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A bufadienolide-loaded submicron emulsion for oral administration: Stability, antitumor efficacy and toxicity

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ABSTRACT

The purpose of this study was to develop an alternative submicron emulsion containing three bufadienolides for oral administration and evaluate its preclinical stability, efficacy, and toxicity. The bufadienolide-loaded oral submicron emulsion (BU-OE) was prepared by high-pressure homogenization. The storage stability, *in vitro* cytotoxicity, *in vivo* antitumor efficacy, acute toxicity, and long-term toxicity of BU-OE were investigated in detail to evaluate the formulation. The stability study suggested that BU-OE was stable at room temperature and could be stored for at least 18 months at 6 ± 2 °C. The cytotoxicity test revealed that BU-OE had marked cytotoxic activities against cancer cells, but no evident inhibitory effects on normal cells. Likewise, BU-OE exhibited significant antitumor efficacy against Hep G2, HCT-8, and EC9706 cell lines and a slight inhibitory effect on BGC 803 cell line in nude mice, while comparable antitumor activity with fluorouracil injection. The LD_{50} of BU-OE in mice was 29.4 mg/kg (male) and 22.8 mg/kg (female), respectively. As for the long-term toxicity, BU-OE showed no apparent toxic effects except minor cardiotoxic effects which were reversible. In conclusion, submicron emulsion is a suitable delivery system for oral administration of bufadienolides, with satisfactory stability, superior antitumor efficacy and low toxicity.

1. Introduction

Toad venom, a traditional Chinese medicine, is originally isolated from the dried secretions of the postauricular and skin glands of Chinese toads (*Bufo bufo gargarzinas* cantor or *Bufo melanostictus* Schneider) (Krenn and Kopp, 1998; Steyn and van Heerden, 1998). It has been widely used in China and other Asian countries for centuries as a cardiotonic, anodyne, local anesthetic, antimicrobial, and antineoplastic agent (Qi et al., 2011). The major biologically active components of toad venom are bufadienolides, a type of C-24 steroid with a characteristic α-pyrone ring at C-17 (Kamano et al., 1998; Ye et al., 2006), principally bufalin (B), cinobufagin (C), and resibufogenin (R). Recently, large numbers of experimental and clinical studies have confirmed that bufadienolides exhibit significant cytotoxic activities against human hepatoma cells (SMMC7221), human colon cancer cells (colon 26-L5), human myeloid leukemia cells (K562, U937, ML1, and HL60), and prostate cancer cells (LNCaP, DU105, and PC3) (He et al., 2006; Nogawa et al., 2001; Yeh et al., 2003; Zhang et al., 1992). These activities are mainly mediated by induction of the differentiation and apoptosis of cells, disruption of the cell cycle, inhibition of cell proliferation and tumor angiogenesis, reversal of multi-drug resistance and regulation of the immune response (Qi et al., 2011; Xie et al., 2013). In particular, B, C, and R all exhibited superior efficacy on digestive system tumors, a class of cancer with a high incidence in Asian countries, mainly involving cancers of the liver, stomach, bowel, and esophagus. However, the currently available drugs able to treat these cancers are very limited in number and toxic, such as fluorouracil, doxorubicin and cytarabine. Therefore, it was of great significance to develop bufadienolide preparations as novel agents for the treatment of digestive system cancers.

However, the optimum administration routes of bufadienolides have not been fully established owing to their poor aqueous solubility and chemical instability (Weng et al., 2010). The main commercial products are Toad Venom Injection and Huachansu Injection. Nevertheless, the clinical applications of these

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formulations are mainly restricted by several problems. Firstly, neither of them can effectively reduce the degradation of bufadienolides in solution, and it is difficult to avoid drug precipitation during injection. Secondly, these commercial preparations usually contain a single active ingredient with a very low purity, consequently producing a variety of adverse reactions and poor efficacy. Thirdly, both of them may cause severe venous irritation thought to be caused by the principal agent. In addition, cardiotoxicity with symptoms similar to those induced by digoxin, including heart block, cardiac dysrhythmia, and ventricular ectopy, may occur following intravenous administration of low dose, and so the use of high doses of these formulations is limited, thus affecting the clinical efficacy. Finally, on account of their short elimination half-life and extensive tissue distribution, frequent administration is often needed. As a result, it is essential to develop an alternative delivery system for bufadienolides with high stability, low toxicity, and potent antitumor activity.

