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Original article

Enhanced effect in combination of curcumin- and ketoconazole-loaded methoxy poly (ethylene glycol)-poly (ε-caprolactone) micelles

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ABSTRACT

In order to enhance water-solubility and realize controlled release while keeping synergistic effects of ketoconazole and curcumin, drug-loaded methoxy poly (ethylene glycol)-b-poly (ε-caprolactone) micelles were prepared through thin membrane hydration method. Transmission electric microscopy and dynamitic light scattering characterization revealed the formation of ketoconazole- and curcumin-loaded micelles with an average size of 44.70 nm and 39.56 nm, respectively. The drug-loaded micelles endowed the two drugs' slow controlled release with water-solubility enhanced to 85 and 82000 folds higher than the corresponding raw drugs, respectively. *In vitro* antifungal activity test, chequerboard test and inhibition zone test indicated that efficacy of ketoconazole-loaded micelles was improved by introduction of curcumin-loaded micelles with a low fractional inhibitory concentration index (0.073). Biofilm formation inhibition assay also demonstrated that participation of curcumin-loaded micelles obviously strengthened the inhibition of fungal biofilms formation induced by ketoconazole-loaded micelles. The high synergistic activity of combinations is encouraging and the MPEG-PCL micelle is a potential drug delivery system for the combination of ketoconazole and curcumin.

1. Introduction

Fighting against *Candida* infections are still a main problem for clinicians. The major pathway to treat this problem includes 1) developing new drug; 2) providing novel formulation; and 3) improving drug's therapeutic efficiency using combinational treatment. Curcumin (CUR) is a natural polyphenolic compound used as a spice and food coloring agent in Asia. It shows several different beneficial biological effects including antioxidant [1], antitumor [2], anti-inflammatory [3], antidiabetic [4], antiasthmatic properties [5] as well as antimicrobial activity [6]. It can be combined with ketoconazole (KCZ), fluconazole or other azole drugs to reduce their minimum inhibition concentration (MIC) value in the *in vitro* treatment of fungi, showing synergistic effect [7]. However, low water solubility of CUR and most of azole drugs' [8–10] will limit their clinical application.

Ketoconazole is now mainly used to treat skin fungal infection via percutaneous delivery. It is stated that azole antifungal drug-loaded MPEG-based amphiphilic copolymeric micelles can enhance drug's accumulation in the skin, showing potential capability of selective drug delivery to skin [11]. It is well-known that amphiphilic copolymer can self-assemble into micelles with a core-shell structure in aqueous medium. The hydrophobic inner core can encapsulate hydrophobic drug via *van der* Waals'force, hydrogen bond or other interactions among them, reducing drug's hydrolysis [12] or improving its stability [13]. Hydrophilic outer shell composed of poly (ethylene glycol) can enhance the water-solubility of drug located in inner core of copolymeric micelles via PEG's hydration, prolong *in vivo* circulation time of drug [14] and protect drug from reticuloendothelial system phagocytosis [15]. In addition, it is reported that the enhanced water-solubility of antifungal drug should be helpful for antifungal drug diffusion through cultural medium and interaction with fungi easy [16], and micellar antifungal drug formulation can enhance the skin deposition of drug when used percutaneously [11].

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In this study, in order to enhance drug water-solubility and investigate synergistic possibility of CUR and KCZ enveloped in copolymeric micelles, we prepared drug-loaded MPEG-PCL micelles by way of thin-membrane hydration method. The effect of PCL chain on the KCZ and CUR encapsulation in drug-loaded micelles was researched, and the drug-loaded micelles were characterized in the respects of particle diameter distribution, infrared spectrum, x-ray diffraction and transmission electron microscopy. We evaluated the *in vitro* activity of the two drug-loaded micelles using broth microdilution test. The synergistic effect of micellar CUR and KCZ was quantitatively investigated by fractional inhibitory concentration index and combination index (CI) calculation using median dose-effect equation through chequerboard titer test. Their synergistic effects in eradicating fungal biofilms were also qualitatively and quantitatively evaluated through biofilm formation inhibition assay.

2. Materials and experiments

2.1. Materials

KCZ was bought from Aladdin Industrial Inc (China). $MPEG_{2000}$ (molecular weight = 2000) was purchased from Sigma-Aldrich (Shanghai, China). ε-Caprolactone was provided by Qingdao Huayuan Polymer Co. Ltd. (Qingdao, China). CUR was kindly provided by Guangye natural pigment Co. Ltd. (Puyang, China). MPEG2000-PCL was prepared on the basis of a ring-opening polymerization procedure reported previously with ethyl ether-hydrochloride as catalyst [17]. Its structure was characterized by ^{1}H NMR (300 M, AVANCE, Bruker BioSpin, Germany) and GPC (in THF, Waters 2690D-2410, Waters, MA, USA). Methylene dichloride was dried by anhydrous magnesium sulfate. All other reagents were analytical grade and used without further purification.

