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Licoricidin inhibits the growth of SW480 human colorectal adenocarcinoma cells *in vitro* and *in vivo* by inducing cycle arrest, apoptosis and autophagy

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ABSTRACT

Licorice (*Glycyrrhiza uralensis* Fisch.) possesses significant anti-cancer activities, but the active ingredients and underlying mechanisms have not been revealed. By screening the cytotoxic activities of 122 licorice compounds against SW480 human colorectal adenocarcinoma cells, we found that licoricidin (LCD) inhibited SW480 cell viability with an IC_{50} value of 7.2 μM. Further studies indicated that LCD significantly induced G1/S cell cycle arrest and apoptosis in SW480 cells, accompanied by inhibition of cyclins/CDK1 expression and activation of caspase-dependent pro-apoptotic signaling. Meanwhile, LCD promoted autophagy in SW480 cells, and activated AMPK signaling and inhibited Akt/mTOR pathway. Overexpression of a dominant-negative AMPKα2 abolished LCD-induced inhibition of Akt/mTOR, autophagic and pro-apoptotic signaling pathways, and significantly reversed loss of cell viability, suggesting activation of AMPK is essential for the anti-cancer activity of LCD. *In vivo* anti-tumor experiments indicated that LCD (20 mg/kg, i.p.) significantly inhibited the growth of SW480 xenografts in nude mice with an inhibitory rate of 43.5%. In addition, we obtained the glycosylated product LCDG by microbial transformation, and found that glycosylation slightly enhanced the *in vivo* anti-cancer activities of LCD. This study indicates that LCD could inhibit SW480 cells by inducing cycle arrest, apoptosis and autophagy, and is a potential chemopreventive or chemotherapeutic agent against colorectal cancer.

1. Introduction

Licorice is a popular herbal medicine derived from the dried roots and rhizomes of *Glycyrrhiza uralensis* Fisch., *G. inflata* Bat., and *G. glabra* L. (Fiore et al., 2005). Among these species, *G. uralensis* is the major source in the herb market, and is recorded in the pharmacopoeia of China, Japan, Europe, and the United States (Chinese Pharmacopoeia Commission, 2010). Licorice shows significant anticarcinogenesis, anti-inflammatory, antiaging, hepatoprotective, and antiviral activities (Jo et al., 2005; Kim et al., 2006; Mae et al., 2003; Oganesyan, 2002). Particularly, the anti-tumor activities of licorice have attracted increasing interests in recent years, and licorice extracts or pure compounds have been extensively reported as anti-cancer or chemopreventive natural agents (Wang and Nixon, 2001).

Colorectal cancer is the third most common cancer in the world and the fourth leading cause of cancer-related death. Researchers have been searching for medicines which could intervene in development and progress of colorectal cancer (Jemal et al., 2011). Licorice has been reported to possess significant anti-colorectal cancer activities, and several effective compounds have been discovered (Kummar et al., 2011; Stewart and Prescott, 2009; Takahashi et al., 2004). In our previous publication, we have reported that isoangustone A, a licorice-derived isoflavone, could induce apoptosis in SW480 human colorectal adenocarcinoma cells by disrupting mitochondrial functions (Huang et al., 2014). Recently, the anti-colorectal cancer activities of other licorice-derived compounds have also been published. Isoliquiritigenin, a major free chalcone in licorice, was able to block M2 macrophage polarization in colitis-associated tumorigenesis through downregulating PGE_2 and IL-6 (Zhao et al., 2014). Dehydroglyasperin D could inhibit the proliferation of HT-29 human colorectal cancer cells through direct interaction with phosphatidylinositol 3-kinase (Jung and Jeong, 2016).