In our previous studies, we attempted to incorporate bufadienolides into liposomes (Li et al., 2009), nanostructured lipid carriers (Li et al., 2010a), lyophilized nanoemulsions and submicroemulsions (Li et al., 2008). However, most of these novel vehicles still restricted clinical application and industrial-scale production due to poor physical stability (except lyophilized nanoemulsions), low drug loading capacity and complicated preparation process. Moreover, these formulations were designed for intravenous administration, and carried risks of venous irritation and cardiotoxicity. Obviously, oral chemotherapy would be better than the intravenous route (DeMario and Ratain, 1998), and could offer improved efficacy and lower toxicity by providing a relatively prolonged systemic exposure with reduced variability. Furthermore, the oral delivery system used clinically is non-invasive, cost-saving, with improved patient compliance and a better quality of life (Kim et al., 2014). Recently, submicron emulsions have been attracting increasing attention as oral delivery systems for poorly soluble drugs (Nicolaos et al., 2003), owing to their advantages in terms of improved physicochemical stability, higher drug loading capacity and suitability for large-scale production (Dong et al., 2013; Venkateswarlu and Reddy, 2001). In addition, by incorporating the drugs into the oil phase and interfacial layer, such carriers could prevent the drugs coming into direct contact with body fluids and tissues, resulting in reduced irritation and toxicity, increased solubility and stability, as well as the possibility of sustained release (Gao et al., 2008; Wang et al., 2006). Therefore, submicron emulsions might be a suitable vehicle for oral administration of bufadienolides and they are expected to exhibit superior antitumor efficacy and low toxicity. Besides, the biggest differences between BU-OE and commercial products are the major components of the formulations. Particularly, BU-OE is an organic combination of three bufadienolides, *i.e.*, B, C, and R, with a higher purity, which may produce lower toxicity, a broader anti-tumor spectrum and better efficacy. Consequently, BU-OE may be a good choice for the clinical studies.

In this study, BU-OE was prepared by high-pressure homogenization and characterized in terms of physical appearance, particle size distribution, zeta potential, pH value, contents, and entrapment efficiency. Moreover, the accelerated and long-term stability were monitored to assess the storage stability of BU-OE. The *in vivo* antitumor activities of BU-OE were evaluated in tumor-bearing nude mice, while the *in vitro* cytotoxicity was determined by microculture tetrazolium (MTT) assay and flow cytometry. Besides, the acute toxicity and long-term toxicity of BU-OE was investigated to determine the toxic effects, target organs and toxic reversibility. These tests will provide evidence for further clinical studies as well as a novel alternative for the oral chemotherapy of digestive system cancers.

2. Materials and methods

2.1. Materials

Bufadienolides were isolated from toad venom by staff in the Department of Pharmaceutics, Shenyang Pharmaceutical University, PR China, mainly consisting of B, C, and R, with a mass ratio of 2:3:5 and a purity of 95% above (Verpoorte and Svendsen, 1980). Medium-chain triglyceride (MCT) was purchased from Tieling Beiya Pharmaceutical Co. (Tieling, China). Soybean lecithin (lipoid S75®, PC72%) and sodium oleate were supplied by Lipoid KG (Ludwigshafen, Germany). Poloxamer 188 (Pluronic F68®) was kindly provided by BASF AG (Ludwigshafen, Germany). Glycerol was obtained from Zhejiang Suichang Glycerol Plant (Zhejiang, China). Fluorouracil injection was from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China). Cisplatin injection and pharmorubicin injection were kindly provided by the Laboratory of Medical Oncology, the First Hospital of China Medical University. All other chemicals and reagents used were of analytical or chromatographic grade.

2.2. Animals and cell lines

The ICR mice used in the acute toxicity study were purchased from the Safety Evaluation Center (Shenyang Research Institute of Chemical Industry, Shenyang, China). The BALB/c-nu nude mice used for *in vivo* antitumor efficacy investigation and the Sprague-Dawley (SD) rats used in the long-term toxicity study were supplied by the Experimental Animal Center of Medical Department (Beijing University, Beijing, China). The Hep G2 human hepatocellular carcinoma cell line, the HCT-8 human colon carcinoma cell line, the BGC 803 human gastric carcinoma cell line and the EC9706 human esophageal carcinoma cell line were obtained from the Department of Pharmacology of the Cancer Institute (Chinese Academy of Medical Sciences, Beijing, China). All the animal experiments mentioned above were evaluated and approved by the local Ethics Committee for the use of experimental animals and complied with the Guidelines for the Care and Use of Laboratory Animals.

2.3. Preparation of BU-OE

The oral submicron emulsion containing bufadienolides was prepared by high-pressure homogenization at a concentration of 1.0 mg/mL (Weng et al., 2010). Firstly, the oil phase, which consisted of 3.0% (w/v) soybean lecithin, 10% (w/v) MCT, and 0.1% (w/v) bufadienolides, was heated at 80 °C with magnetic stirring until it was uniformly dissolved. At the same time, 0.4% (w/v) F-68, 2.5% (w/v) glycerin, and 0.05% (w/v) sodium oleate were dispersed in double-distilled water with agitation at 70 °C to obtain the aqueous phase. Subsequently, the oil phase was slowly added to the aqueous phase with continuous stirring using a high speed shear mixer (ULTRA RURRAX® IKA® T18 basic, Germany) at 12,000 rpm for 3 min to obtain a coarse emulsion. After that, the pH was adjusted to 6.5 with 0.1 mol/L HCl and the volume was made up to 100 mL with double-distilled water. Then, the coarse emulsion was subjected to high-pressure homogenization (Pharmaceutical ultra-high-pressure homogenizer of AH100D, ATS Engineering Inc. Shanghai, China) at 800 bar for 8 cycles to obtain the final emulsion. The temperature of the whole homogenization process was maintained at 30 °C using an ice-water bath. Finally, the emulsion was transferred to vials after flushing with nitrogen gas and sterilized by autoclaving at 121 °C for 10 min. The blank submicron emulsion was prepared in the same way as described above without drug incorporation.