2.2. Preparation of KCZ- and CUR- loaded micelles

10 mg of KCZ and 70 mg of copolymer were co-dissolved in 2 mL of methylene dichloride. The mixture was evaporated in vacuum to form thin membrane which was hydrated in 10 mL of water for 30 min at 60 °C to obtain KCZ-loaded micelle solution (KCZ-M). The solution was filtered through 0.22 μm filtration membrane to remove undissolved drug and other impurities. The content of KCZ in drug-loaded micellar solution was determined through high pressure liquid chromatography (HPLC) under a condition of methanol-water (4:1, V/V) as mobile phase and UV detection at 243 nm [18,19]. The flow rate of mobile phase was 1 mL/min.

In order to prepare CUR-loaded micelles (CUR-M), 10 mg of CUR and 70 mg of copolymer was co-dissolved in 2 mL of acetone and evaporated in vacuum to form thin membrane which was treated via similar procedure described above. A certain volume of CUR-loaded micelle solution was added into ethanol in volumetric flask to destroy micelles and dissolve CUR. CUR's content was determined by UV–vis spectrophotometer at 425 nm (T6 New Century, Purkinje General, Peking, China). The drug loading (DL) and entrapment efficiency (EE) of the two drug-loaded micelles were calculated by way of Eqs. (1) and (2), respectively [20]:

$$EE = \frac{\text{weight of drug in micelles}}{\text{weight of the initial drug}} \times 100\% \quad (1)$$

$$DL = \frac{\text{weight of drug in micelles}}{\text{weight of micelles containing drug}} \times 100\% \quad (2)$$

The two blank micelles were also prepared without adding the corresponding drug according to the above preparation procedure of drug-loaded micelles, respectively. All of them were used in further analysis, characterization or lyophilization for IR or XRD analysis.

2.3. Characterization of micelles

2.3.1. Particle diameter distribution and TEM

The particle size and zeta potential of drug-free and −loaded micelles were measured by dynamic light scattering (DLS, Zetasizer ZS90, Malvern Instruments Ltd., UK). Transmission electron microscope (TEM, JEM-1200EX, JEOL, Tokyo, Japan) was used for shape and morphology observation.

2.3.2. IR and XRD

The IR of crude drug, blank and drug-loaded micelle freeze-dried powder were recorded in the range from 400 to 4000 cm^{-1} by way of KBr pellet method on a FT-IR spectrometer (Spectrum One, PerkinElmer, USA).

The XRD of crude drug, blank and drug-loaded micelle freeze-dried powder were performed on an X-ray diffractometer (Bruker D8 Focus, Bruker, Germany) using Cu K α radiation (40 kV, 30 mA).

2.4. In vitro *release of CUR and KCZ-loaded micelles*

To determine release behavior of KCZ and CUR from the combined micelles, the *in vitro* release experiment was done in 40% ethanol solution in saline under sink condition with KCZ and CUR solution in methanol as control. The 6 mL of drug-loaded micellar and control solutions (equivalent to 6.9 mg of CUR and 1.2 mg of KCZ) was added into dialysis bags, sunk into beakers filled with 94 mL of release medium and then vibrated with 100 rpm at 37 °C, respectively. At each determined time point, 1 mL of release medium was extracted, and the same volume of fresh release medium was complemented into the beakers. The content of KCZ was analyzed using HPLC method with methanol-PBS (10 mmol/L, pH 6.0) (3:1, V/V) as flow phase at a wavelength of 243 nm. The content of CUR was determined using UV spectrometer at 425 nm. All of the tests were carried out in four parallel samples. The accumulative release percentage of CUR and KCZ was calculated in accordance with a reported formula [21].

2.5. In vitro *antifungal experiment*

2.5.1. Determination of MIC

Candida albicans strains (*C. albicans*, CMCC 98001) suspended in Sabouraud's liquid medium at a final density of 0.5-2.5 × 10^3 CFU/mL were added into each hole of the 96-well plate. Then, a series of dilution solutions of KCZ-M and CUR-M were mixed with the strains suspension to afford final solution ranging from 32 to 0.25 μg/mL and 256 to 2 μg/mL, respectively. The mixed solution was cultured at 28 °C for 48 h. The medium containing fungi and sterile medium were used as growth control and sterility control, respectively. Absorbance of each plate was determined in a microplate reader (1510, Thermo Fisher Scientific). The minimum inhibitory concentration (MIC_{80}) values, defined as the lowest concentration required to inhibit the growth of eighty percent of the fungi [7,22,23], were determined. The experiments were performed in triplicates.

2.5.2. Chequerboard titer test

The *C. albicans* strains suspended in Sabouraud's liquid medium at a final density of 0.5–2.5 × 10^3 CFU/mL added into the 96 well plates, respectively. KCZ-M was added to the rows of 96-well plate in diminishing concentrations and CUR-M was added to the columns in diminishing concentrations to form a series of diluted KCZ-M and CUR-M solutions ranging from 8 to 0.25 μg/mL and 180