Abbreviations: LCD, licoricidin; LCDG, the glycosylated product of licoricidin; AMPK, adenosine 5′-monophosphate (AMP)-activated protein kinase; Caspase, cysteinyl aspartate specific proteinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; PGE_2, prostaglandin E_2; IL-6, interleukin-6; NMR, nuclear magnetic resonance spectroscopy; MS, mass spectrometry; HPLC, high-performance liquid chromatography; UV, ultraviolet; FBS, fetal bovine serum; MEM, minimum essential medium; MTT, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; CDK, cyclin-dependent kinase; Parp-1, poly[ADP-ribose] polymerase 1; Bcl-2, B-cell lymphoma-2; ACC, acetyl-CoA carboxylase; LC3, microtubule associated protein 1A/1B-light chain 3; ULK, uncoordinated 51-like kinase; BCA, bicinchoninic acid; 3-MA, 3-methyladenine; PMSF, phenylmethanesulfonyl fluoride.

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Glycyrrhizic acid, the most abundant saponin in licorice, could suppress the development of precancerous lesions *via* regulating the hyperproliferation, inflammation, angiogenesis and apoptosis in the colon of Wistar rats (Khan et al., 2013). All these observations suggest that licorice-derived compounds are potentially effective as chemopreventive or adjuvant chemotherapeutic agents against colorectal cancers. At least 400 compounds have been isolated from licorice, thus far (Zhang and Ye, 2009). Few reports are available on the anti-colorectal cancer activities of other licorice compounds.

In our previous report, the chemical constituents of licorice (*Glycyrrhiza uralensis* Fisch.) were systematically studied, and a total of 122 compounds were obtained (Ji et al., 2016a). We screened the cytotoxic activities of all the compounds against SW480 human colorectal adenocarcinoma cells, and found that a number of prenylated flavonoids could remarkably inhibit SW480 cell viability. In this paper, we report the *in vitro* and *in vivo* anti-colorectal cancer activities of licoricidin (LCD), an isoflavan with two isoprenyl groups, and investigate the cellular and molecular mechanisms. Moreover, we obtained the glycosylated product of LCD (LCDG) by microbial transformation, and found that LCDG showed slightly more potent *in vivo* anti-cancer activities than LCD. Licoricidin (LCD) had been reported to inhibit metastatic capacity of DU145 human prostate cancer cells and 4T1 murine mammary carcinoma cells (Park et al., 2010, 2016). The anti-colorectal cancer activities of LCD were reported here for the first time.

2. Material and methods

2.1. General experimental procedures

Licoricidin (LCD) was isolated from *Glycyrrhiza uralensis* Fisch., and its glycosylated product (LCDG) was obtained by microbial transformation with the filamentous fungus *Mucor hiemalis* as we had previously reported (Ji et al., 2016a,b). Their structures were identified by NMR and MS spectral data analyses, and the purities were above 95% according to HPLC/UV analysis (Fig. S1). The negative optical rotation ($[\alpha]^{25}_{D}$) of −19.8 (*c* 0.1, MeOH) for LCD confirmed the *R*-configuration of C-3 (Zeng et al., 1998). Fetal bovine serum (FBS) was purchased from Thermo Scientific Hyclone (Logan, UT). Minimum essential medium (MEM) and other cell culture supplements were purchased from Life Technologies Invitrogen (Carlsbad, CA). 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO). PI-Annexin V apoptosis assay kit was purchased from Beyotime Institute of Biotechnologies (Jiangsu, China). Antibodies against cyclin B, cyclin A, p-p21, CDK1, Parp-1, Bcl-2, p-AMPKα T172, p-ACC S79, p-Akt S473, β-actin, caspase-3 and cleaved caspase-3, LC3 I/II, p62, p-mTOR and p-ULK1 were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). All other chemicals were purchased from Beijing Chemical Corporation (Beijing, China).

2.2. Cell culture

SW480 human colorectal adenocarcinoma cells were obtained from American Type Culture Collection (ATCC) and cultured in MEM medium supplemented with 10% FBS, 2.0 mg/mL sodium bicarbonate, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in a humidified 5% CO_2 atmosphere at 37 °C. All experiments were performed using cells in exponential growth within 20 passages.

