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Self-reporter shikonin-Act-loaded solid lipid nanoparticle: Formulation, physicochemical characterization and geno/cytotoxicity evaluation

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ABSTRACT

Shikonin and some of its derivative have approved apoptotic potential in different human cancer cell lines, and moreover have a dominant fluorescent emission at ~600 nm. Here, to enhance shikonin-Act anti-proliferation properties, it was successfully incorporated in Solid Lipid Nanoparticles (SLNs) by the hot homogenization and entrapment efficiency (EE) of drug in SLNs was determined by ultrafiltration method. Scanning electron microscopy (SEM), laser diffractometry and zeta-sizer indicated that shikonin-Act-SLN were spherical and regular particles in the range of 70–120 nm with polydispersity index (PI) of less than 0.10. The physical stability of shikonin-Act-loaded SLN in aqueous dispersion was evaluated in terms of size, PI, EE and drug leakage and the results showed that SLNs were stable upon storing three month. Long term *in vitro* release of the shikonin-Act was also approved. Cellular uptake of the shikonin-Act-SLN was examined by the *in vitro* fluorescent microscopy and facs flow cytometry analyses. *In vivo* rat imaging approved the penetrating capability of shikonin-Act-SLN emission through living tissues. *In vitro* anti-proliferation and genotoxicity evaluation by MTT and comet assay confirmed that shikonin-Act-SLN showed higher cytotoxic/antitumor potential than intact shikonin in terms of IC_{50} and DNA damage. This work provide sufficient information about improving of the therapeutic efficacy of the shikonin-Act, and also using of the shikonin-Act-SLN in bio-distribution studies during drug delivery investigation by incorporating in lipidic and colloidal drug delivery particles such as SLNs.

1. Introduction

Shikonin (beta-alkannin) and its ester derivative are red self-fluorescent naphthoquinone pigments (Fig. 1) that are accumulated in the roots of various species including *Echium italicum* L. These compounds derived from two precursors, 4-hydroxybenzoate and geranyl diphosphate and have been used as food colorants, cosmetic, textile dyes and burn healing agents. Shikonin and its derivatives have been verified to possess different pharmaceutical properties. Acetylshikonin (shikonin-Act), a shikonin derivative, versus to the shikonin has the acetyl radical in its chemical structure and showed slightly different pharmacological effects.

Shikonin is well known for a wide spectrum of bioactivities, including anti-inflammatory (Andujar et al., 2012; Liang et al., 2013; Xiong et al., 2013), anti-HIV (Chen et al., 2001, 2003), anti-bacterial (Shen et al., 2002), anti-oxidant (Han et al., 2008), anti-inflammatory (Singh et al., 2003) and anti-tumor. While, during previous non-comprehensive investigation, shikonin-Act

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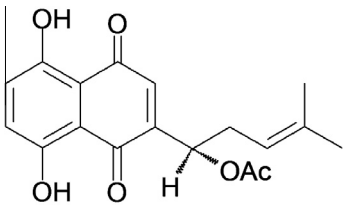

Fig. 1. Chemical structure of shikonin-Act.

showed some bioactivities including: adipogenesis inhibiting (Gwon et al., 2012) through inhibition of 5-lipoxygenase activity (Hsu et al., 2009), inhibition of the generation of NADPH oxidase complex (Kawakami et al., 1996) and inhibition of phosphatidylinositol signaling pathway (Wang and Kuo, 1997). Shikonin and shikonin-Act represents potent inhibiting effects against diverse tumors via special molecular pathways. Shikonin can suppress cancer cell proliferation through blocking of phosphorylation of extracellular signal-regulated kinase (pERK) (Chang et al., 2010; Kim et al., 2001; Wang et al., 2013). It could also induce apoptosis via activating of p53 protein via caspase-9/3 dependent mechanisms (Wu et al., 2004a, 2004b). Shikonin also may be lead to cancer cell death through other well-known mechanisms including: inactivation of NF-kappaB (Chiu and Yang, 2007; Lu et al., 2011; Min et al., 2008, 2011; Ruan et al., 2010), inhibiting of tumor-specific pyruvate kinase-M2 (PKM2) function (Chen et al., 2011), and inactivating of topoisomerase (Yang et al., 2006). However, acetylated shikonin (shikonin-Act) could induce apoptosis via activating the pro-apoptotic bcl-2 family and caspase-3 (Liu et al., 2008; Xiong et al., 2009; Zeng et al., 2009) and inhibition of angiogenesis in solid tumors (Lee et al., 2008; Pietrosiuk et al., 2004).

