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Dihydromyricetin induces autophagy in HepG2 cells involved in inhibition of mTOR and regulating its upstream pathways

Juan Xia [a,1], Shiwei Guo [b,1], Tao Fang [a], Du Feng [c], Xingli Zhang [c], Qingyu Zhang [a], Jie Liu [a], Bin Liu [a], Mingyi Li [a,*], Runzhi Zhu [a,*]

[a] Laboratory of Regenerative Medicine, Department of Hepatobiliary Surgery, Affiliated Hospital of Guangdong Medical College, Zhanjiang 524001, China
[b] Tumor Immunology and Gene Therapy Center, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University, Shanghai 200433, China
[c] Key Laboratory of Age-associated Cardiac-cerebral Vascular Disease of Guangdong Province, Institute of Neurology, Affiliated Hospital of Guangdong Medical College, Zhanjiang 524001, China

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ABSTRACT

Dihydromyricetin (DHM), a bioactive flavonoid compound extracted from the stems and leaves of *Ampelopsis grossedentata*, has oxidation resistance, anti-tumor and free radical scavenging capabilities. In this study, we found that DHM-induced autophagy inhibited the cell proliferation in HepG2 cells. The transmission electron microscopy results showed that DHM induced significantly autophagosome characteristics like autophagolysosome containing degraded cellular content. GFP labled LC3 plasma transfection showed that LC3 largely diffused to punctate structures with DHM treatment, while lysosomal-rich/acidic compartments detected using LysoTracker Red staining. In addition, DHM promoted the expressions of LC3-II and Beclin-1 in a dose- and time-dependent manner. Further study showed that DHM suppressed the activation of mTOR (mammalian targets of rapamycin) involved in regulating its upstream signaling pathways including extracellular signal-regulated kinase 1/2 (ERK1/2), AMPK (AMP-activated kinase) and class III phosphatidylinositol 3-kinase/phosphoinositide-dependent protein kinase 1/protein kinase B (PI3K/PDK 1/Akt) pathways. Taken together, all the results demonstrated that DHM-induced autophagy inhibited the cell proliferation in HepG2 cells, the possible mechanism involved in inhibition of mTOR activation and regulating the related upstream signaling pathways.

1. Introduction

Autophagy, an evolutionarily conserved catabolic process, plays a role as homeostasis regulator by removing excessive or unnecessary proteins and damaged or aged organelles (Kondo and Kondo, 2006). In the process of tumor development, autophagy is activated under multiple stresses, such as hypoxia, endoplasmic reticulum (ER) stress and nutrient starvation. In addition, autophagy is always observed in cancers when treated with broad-spectrum or targeted chemotherapeutic agents (Chen and Debnath, 2010; Kondo et al., 2005). LC3, widely used to monitor autophagy, exists in two forms soluble LC3-I and lipidated LC3-II, LC3-II is associated with autophagosome undergoing autophagy (Fulda et al., 2010; Hu et al., 2012). Beclin 1, another biomarker of autophagy, which also has a key role at the beginning of autophagy (Capparelli et al., 2012; Pattingre et al., 2005).

mTOR is considered as a master regulator of autophagy induction, which integrates related upstream signaling pathways into a complex cellular network for regulating autophagy (Chen and Karantza, 2011; Jung et al., 2010). mTOR belongs to the PI3K -related kinase family and impacts most major cellular functions, giving it an outsized role in regulating basic cell behaviors such as growth and proliferation (Laplante and Sabatini, 2012). PI3K activates the serine/threonine kinase Akt, which in turn induces the phosphorylation and activation of mTOR through a cascade of regulators (Xie et al., 2013). Previous studies also reported that kinases ERK 1/2 and AMPK were involved in signal to raptor-mTOR by phosphorylating tuberous sclerosis complex 1 (TSC2) and regulating the stability or GAP activity of the tuberous sclerosis complex 1/2 (TSC1/2) heterodimer (McCubrey et al., 2012; Sarbassov et al., 2005). Inhibition of the phosphorylation of mTOR down -regulated phosphorylation of ribosomal S6 kinase (S6K), direct

Abbreviations: DHM, dihydromyricetin; mTOR, mammalian targets of rapamycin; ERK1/2, extracellular signal-regulated kinase 1/2; AMPK, AMP-activated kinase; PI3K, class III phosphatidylinositol 3-kinase; PDK 1, phosphoinositide-dependent protein kinase 1; Akt, protein kinase B; p70S6k/p85S6k, phosphorylation of ribosomal p70S6/p85S6k kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; GFP, green fluorescent protein; TEM, transmission electron microscopy.

* Corresponding authors. Tel./fax: +86 759 2387596.
E-mail addresses: hepatolab@163.com, hepatolab@gmail.com (R. Zhu).

[1] Juan Xia and Shiwei Guo contributed equally to this work.

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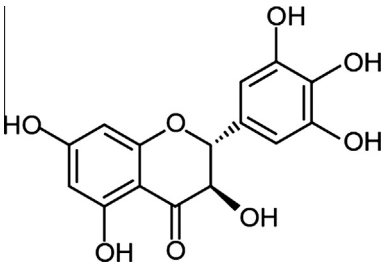

Fig. 1. The chemical structure of DHM.

targets of mTOR, which is crucial for conducting protein translation associated with cellular functions (Feng et al., 2005). Understanding the roles of autophagy in cancers, which has led to recent advances in the pharmacologic manipulation of autophagic pathways as a therapeutic strategy for cancer (Amaravadi et al., 2011; Ding et al., 2011).

