Dual Role of Photosensitizer and Carrier Material of Fullerene in Micelles for Chemo–Photodynamic Therapy of Cancer

XINHONG GUO, RUI DING, YING ZHANG, LING YE, XIN LIU, CAIPING CHEN, ZHENZHONG ZHANG, YUN ZHANG

School of Pharmaceutical Sciences, Zhengzhou University, Zhengzhou 450001, People's Republic of China

Received 23 April 2014; revised 6 July 2014; accepted 23 July 2014

Published online 29 August 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24124

ABSTRACT: Derivatives of fullerene (C60) as photosensitizers have rarely been studied as delivery carrier materials. The focus of this study was to explore the potential advantages of diadduct malonic acid-fullerene (DMA-C60) as delivery carrier materials and combination of chemo–phototherapy of some tumors. In this study, DMA-C60 and docetaxel (DTX) were coentrapped in micelles (MCs) (DMA-C60/DTX-MC). The addition of DMA-C60 could obviously improve static stability and decrease critical MC concentration of DTX-MC without hemolysis. The sustained release of DTX and DMA-C60 could be achieved, following Higuichi and first-order model, respectively. DMA-C60 could still produce reactive oxygen species efficiently in HeLa cells after encapsulation in MC. The addition of DMA-C60 under irradiation caused DTX-MC more stronger cytotoxicity, cell cycle changes, and more early apoptotic cells *in vitro*. More importantly, after intravenous injection, the addition of DMA-C60 in DTX-MC could result in 2.25-fold and 4.57-fold longer mean residence time compared with DTX-MC and Duopafei®, increase drug intratumoral distribution and decrease drug distribution in heart and kidney, and enhance antitumor effect under irradiation without body weight loss. These results suggested tremendous promise of DMA-C60 as carrier materials of MC and significant advantages in combination of chemo–phototherapy of some tumors. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3225–3234, 2014

Keywords: diadduct malonic acid-fullerene; micelles; photosensitive; excipients; cellular mechanisms; pharmacokinetics; cancer chemotherapy; controlled release/delivery; antitumor effect

INTRODUCTION

Many water-soluble derivatives of fullerene (C60) have showed many potential biological activities[1–5] such as enzymatic inhibition, antihuman immunodeficiency virus activity, antibacterial activity, antitumor activity, DNA cleavage, photodynamic therapy (PDT) and acting as drug carriers for selective tissue targeting because of their unique electrochemical, optical, and physical properties.[6–12] Malonic acid derivatives of C60 (DMA-C60) were found to make cells death upon exposure to ultraviolet (UV) or plain visible light by generating reactive oxygen species (ROS) through PDT.[13] However, few studies on fullerenes derivatives such as DMA-C60 as a delivery carrier material and their potential advantage have been carried out so far.

Depending on different mechanisms, cytotoxic drugs can combine with photosensitizer (PS) for tumor killing, achieving potentiated therapeutic outcome through an anticancer synergistic effect.[14] This approach is demonstrated to be a promising strategy to overcome tumor drug resistance in a mouse tumor model.[15] Moreover, the layout of two drugs in copolymer micelles (MCs) being able to release slowly their drug cargo allows chronic administration of chemotherapeutics and PS at relatively low and minimally toxic doses, and showing with no prolonged drug-free breaks. The potential of this method has been recently improved on the basis of clinical tests[16] and considered a quite promising novel strategy also in cancer PDT.[17] Docetaxel (DTX) is widely used in the treatment of many cancers such as cervical cancer.[18] However, the poor aqueous solubility, low bioavailability, and high toxicity caused by DTX have considerably overshadowed its clinical use. Duopafei (Jinan, People's Republic of China), a listed preparation of DTX for intravenous injection for clinical treatment of tumor in People's Republic of China, could result in serious adverse reactions such as hemolysis because of it containing a high concentration of Tween 80.[19] Furthermore, the multidrug resistance of DTX has already been a major clinical problem, which limits its effectiveness and other anticancer agents.[20] Thus, design of new targeting delivery carrier combining multiple mechanisms of tumor therapy is one of the most promising strategies to improve the treatment of cancer.

