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Research paper

Doxorubicin and curcumin co-delivery by lipid nanoparticles for enhanced treatment of diethylnitrosamine-induced hepatocellular carcinoma in mice

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ABSTRACT

At present, the treatment of hepatocellular carcinoma (HCC) is an international problem. The delivery of a chemotherapeutic agent and chemosensitizer using nanocarriers has been suggested as a novel and promising strategy in cancer treatment. However, such studies in HCC remain very limited. In this study, we developed doxorubicin (DOX) and curcumin (Cur) co-delivery lipid nanoparticles (DOX/Cur-NPs) and examined its inhibitory effect on diethylnitrosamine (DEN)-induced HCC in mice. DOX/Cur-NPs displayed the physicochemical characterizations with uniform particle size, high encapsulation efficacy and sustained release profile. In DNE-induced HCC mice treated with DOX/Cur-NPs, we observed decreased liver damage assessed by serum ALT and AST levels, liver/body weight ratio, and histopathological analysis. Compared with DOX-loaded nanoparticles (DOX-NPs), DOX/Cur-NPs induced increased Caspase-3 and Bax/Bcl-2 ratio, and decreased C-myc, PCNA and VEGF. The results revealed the synergistic effect of DOX/Cur-NPs on the apoptosis, proliferation and angiogenesis of HCC. The mRNA levels of *MDR1*, *bcl-2* and *HIF-1α*, and protein levels of P-gp, Bcl-2 and HIF-1α were decreased in DOX/Cur-NPs than those in DOX-NPs, indicating that Cur might reverse multidrug resistance (MDR) through these pathways. In HCC cells, enhanced cytotoxicity and decreased IC_{50} and resistant index further confirmed the synergistic effects of DOX/Cur-NPs than DOX-NPs. Our studies suggest that simultaneous delivery of DOX and Cur by DOX/Cur-NPs may be a promising treatment for HCC.

1. Introduction

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is a malignant tumor treated with difficulty. Since most patients are diagnosed at advanced stages, there is an urgent need for effective nonsurgical therapies, such as systemic chemotherapy [1]. Currently, traditional chemotherapeutic agents, such as sorafenib, doxorubicin (DOX), 5-fluorouracil and cisplatin, play a very limited role in the management of HCC. The development of multidrug resistance (MDR) to chemotherapy is a major obstacle to the effective treatment of human malignancies, including HCC [2].

Several strategies have been applied to improve the efficacy of chemotherapeutic agents, such as chemical modification, development of new drugs not recognized by MDR efflux pumps, combination of cytotoxic agent with chemosensitizer, and application of nanoparticle-based targeted drug delivery [3,4]. Some chemosensitizers have been demonstrated to decrease MDR by inactivation of MDR-related mRNAs via silence or breakdown, or by inhibition of MDR efflux transporters. Drug resistance at the tumor level is a complicated process involving multiple and dynamically acquired MDR mechanisms, which result in the expression of drug efflux pumps (e.g. P-glycoprotein (P-gp)) [5],

Abbreviations: HCC, hepatocellular carcinoma; MDR, multidrug resistance; DOX, doxorubicin; Cur, curcumin; DOX/Cur-NPs, doxorubicin and curcumin co-delivery lipid nanoparticles; DEN, diethylnitrosamine; ALT, alanine aminotransferase; AST, aspartate transaminase; P-gp, P-glycoprotein; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; RI, resistant index.

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antiapoptotic proteins (e.g. Bcl-2) [6], oncogenes (e.g. C-myc) [7], and hypoxia-inducible factor (e.g. HIF-1α) [8]. Therefore, combination of chemotherapeutic agents with chemosensitizers has been shown to modulate multiple signaling pathways in cancer cells, which is helpful to overcome MDR, maximize the therapeutic effect, and reduce side effects [9].

Despite the benefits, drug resistance, altered biodistribution, biotransformation, and drug clearance still remain as common problems in combination therapy. The co-delivery of chemotherapeutic agents and chemosensitizers by nanoparticle-based targeted drug delivery, has been designed to overcome MDR and increase drug accumulation at tumor sites to improve permeability and retention [10]. Therefore, delivery of chemotherapeutic agents and chemosensitizers using nanocarriers has been suggested as a novel promising strategy in cancer treatment [11].

Curcumin (Cur), the dietary polyphenol constituent from *Curcuma longa*, exhibits antioxidant, anti-inflammatory, anti-angiogenic, antimicrobial and anti-cancer activities [12]. It shows potential in terms of the prevention and treatment of cancer. For example, Chuang et al. observed chemopreventive effect in diethylnitrosamine (DEN)-induced HCC in mice treated with Cur [13]. Cur acts as a chemosensitizer to suppress the overexpression of P-gp, Bcl-2, HIF-1α to reverse MDR in various cancer cells and xnografts of hepatoma [14,15]. Recently, in human HCC cells, Cur has been reported to inhibit cell proliferation and induce apoptosis in a dose-dependent manner [16]. Therefore, Cur has been selected to deliver simultaneously with several chemotherapeutic agents, such as DOX, paclitaxel and 5-fluorouracil for enhanced treatment of cancers. However, the extremely low water solubility and poor bioavailability have impeded its clinical use [17].

