Biomedicine & Pharmacotherapy 98 (2018) 709–718

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Alpha, 2'-dihydroxy-4,4'-dimethoxydihydrochalcone inhibits cell proliferation, invasion, and migration in gastric cancer in part *via* autophagy

Boshun Wan[a,b,1], Junqiu Zhu[c,1], Qing Chang[b], Haihua Zhou[b], Zhan Shi[c], Li Min[d], YueJiao Cai[e], Honggeng Guan[a,*]

[a] Department of General Surgery, The First Affiliated Hospital of Soochow University, Soochow, 215000, PR China
[b] Department of General Surgery, JiaDing District Central Hospital, Shanghai University of Medicine & Health Sciences, Shanghai, 201800, PR China
[c] Department of Oncology, HuaDong Hospital, FuDan University, Shanghai, 200040, PR China
[d] Department of Anorectal, JiaDing Traditional Chinese Medicine Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, 201800, PR China
[e] No.2 Department of Oncology, LongHua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, 200030, PR China

ARTICLE INFO

Keywords:
Alpha, 2'-dihydroxy-4,4'-dimethoxydihydrochalcone
Autophagy
MKN45
PDX

ABSTRACT

Gastric cancer is a leading cause of mortality worldwide. Alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone is a type of limonoid mainly isolated from *Cedrela odorata* (Meliaceae) that has been shown to suppress cell proliferation in several human carcinoma cell lines. In this study, we investigated the anti-cancer ability of alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone and its underlying mechanism in MKN45 cells. Alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone induced excess reactive oxygen species (ROS) accumulation. Transwell and wound healing assays demonstrated that alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone inhibited the invasion and migration ability of MKN45 cells. Moreover, autophagy-related proteins Beclin-1, Atg5, and Atg7 were up-regulated. Light chain 3 (LC3)-I protein was converted into LC3-II under alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone exposure. Transmission electron microscopy demonstrated that alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone treatment resulted in the formation of autophagosomes. Immunofluorescence assays suggested that alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone treatment elicited dot formation of green fluorescent protein (GFP)-LC3. 3-methyladenine (3-MA), an autophagy inhibitor, demonstrated that autophagy promoted death in MKN45 cells. Western blotting showed that ROS/mitogen activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways play crucial roles in the intrinsic mechanism of alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone's activity. The combined use of *N*-acetyl-$_{L}$-cysteine (NAC) or U0126 validated the regulatory role of ROS/MEK/ERK signaling pathways. Alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone administration inhibited the growth of MKN45 xenograft tumors in nude mice and suppressed Ki67 expression. More importantly, a similar effect was achieved in a patient-derived xenograft (PDX) model, which is more relevant to clinical application. Taken together, alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone has the potential to be further developed into an anti-tumor agent for clinical treatment of gastric cancer.

1. Introduction

Cancer is a leading cause of mortality globally, and gastric cancer is one of the most lethal malignancies and the third leading cause of tumor-related death [1]. The clinical outcome for gastric cancer sufferers remains poor, with a 5-year survival rate of only about 20% [2]. Its tumors are characterized by abnormal reproduction and reduced death. Most anti-cancer drug research and development has thus focused on promoting apoptosis as a means of eliciting tumor cell death. Cancer cell apoptosis is commonly triggered by outside stimulation or drugs via related signaling pathways and consequent cytochrome C release, caspase family activation, and finally DNA destruction [3]. However, the effects of anti-cancer drug are still limited, and side-effects are often uncontrollable. As another promising means to promote

Abbreviations: TEM, transmission electron microscope; DCFDA, dihydrodichlorofluorescein diacetate; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; ROS, reactive oxygen species; PDX, patient derived xenograft; MMP, matrix metalloproteinases; GFP, green flurorescent protein; 3-MA, 3-methyladenine; NAC, N-acetyl-L-cysteine; LC-3, Light chain 3; IHC, Immunohistochemistry

* Corresponding author.
E-mail address: guanhonggeng@suda.edu.cn (H. Guan).
[1] These authors contributed equally for this work.

https://doi.org/10.1016/j.biopha.2017.12.081
Received 11 September 2017; Received in revised form 26 November 2017; Accepted 18 December 2017

cell death, autophagy regulation by anti-cancer agents has attracted extensive attention [4]. Although still controversial, it is commonly acknowledged that autophagy can regulate tumorigenesis, survival, and proliferation [5]. Compounds that affect autophagy regulation are promising anti-tumor regents that can kill cancer cells via non-apoptotic pathways [6].

The Meliaceae is a plant family cultivated worldwide, and some genera are used for medicinal purposes to treat malaria, infections, and cancer [7,8]. A number of natural agents and secondary metabolites have been isolated from Meliaceae, for example limonoids, flavonoids, and steroids [9]. Among these, limonoids are highly oxidized secondary metabolites of triterpenes with numerous pharmacological activities, such as anti-inflammation, anti-diabetic, anti-cancer, and anti-septic activities [10,11]. Alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone, with another more famous name odoratol, is a type of limonoid mainly isolated from *Cedrela odorata* (Meliaceae). Compounds with structures highly similar to alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone include hispidol A, piscidinol A, pentaol, nilocetin, iso-alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone, odoratone, and 22S,3α-dihydrotirucala-7,24-dien-23-one [12]. Alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone promotes the recovery of the photosystem II donor side [13]. In 2010, Caza et al. revealed that alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone exhibits cytotoxicity against breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460), and melanoma (A375-C5) cells via cell cycle arrest but not apoptosis [14]. In addition, a compound with a similar structure has been found to exert cytotoxicity against human carcinoma cells [15,16]. Therefore, alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone is a bioactive limonoid with anti-cancer potential. However, although limonoids induce cell cycle arrest and exert cytotoxicity against numerous human cancer cell lines, whether limonoids can elicit autophagy and other cellular responses in carcinomas cells is still unclear.