The MCs have been widely studied for their prominent superiorities such as drug solubilization, controlled drug release, escaping from reticuloendothelial system uptake, and tumor targeting by enhanced permeability and retention effect.[21,22] In this study, MCs comprising methoxy poly (ethylene glycol)–poly (lactide) polymer (mPEG–PLA) were designed and prepared for codelivery of DTX and DMA-C60 for combination of chemo–phototherapy of some tumors. *In vitro* and *in vivo* characteristics of DMA-C60/DTX-MC including static stability, hemolytic properties, critical MC concentration (CMC), *in vitro* release, cytotoxicity and cellular mechanisms, pharmacokinetics, tissue distribution, and antitumor efficacy were investigated. The focus of this study was to explore the potential advantage of the combination of chemo–phototherapy of some tumors and DMA-C60 as a delivery carrier material.

MATERIALS AND METHODS

Materials

Docetaxel (DTX, purity >99%) was purchased from Beijing Yi-He Biotech Company Ltd (Beijing, China). Fullerence (C60,

Correspondence to: Zhenzhong Zhang (Telephone: +86-371-67781910; Fax: +86-371-67781908; E-mail: 15237155029@126.com); Yun Zhang (Telephone: +86-371-67781910; Fax: +86-371-67781908; E-mail: Zhang_yun@ymail.com)

Journal of Pharmaceutical Sciences, Vol. 103, 3225–3234 (2014)

purity >99%) was obtained from Henan Fengyuan Chemicals Company Ltd. (Puyang, China). Duopafei® was provided by Qilu Pharmaceutical Company Ltd. (Jinan, China). Methoxy poly (ethylene glycol) (MW 2000 Da)–b-poly (D,L-lactic acid) (MW 2000 Da) diblock copolymer (MW 4000Da) was obtained from Daigang Biomaterial Company (Jinan, China). Fluorescein isothiocyanate (FITC), dimethyl sulfoxide, and sulforhodamine B (SRB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The HeLa cell line was purchased from the Chinese Academy of Sciences Cell Bank (Beijing, China). RPMI Culture medium 1640, fetal bovine serum, penicillin, and streptomycin were purchased from Beijing Solar Bioscience and Technology Company Ltd (Beijing, China).

Preparation and Characterization of DMA-C60/DTX-MC

DMA-C60 was added to phosphate buffer solution (PBS, pH 7.4) followed by sonication at room temperature for 1 h. A solvent evaporation method was used for the self-assembling of mPEG–PLA copolymers and drug encapsulation.[23] Briefly, mPEG–PLA block copolymer (100 mg) was added to acetonitrile (20 mL) containing DTX and stirred for 10 min. After evaporation to remove acetonitrile, the film was dispersed by the previously prepared PBS containing DMA-C60, and then stirred at 45°C for 10 min. The copolymer MCs solution was filtered through a 0.22-μm membrane filter to remove free DTX.

The morphology of the copolymer MCs was examined by transmission electron microscopy (TEM) (Tecnai G220)(FEI, Hong Kong). The hydrodynamic diameter and polydispersity index of copolymer MCs were determined by DLS ((Malvern Zetasizer Nano ZS-90) (Malvern, Britain).

The encapsulation efficiency (EE) and drug loading (DL) of DTX and DMA-C60 in the MC were quantified by ultrafiltration 9. After centrifugation at 1062.5×g for 30 min, DMA-C60-MC (or DTX-MC) along with the encapsulated drug remained in the outer chamber, and the dispersion medium was filtered to the sample recovery chamber through the filter membrane with molecular weight cut-off of 10,000 Da (Reili Separation Instrument Factory, Shanghai, People's Republic of China). The DTX content in the dispersion medium and the total amount of DTX in DMA-C60-MC after demulsification with methanol in 5 mL volumetric flask was measured by HPLC (Agilent 1200) with the mobile phase of methanol and ultrapure water (75:25, v/v) on a C18 column (Agilent Eclipse XDB-C18, 4.6 mm × 150 mm) using the UV detector at 229 nm, column temperature at 30°C, flow rate at 1.0 mL/min, and injection volume at 10 μL. The DMA-C60 content in the dispersion medium was measured by a UV detector (Shimadzu 2550) (Kyoto, Japan) at 340 nm.[24] The total amount of DMA-C60 in DTX-MC was input amount. The EE and DL of DTX or DMA-C60 were calculated as follows: EE (%) = $[(W_{total}-W_{free})/W_{total}] \times 100\%$, DL (%) = $[(W_{total}-W_{free})/W_{lipid}] \times 100\%$.

