simulating it with approaches of molecular mechanics (MM) and molecular dynamics (MD). At first, the initial structure of MPEG-PCL was built by using HyperChem software (HyperChem Professional 80, Hypercube, USA). Then the built structure experienced a series of geometrical optimization at MM level with OPLS method [29] using steepest descent algorithm and Flether-Rooves algorithm [30]. It was optimized until the root mean square gradient is less than 0.10 kcal/(mol·Angstrom). After that, a series of MD

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