DHM, one kind of flavonoids compound, is extracted from the stems and leaves of *Ampelopsis grossedentata* (Fig. 1). Ampelopsis, widely distributed in tropical and subtropical regions, is a member of the Vitaceae family and used in Chinese traditional medicine for treating liver disorders caused by HBV (Pang et al., 2011). Previous studies documented that DHM has oxidation resistance, anti-tumor, free radical scavenging capabilities and so forth (Blackman, 2013; Li et al., 2011; Liu et al., 2009). In our lab, we have found that DHM inhibited the cell proliferation in HepG2 cells (Wu et al., 2013). In order to further study the mechanisms of anti-tumor effect by DHM on HepG2 cells. We explore whether DHM induced autophagy for the first time to guide further study of its antitumor effect.

2. Materials and methods

2.1. Reagents and antibodies

DHM, purchased from Sigma (St. Louis., USA), was dissolved at a concentration of 50 mmol/L in dimethysulfoxide (DMSO) as a stock solution, stored at −20 °C, and diluted with RPMI-1640 medium to the desired working concentrations. The final concentration of DMSO did not exceed 0.4% (V/V) throughout the study. Bafilomycin A1 was from Sigma (St. Louis., USA) and diluted to a final concentration of 100 ng/mL. LysoTracker Red and hochest33342 were obtained from Beyotime (Haian, China).

The primary antibody to LC3 was obtained from abcam and Beclin 1 was from Santa Cruz Biotechnology (Texas, USA). Other Primary antibodies to mTOR, p-mTOR, p70S6K/p85S6K, p-p70S6K/p-p85S6K, PI3K P85, PI3K P110, Akt, p-Akt, ERK1/2, p-ERK1/2, AMPK, p-AMPK, GAPDH were all obtained from Cell Signaling technology (Danfoss, USA). The horseradish peroxidase-conjugated (HRP) anti-rabbit IgG secondary antibody was from EarthOx (San Francisco, USA).

2.2. Cell culture

HepG2 cells were obtained from Cancer Cell Repository (Shanghai Cell Bank). Cells were maintained in RPMI-1640 (Gibco, California, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) (Gibco), at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Cell proliferation assay

The cells were plated at a density of approximately 10×10^3 viable cells per well in 96-well microtiter plates. After incubation with Bafilomycin A1 with or without DHM for the indicated time, 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diph-enyltetrazoliumbromide

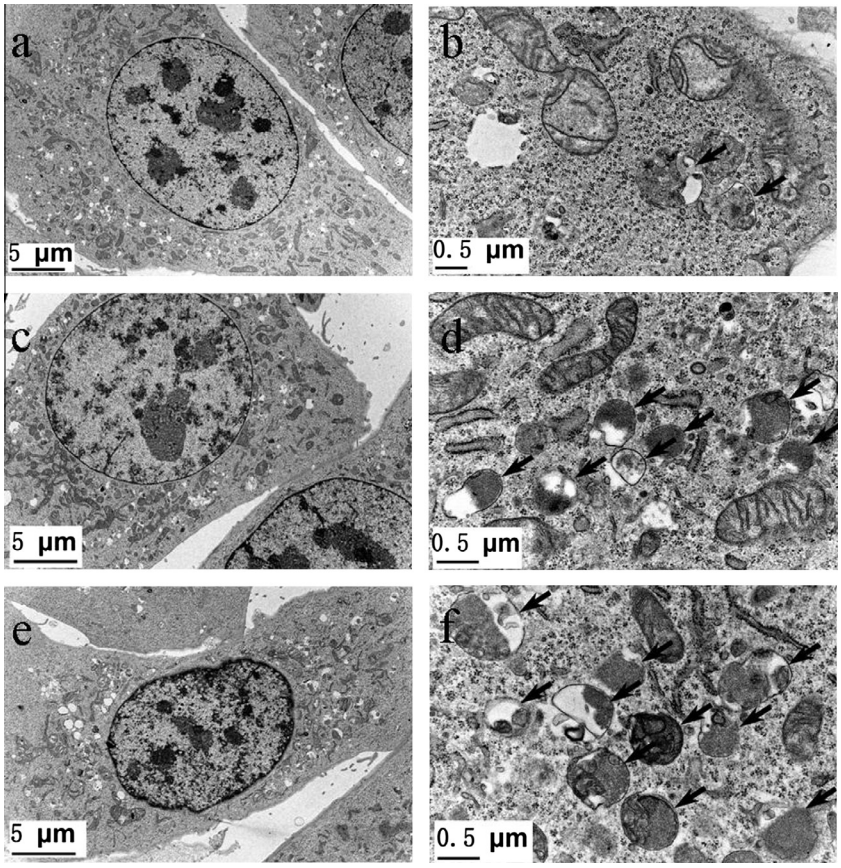

Fig. 2. Transmission electron microscopy images of HepG2 cells after treated with DHM. HepG2 cells were treated with 0, 10, 50 μmol/L DHM for 24 h. Cells were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetraoxide. Then the cell pellets were embedded in Epon. Representative areas were chosen for ultrathin sectioning and observed on a JEM1400 (Japan) transmission electron microscope at 80 kV. (a and b) Control; (c and d) 10 μmol/L; (e and f) 50 μmol/L. Bars: (a, c, and e) 5 μm; (b, d, and f) 0.5 μm. Black arrows, autophagosomes or autolysosomes.