Appearance, particle size, and EE of copolymer MCs stored in the dark at 4°C and 25°C for 1 month were monitored.

The CMC was determined using pyrene as a fluorescent probe.[25] MC solutions with different concentrations ranging from 0.1 to 400 μg/mL were equilibrated with a fixed concentration of 2×10^{-6} mol/L of pyrene. Fluorescence measurements were carried out using a steady-state fluorescence spectrometer (RF-5301 PC; Shimadzu). The excitation wavelength was 339 nm, and the fluorescence emission was measured from 300 to 350 nm for all tests. The intensity ratio of the peak at 335 nm to that at 333 nm was plotted against the log of the polymer concentration. The CMC was determined by taking the flexion point of the sigmoidal curve.

Table 1. The Sample List of *In Vitro* Hemolysis Experiments

Component (mL)	Tube 1 (Negative Control)	Tube 2 (Sample)	Tube 3 (Duopafei®)	Tube 4 (Positive Control)
2% rabbit BRC	2.5	2.5	2.5	2.5
DMA-C60/DTX-MC	–	0.5	–	–
Duopafei®	–	–	0.5	–
Saline	2.5	2.0	2.0	–
Distilled water	–	–	–	2.5

In Vitro Hemolytic Experiments

Briefly, 2% (v/v) red blood cells (RBCs) were prepared from rabbits by conventional method. Various ingredients were added to different centrifuge tubes according to Table 1, shaken gently and then incubated at 37°C for 4 h. Samples were placed in ice to stop hemolysis. After centrifugation at 2000 rpm for 10 min, the supernatant of each tube was collected and analyzed for hemoglobin by UV spectroscopy at 576 nm (Shimadzu UV-2550) (Kyoto, Japan). The percentage of hemolyzed RBC was determined using the equation: hemolysis (%) = 100 (Abs-Abs_0)/(Abs_{100}–Abs_0), where Abs, Abs_0, and Abs_{100} are the absorbance of sample, negative control, and positive control, respectively.

In Vitro Release

The release profile of DTX and DMA-C60 from MC was evaluated according to an earlier report with slight modification.[26] DTX-DMA/C60-MC and control (equivalent to 50 μg DTX or 50 μg DMA-C60) were placed in a mini dialysis bags (MWCO, 8000–14,000Da; Sigma–Aldrich) (St.Louis, MO, USA) and the bags were then placed into 30 mL of PBS (pH 7.4) or plasma from a rabbit (5 kg) containing 0.5% (w/v) Tween 80 as a release medium. The system was maintained at 37.0 ± 0.5°C under a stirring condition at a rate of 100 times/min. The release media (0.2 mL) was sampled at predetermined time intervals and replaced with equal volume of fresh medium. The samples were analyzed for DTX content by HPLC.

Cytotoxicity Assay

Human cervical cancer HeLa cells were obtained from the Chinese Academy of Sciences Cell Bank (Beijing, China). HeLa cells were cultured in RPMI-1640 medium containing with 10% FBS and 1% penicillin/streptomycin in 5% CO_2 and 95% air at 37°C in a humidified incubator.

The cytotoxicity of DMA-C60/DTX-MC to HeLa cells was assessed by using SRB method. The cells were cultured and lifted as described above before being seeded (1×10^4) into 96-well plates and incubated for 24 h. The medium was then replaced with fresh medium containing different formulations. The cells were further incubated for 24 or 72 h. After incubation, cells were fixed by adding 20 μL cold 50% trichloroacetic acid (w/v, 4°C) and then incubated at 4°C for 1 h. After the plates had been washed with water and dried in air, an aliquot of 50 μL 4% (w/v) SRB in 1% acetic acid (v/v) was added and stained for 30 min. Excess SRB was removed by washing with 1% acetic