DOX, an anthracycline antibiotic, is one of the most efficacious drugs in the treatment of HCC. However, clinical application of DOX has been severely hindered because of its narrow therapeutic window, and the development of MDR [18]. In several cancer cell studies, reversed MDR and increased apoptosis have been reported with the combination therapy of DOX and Cur loaded in nanocarriers, including liposome [19], PLGA nanoparticles [20], MPEG-PCL micelles [21], and chitosan/poly(butyl cyanoacrylate) nanoparticles [22].

To date, several cell and animal studies have demonstrated the enhanced anti-cancer efficacy by co-delivery of DOX and Cur in lung cancer, breast cancer and human chronic myelogenous leukemia [20–22]. However, study in HCC remains very limited. Qian et al. reported that Cur enhanced DOX-induced cell death in HepG2 human liver-derived hepatoma cells [23]. Similarly, in our previous work, we observed enhanced growth inhibition in HepG2 cells treated with DOX and Cur co-delivery lipid nanoparticles (DOX/Cur-NPs) [24]. However, the anti-cancer effect of co-delivery nanoparticles in HCC animal model and the underlying mechanisms of the enhanced efficacy have been poorly reported.

In the present work, we developed DOX/Cur-NPs and examined their efficacy in DNE-induced HCC in mice model assessed by tumor growth, hepatotoxicity markers and histopathological examination. We further investigated the underlying mechanisms on how Cur can efficiently enhance anti-cancer efficacy and reverse the MDR in mice and HCC cell lines.

2. Materials and methods

2.1. Chemicals and regents

Doxorubicin hydrochloride was purchased from Beijing Huafeng United Technology Co., Ltd. (Beijing, China). Cur was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Glyceryl distearate (Precirol ATO 5) and triglycerides medium-chain (Labrafac Lipophile WL 1349) were kindly provided by Gattefossé (Genas, France). Polyoxyl 40 Hydrogenated Castor Oil (Cremophor RH 40) was obtained from BASF, Germany. Soybean lecithin (Lipoid S75) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Glycerin (injection grade) was obtained from Jiangxi Ipsen Pharmaceutical Co., Ltd. (Jiangxi, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) of analytical reagents grade and DEN were obtained from Sigma–Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was bought from Millipore (Burlington, MA, US). Primary antibodies used in this study included antibodies against P-gp, HIF-1α (Abcam Inc., MA, USA), Bcl-2, C-myc, PCNA, GAPDH (Santa Cruz Biotechnology, CA, USA), Bax, VEGF and Caspase-3 (Boster, Wuhan, China).

2.2. Ethics statement

Male Kunming mice (18–22 g) were purchased from the Laboratory Animals Center at Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All mice were fed in College of Pharmacy of South-central University for Nationalities (Wuhan, China) with free access to food and water. Animal experiments were performed in strict accordance with the recommendations in the Guide for Animal Experimentation of the South-Central University for Nationalities and the Committee of Research Facilities for Laboratory Animal Sciences, South-Central University for Nationalities, China. The protocols were approved by the Committee on the Ethics of Animal Experiments of the South-Central University for Nationalities, China (Permit Number: 2012-SCUEC-AEC-005).

2.3. Preparation of drug loaded nanoparticles

Drug-free, Cur-loaded (Cur-NPs), DOX-loaded (DOX-NPs), DOX and Cur-loaded nanoparticles (weight ratio of DOX and Cur was 1:1) were prepared using high pressure microfluidics technique. Briefly, drugs were dissolved in the melted oil phase (3% Precirol ATO 5, 1% Labrafac Lipophile WL 1349, 1% Lipoid S75) heated to 75 °C. Then oil phase was mixed with water phase (2.5% Cremophor RH 40, 2.25% glycerin) under high speed shearing at 10,000 rpm for 30 s. The formed coarse oil-in-water emulsion was further homogenized in a six cycles homogenizing regime at 1000 bar. Finally, the hot dispersion was cooled down at 4 °C and sterilized with a 0.45 μm cellulose acetate filter.

2.4. Characterization of DOX/Cur-NPs

The particle size and zeta potential of DOX/Cur-NPs were measured using a Zetasizer (Nano ZS 90, Malvern, U.K.). The morphology of DOX/Cur-NPs was observed by a 3H-7000FA transmission electron microscope (TEM, Hitachi, Japan). Diluted NPs were placed on a carbon-coated copper grid, negatively stained with 2% phosphotungstic acid, and then observed with TEM.

The encapsulation efficacy (EE) of DOX and Cur was determined using ultra filtration method described previously [25]. The amount of DOX was determined with F-4500 fluorescence spectrophotometer (emission wavelength: 480 nm, excitation wavelength: 556 nm, Hitachi, Japan). The amount of Cur was measured with a HPLC method at 420 nm using Agilent 1260 Infinity LC (Agilent Technologies, USA). HPLC analyses were performed on a Hypersil ODS2 C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase used was acetonitrile: 4% (V/V) glacial acetic acid in water (45:55). The run temperature was 30 °C.

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