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In vitro and in vivo anticancer activity of a novel puerarin nanosuspension against colon cancer, with high efficacy and low toxicity

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ABSTRACT

The present study aims to evaluate the anticancer activity of puerarin nanosuspensions in human colon cancer HT-29 cell line in vitro and in vivo. Puerarin nanosuspensions were prepared by the high-pressure homogenization (HPH) technique. The HT-29 cells were incubated with increasing concentrations of puerarin solution and nanosuspensions for indicated times. MTT evaluated cellular viability and investigated the effect of puerarin on cell proliferation of HT-29. Annexin V-FITC/PI staining method was conducted to determine the influences of the puerarin nanosuspensions on cell cycle and apoptosis. The in vivo anticancer activity of the puerarin nanosuspensions was observed in HT-29 cancer bearing mice. The puerarin nanosuspensions were well re-dispersed in aqueous media a mean diameter about 400–500 nm. Cytotoxicity assay, observation of morphological changes and early apoptosis revealed that the puerarin nanosuspensions could significantly enhance the in vitro anti-proliferation against HT-29 cells compared to the puerarin free solution. The prepared puerarin nanosuspensions in vivo evaluation showed higher anticancer efficacy and lower toxicity compared to the free solution, as shown by changes in tumor volumes, body weights, and survival rates. Based on these data, the potential of the puerarin nanosuspensions to serve as a cancer chemotherapeutic agent for colon cancer could be suggested.

1. Introduction

Puerarin, a well-known isoflavone-*C*-glucoside (Fig. 1), has been identified as a major constituent in *Pueraria radix* (Tian et al., 2011). It has been shown to have beneficial effects on liver disease (cirrhosis) (Liu et al., 2010a; Xiao et al., 2011), cardiovascular (Wu et al., 2007; Zhang et al., 2008), neurological (Gu et al., 2010; Zhu et al., 2008), anti-platelet aggregation (Hu et al., 2010b) and hyperglycemic disorders (Meng et al., 2009). Furthermore, numerous studies demonstrated the anticancer activity of puerarin in animal models as well as proliferation inhibition and apoptosis induction in a variety of cancer cell lines in vitro (Hien et al., 2010; Kim et al., 2008b; Liu et al., 2010b; Yu and Li, 2006). Previous study has indicated that puerarin suppresses the growth of Human colon carcinoma cell line HT-29 and some of the mechanisms were demonstrated (Yu and Li, 2006).

Colon cancer, a serious health problem in most developed countries, is the third most common form of cancer and the second-leading cause of cancer-related death in the Western world (Go et al., 2010; Yu et al., 2004). A key regulator of tissue homeostasis is the apoptosis or programmed cell death. And the imbalances between cell death and proliferation may result in tumor formation (Sheng et al., 2008). To induce apoptosis-related signaling in cancer cells while disrupting their proliferation were the objective of using anticancer agents (Hu et al., 2010a).

Due to the low solubility of puerarin in water, the current puerarin injection formulation contained 50% (v/v) 1, 2-propanediol which served as cosolvent. The 1, 2-propanediol and its metabolism may be one of the allergize agents (Budden et al., 1979; Morshed et al., 1988; Ruddick, 1972). Given the interesting results obtained in recent work with silybin and deacety mycoepoxydiene nanosuspensions, in vitro and in vivo antitumor evaluation of the puerarin nanosuspensions was carried out (Wang et al., 2010, 2011).

Nanosuspensions, a new approach for the formulation for the poorly soluble drugs, are sub-micron colloidal dispersions of pure particles of drugs, which are stabilized by surfactants (Rabinow, 2004). The nanosuspensions can be obtained either by top-down approach or by bottom-up technique. The top-down techniques for nanosuspensions production comprise high-pressure homogenization (HPH) and media milling. The bottom-up technologies

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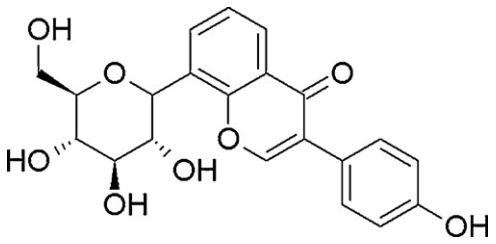

Fig. 1. Structural formulas of puerarin.

start from the molecules which are dissolved and precipitate them by adding the solvent to a non-solvent (Mauludin et al., 2009). The HPH technique is a frequently used method for preparing the drug nanosuspensions. The basic advantages of HPH technique are including simplicity of the process, ease of large-scale production and a reduced product contamination (Wang et al., 2010). In the present study, we employed the HPH method to prepare small and uniform nanosuspensions particles.