2.3. Cell viability assay

The cells were seeded at a density of 5×10^4 cells/mL in 96-well plates and were allowed to attach to the well overnight. After incubation, the media were replaced by serum-free media containing various concentrations of LCD for indicated time (five parallel wells for each concentration of LCD), and 0.1% DMSO was employed as vehicle control. After that MTT (final concentration 0.5 mg/mL) was added into each well and incubated for further 4 h. The supernatant was subsequently removed, and MTT crystals were dissolved in 200 μL/well DMSO. Thereafter, optical density was measured at 492 nm using a MultiSkan MK3 microplate reader (Thermo Fisher Scientific, Waltham, MA). Experiments were performed for at least three times.

2.4. Flow cytometric analysis for cell cycle

The cells were cultured in 6-well plates and treated with LCD for indicated time or concentration. After treatment the cells were trypsinized, and both the floating and attached cells were collected by centrifugation. The cells were then washed twice with ice-cold phosphate buffered saline (PBS) and fixed in 70% ice-cold alcohol overnight. After treated with 100 μg/mL DNase-free RNase A, the fixed cells were stained with 5 μg/mL PI at 4 °C for 30 min. Finally, the cellular DNA content was tested on a BD FACScalibur flow cytometer (BD bioscience, San Jose, CA), and the cell cycle distribution and percentage of apoptotic cells were calculated.

2.5. Flow cytometric analysis for apoptosis

The cells were cultured in 6-well plates and treated with LCD for indicated time or concentration, and then the cells were trypsinized and collected as described above. After the cells were resuspended in 200 μL of binding buffer (BD Biosciences, San Diego, CA, USA), an amount of 5 μL Annexin V-FITC was added to the cells and incubated for 15 min at 37 °C in the dark. Next, 10 μL PI was added and incubated on ice for 10 min in the dark. The population of Annexin-V-positive cells was analyzed by flow cytometry using the green and red fluorescence channels within 1 h, and the percentages of apoptotic cells were calculated.

2.6. Dominant negative mutation of AMPKα2

The dominant negative AMPKα2 (T172A, termed as "dnAMPKα2") had been described in our previous publication (Yu et al., 2008). Cells were seeded onto 6-well plates for 24 h and transfected with 1 μg of dnAMPKα2 cDNA/well using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Briefly, the cell culture media were changed to serum-free media 1 h prior to transfection, and 1 μg of dnAMPKα2 cDNA and 2.5 μL of transfection reagent were diluted in 200 μL of serum-free medium, respectively. The two solutions were then gently mixed and incubated at room temperature for 20 min. After that the mixture was added into cell cultures, and they were replaced with complete media after 6 h. Control cells were transfected with the empty vector, and the following experiments were performed 24 h after transfection.

2.7. Western blotting analysis

The cells were cultured in 6-well plates and were treated with LCD for indicated time or concentration as described above. Cells were then harvested, lysed using RIPA cell lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 3 mM sodium pyrophosphate, 1 mM EGTA, 1 mM Na_2EDTA, 50 mM NaF, 1% NP-40, 1% sodium deoxycholate, 1 mM glycerol phosphate, 1× protease/phosphatase inhibitor cocktail, 1 mM PMSF (phenylmethanesulfonyl fluoride), and 1 mM Na_3VO_4). The lysates were centrifuged at 12,000*g* for 10 min at 4 °C, and the resulting supernatant was used as the cytosolic fraction. The total protein concentration was determined by BCA (bicinchoninic acid) protein assay kit (Beyotime, Jiangsu, China). Into equal amounts of protein, 5× loading buffer was added and the mixture was boiled at 90 °C for 5 min. A 20-μg aliquot of protein was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and the proteins in SDS-polyacrylamide gel were electro-transferred to polyvinylidene difluoride