Poor solubility in aqueous medium and short half-life of the shikonin-Act are the two main problems during drug screening and treatment. Recently, drug delivery using different nano-carriers; as a promising strategy have been attracted the attention of both phytochemist and pharmaceutical scientist to overcome these issues. Biocompatible colloidal lipidic nanoparticles such as Solid Lipid Nanoparticles (SLN) have emerged as promising therapeutic strategy to improve bioavailability and bioactivity of lipophilic bioactive natural compounds (Yang et al., 2012). Homogenization, solvent emulsification/evaporation, and microemulsion are the three main methods that routinely have been used for SLN preparation. Among these methods, simple homogenization and solidification procedure would enable effective scale up SLNs formulation for industry. There are many reports have shown that ingredient composition including type of solid lipid, proportions of emulsifier and water influence on the physiochemical characteristics of final product. However, avoidance of organic solvent during formulation is the main advantage of SLNs compared to the other colloidal carriers (Mehnert and Mäder, 2012). Due to their adaptability in loading drugs in the matrix of solid lipid, SLNs depict the capability to prolong and sustain the release profile of the loaded compounds. Possibility of controlled drug release, passive drug targeting, and protection of incorporated compound against chemical degradation are the other advantages of the SLNs (Souto and Doktorovova, 2009; Tiyaboonchai et al., 2007). Different advantages of the SLNs candidate it as a robust carrier for drug delivery and targeting in various routs of administration such as parenteral, oral, ophthalmic and topical during last decades (Uner and Yener, 2007).

Although, there have been few studies related to the shikonin delivery to cancer cells by different nanodrug delivery systems, but no investigation have been done in direct to the delivery of the shikonin-Act until now. Xia et al. (2013)were prepared shikonin-containing liposome and assessed its angiogenesis activity and demonstrated its cytotoxicity. Other study formulated shikonin in different branched denderimer as well as chimeric drug delivery nanosystems (chi-aDDnSs) comprised their *in vitro* characterization (Kontogiannopoulos et al., 2012) while the biological activities was not included.

The primary goal here was to investigate the anti-tumor activity of sparingly aqueous soluble shikonin-Act and also, improve its bioavailability through preparation in SLN for the first time. Additionally, the processing factors affecting the characteristics of the shikonin-Act-SLN including size and morphology of the SLNs were studied, as well as the efficiency of drug incorporation. The fluorescent capability of shikonin-Act-SLN was assessed, as a robust and novel tool in bio-distribution studies by *in vitro* fluorescent detection, facs flow cytometry analyses and *in vivo* fluorescent whole rat imaging. Finally, cytotoxic and genotoxic effects of shikonin-Act-SLN on A549 human alveolar epithelial cancer cell lines using MTT and comet assay, respectively were also involved in this study and compared with the intact shikonin-Act.

2. Materials and methods

2.1. Materials

RPMI1640 medium, fetal bovine serum (FBS) and low and normal melting point agarose were purchased from Gibco, Invitrogen (Paisley, UK) Cell culture flasks and plates were attained from IWAKI, Japan. Trypsin–EDTA (0.02–0.05%), was purchased from Sigma Aldrich Co., (Poole, UK). Chemical solvents were obtained from Caledon Co., (Georgetown, Ont, Canada). The other chemical materials were obtained from Sigma Aldrich and Merck Co.

2.2. Plants materials and shikonin-Act extraction

The root samples of *Echium italicum* L. (Boraginaceae) were collected from Alavian village – Maragheh, Iran in 2012 and dried at room temperature. Collected samples were established and deposited in the Herbarium of the Faculty of Pharmacy (Voucher No: Tbz-FPh 737), Tabriz – Iran. Total *n*-hexane extraction using soxhlet instrument was carried out and shikonin/its ester derivative were extracted and purified based on a methods according to the our previous studies (Zare et al., 2011, 2010).

2.3. Preparation of shikonin-Act loaded solid lipid nanoparticles

Shikonin-Act-SLN were prepared by simple hot homogenization method according to our previous work (Dolatabadi et al., 2014). The amount and proportions of the elements used for the preparation of the SLNs are listed in Table 1. In brief, the lipid phase, consisting of different concentration of precirol® ATO 5, shikonin-Act and Tween 80 was heated to 65 °C (10 °C above the melting point of precirol® ATO 5). The aqueous phase containing poloxamer 407 in double-distilled water was simultaneously prepared at the same temperature. An emulsion was obtained by adding the aqueous phase to the lipid phase gradually under homogenizing at different rpms (14,000–18,000) for 45 min at a temperature above the melting temperature. The shikonin-Act-SLN was formed after cooling the mixture to room temperature and finally in an ice bath.

2.4. Measurement of physicochemical properties of shikonin-Act-SLN

2.4.1. Particle size and scanning electron microscopy

In order to gain the suitable system for SLN preparation, the particle size analyses were carried out immediately after preparation by laser diffractometry (SALD-MS30, Schimadzu, USA). Prior to the analysis, SLN dispersions were diluted with purified water to