Nanosuspensions, a drug delivery platform, has been shown to provide numerous advantages over conventional ocular dosage forms, including improved bioavailability, reduced in systemic toxicity of drug, enhanced stability, increased pharmacodynamic action, targeting effect (Juhnke et al., 2012; Pu et al., 2009). However, few studies of puerarin nanosuspensions have been reported for its anti-tumor activity and apoptotic mechanism. Therefore, the aim of the present study was to evaluate the effect of puerarin nanosuspensions for colon cancer compared to the free solution formulation. An Annexin V-FITC-/PI-stain apoptosis assay was conducted to determine whether the decrease in viability of cell growth observed after treatment with the puerarin nanosuspensions was the result of enhanced apoptosis in HT-29 cells. Finally, the antitumor efficacy of puerarin nanosuspensions was evaluated by measuring the in vivo antitumor effects in HT-29 bearing nude mice after intravenous (i.v.) administration of the puerarin nanosuspensions or solution formulations. The safety of the puerarin nanosuspensions was assessed by observing changes in the body weights and survival rates of tumor-bearing mice treated with the nanosuspensions.

2. Materials and methods

2.1. Materials

Puerarin was obtained from Nanjing Zelang Pharmaceutical Co., Ltd, China. Lecithin, injection grade, was provided by Shanghai Taiwei Medicine Co., Ltd, China. MTT and DMSO were purchased from Sigma Chemical (St. Louis, MO, USA). Trypsin was purchased from GIBCO BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was supplied by FMG Biotech Co., Ltd. Propidium iodide (PI), RNase A and Annexin V-FITC was obtained from KeyGen Biotechnology (Nanjing, China). Water was purified on a MilliQ Plus system, Millipore (Schwalbach, Germany). Lactose was purchased from Sigma and used as a cryoprotectant during freeze-drying. Stock solutions of the puerarin were prepared by dissolving in DMSO and the aliquots stored at −20 °C prior to use. All the other chemicals and solvents were of chromatographic and pharmaceutical grade and used without additional treatments.

2.2. Animals and cell line

BALB/c nude mice (20 ± 2 g) were obtained from the Shandong University Laboratory Animal Center. The animals were acclimatized at an ambient temperature of 25 ± 2 °C and a relative humidity 75 ± 5% under natural light/dark conditions for 1 week with food and water ad libitum. The animal care and all the experimental procedures were performed in accordance with the Guidelines for Ethics and Regulations for Animal Experiments as defined by the Department of Pharmaceutical Sciences, Shandong University, China. Human colon carcinoma cell line HT-29 was obtained from obtained from Institute of Biochemistry and Cell Biology, Institute for Biological Sciences, Chinese Academy of Science (Shanghai).

2.3. Formulation of puerarin nanosuspensions

The puerarin nanosuspensions were produced by high pressure homogenization (HPH) technique. Briefly, puerarin powder (1%, w/v) was dispersed in an aqueous surfactant solution, containing 0.5% (w/v) lecithin and 0.25% (w/v) HPMC under magnetic stirring. The obtained mixture was firstly disintegrated into microparticles by high shear homogenizer using Ultra-Turrax® T25 (IKA, Germany) at 20,000 rpm for 5 min. The suspensions were further processed via HPH by an EmulsiFlex-C3 (Avestin Inc., Ottawa, Canada) equipped a heat exchanger applying 6 homogenization cycles at 800 bar, and then by 15 homogenization cycles at 1500 bar for the puerarin nanosuspensions.

For long-term stability, the prepared nanosuspensions were dried using freeze-drying by a FD5-series freeze dryer (SIM, USA). Lactose 5% (w/v) was served as cryoprotectant. In a 20 ml vial 2 ml of the puerarin nanosuspensions were frozen at −80 °C (DW-HL218, Shanghai Huayan Co., Ltd, China). The frozen nanosuspensions were freeze dried for 48 h at −55 °C under vacuum (pressure < 15 mTorr). The freeze-dried nanosuspensions were re-dispersed with water before using.

2.4. The particle size and morphology analysis of puerarin nanosuspensions

Before and following the freeze-drying, the particle size and distribution of the puerarin nanosuspensions were evaluated. The particle size and polydispersity index (PI) of the nanosuspensions were determined by photon correlation spectroscopy (PCS, Zetasizer Nano ZS, Malvern Instruments, UK) following the freeze-dried powder were rehydrated with puerarin saturated water to obtain a properly scattering intensity and re-dispersed by hand agitation before measurement. The photon correlation spectroscopy (PCS) value is the mean diameter of the bulk population (z-average) and the PI is a measure of the width of the particle size distribution. Light microscopy was conducted using an optical microscope (Leica S6E, Germany). The employed magnification was 400 folds and each sample was investigated 10 times.

2.5. In vitro cytotoxicity of puerarin nanosuspensions against HT-29 cells

2.5.1. Cell culture

HT-29 colon cancer cell line was maintained in RPMI 1640 medium (GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% antibiotic solution (penicillin 100 U ml^{-1} and streptomycin 100 μg ml^{-1}) at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every second day, and cells were subcultured when confluency reach to 95% by 0.25% trypsin containing 0.02% ethylene-diaminetetraacetic acid (EDTA) in PBS for 3 min at 37 °C.

2.5.2. Estimation of cell proliferation

Cell proliferation was quantified by an MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay). HT-29 cells were seeded at a density of 1×10^5 per well onto 96-well cell plates with a 200 μl culture medium for 24 h. After 24 h of incubation, the medium was removed and the cells were