Here, we explored the activities of alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone against human gastric cancer. MKN45 human stomach cancer cells were employed as a model *in vitro*. MKN45 cell xenograft tumors and patient-derived xenograft (PDX) tumors were employed as *in vivo* models. Additionally, we investigated the underlying mechanism of alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone's anti-cancer effect.

2. Materials and methods

2.1. Reagents and standard preparation

A standard alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone preparation (purity > 98%) was purchased from RoChen Pharmaceutical and Biological Products (Rochen, Shanghai, China). A CCK-8 kit was purchased from Beyotime Institute of Biotechnology (Beyotime, Haimeng, Jiangsu, China). The SYTO™ green death cell kit was purchased from Invitrogen (Grand Island, New York, USA). An Annexin V&PI Kit was provided from Selleck (Selleck Chemicals, Houston, TX, USA). Matrigel matrix was from BD (BD Bioscience, Bedford, MA, USA). All other chemicals unless otherwise stated were provided from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Primary antibodies and HRP-labeled secondary antibodies were provided by Cell Signaling Technology (CST, Boston, MA, USA).

2.2. Cell culture

MKN45 human gastric cancer cells were purchased from the American Type Culture Collection (ATCC, VA, USA). Cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, USA) with 10% fetal calf serum containing antibiotics (100 IU/mL penicillin and 100 IU/mL streptomycin) at 37 °C in an atmosphere containing 5% CO_2. Cells were divided into vehicle group (DMSO), three alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone groups grown under alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone exposure (8, 16 and 32 μM), and two alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone combination groups (combined with *N*-acetyl-$_L$-cysteine (NAC) or U0126). The standard alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone preparation was dissolved in DMSO at a final DMSO concentration of less than 0.1% for cell experiments. DMSO of the same volume was added to the vehicle group.

2.3. Cell viability and cell death assays

To measure cytotoxicity and proliferation inhibition of alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone against MKN45 cells, CCK-8 assay was employed using WST-8 reagents as previously reported [17]. Cells were treated with increasing concentrations of alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone for specified times. Ten microliters of CCK-8 solution for per well were added followed by incubation for another 4 h. The absorbance at 450 nm was recorded with a microplate reader (PerkinElmer Inc, Waltham, MA, USA). Furthermore, to identify whether inhibited proliferation was due to cell death, Sytox™ green dye was added to treated MKN45 cells at 1 μM final concentration per well following the protocol of Daniel et al. [18]. After a 10 min incubation, fluorescence was probed using a Spectramax M5 fluorescence plate reader at excitation of 488 nm and emission of 530 nm using a Corona MTP 601F fluorescence micro-plate reader (HITACHI, Tokyo, Japan).

2.4. Apoptosis assays

The cell apoptosis rate in alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone-treated cells was determined as reported previously [3]. Cell apoptosis was analyzed according to the manufacturer's direction. Approximately 2-5 × 10^5 MKN45 cells were washed and centrifuged, then cells were suspended in 100 μL Binding Buffer. After that, 5 μL Annexin V-FITC and 5 μL PI staining solution were added into each cell suspension per 100 μL. After a 15-min incubation, 400 μL Binding Buffer was added to cell suspensions. Finally, the apoptosis ratio was determined using FACS-Calibur flow cytometry (BD Biosciences, San Jose, CA, USA).

2.5. Wound healing migration assays

Wound healing assays were performed to investigate migration under alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone treatment following an established method [19]. When cell density in 6-well plates was approximately 80%, a sterile 200 μL pipette tip was used to make a linear wound about 1 mm wide. Cells were washed to remove superfluous floating cells and debris, then co-incubated with mitomycin-C (5 μg/mL) to inhibit cell proliferation [20]. Wound closure was photographed at 0 and 24 h after wound induction using an inverted microscope (D5100, Nikon, Tokyo, Japan). Wound closure was defined using Image J software (Image J 1.47v, NIH, Thornwood, Bethesda, MD, USA) by comparing average gaps in each group between migrating cells of opposing wound edges. The effect of alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone on the wound healing ratio was quantified by comparing the ratio of cell area at 0 h and 24 h.

2.6. Matrigel invasion assay

Invasion assays were performed using transwell chambers precoated with 40 μL of 1 mg/ml Matrigel matrix [21]. Vehicle- or alpha, 2′-dihydroxy-4,4′-dimethoxydihydrochalcone-induced cells at the proper density were trypsinized and suspended in 500 μL DMEM FBS-free medium and plated in the upper wells. Meanwhile, 500 μL DMEM containing 10% FBS was added to the lower well and employed as an attractive stimulus. After 24 h, cells were fixed and stained using Cresyl