Phytomedicine 22 (2015) 902–910

Contents lists available at ScienceDirect

Phytomedicine

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

Guttiferone K induces autophagy and sensitizes cancer cells to nutrient stress-induced cell death

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ARTICLE INFO

Article history:
Received 12 December 2014
Revised 1 June 2015
Accepted 12 June 2015

Keywords:
Apoptosis
Autophagy
Guttiferone K
Natural compound
Starvation

ABSTRACT

Background: Medicinal plants have long been an excellent source of pharmaceutical agents. Autophagy, a catabolic degradation process through lysosomes, plays an important role in tumorigenesis and cancer therapy.
Purpose: Through a screen designed to identify autophagic regulators from a library of natural compounds, we found that Guttiferone K (GUTK) can activate autophagy in several cancer cell lines. The objective of this study is to investigate the mechanism by which GUTK sensitizes cancer cells to cell death in nutrient starvation condition.
Methods: Cell death analysis was performed by propidium iodide staining with flow cytometry or Annexin V-FITC/PI staining assay. DCFH-DA staining was used for intracellular ROS measurement. Protein levels were analyzed by western blot analysis. Cell viability was measured by MTT assay.
Results: Exposure to GUTK was observed to markedly induce GFP-LC3 puncta formation and activate the accumulation of LC3-II and the degradation of p62 in HeLa cells, suggesting that GUTK is an autophagy inducer. Importantly, hydroxychloroquine, an autophagy inhibitor, was found to significantly prevent GUTK-induced cell death in nutrient starvation conditions, suggesting that the cell death observed is largely dependent on autophagy. We further provide evidence that GUTK inhibits Akt phosphorylation, thereby inhibiting the mTOR pathway in cancer cells during nutrient starvation. In addition, GUTK causes the accumulation of reactive oxygen species (ROS) and the phosphorylation of JNK in EBSS, which may mediate both autophagy and apoptosis.
Conclusion: These data indicate that GUTK sensitizes cancer cells to nutrient stress-induced cell death though Akt/mTOR dependent autophagy pathway.

Introduction

Autophagy (type II cell death) refers to morphologically distinctive modes of programmed cell death. The role of autophagy in cancer remains controversial. On the one hand, autophagy can act as a survival mechanism that provides energy during metabolic stress and protects cancer cells from apoptotic or necrotic cell death induced by various anticancer treatments (Rubinsztein et al., 2012; Maes et al., 2013); on the other hand, recent studies have shown that autophagy is also a cell death mechanism and a response to various anticancer therapies in many types of cancer cells (Gozuacik et al., 2007; Fulda, 2012; Green et al., 2014). Autophagy in response to cellular stress states serves as a potent death signal, as in the case of chemotherapy-induced autophagy, a specific non-apoptotic death pathway has been triggered off. Autophagy upregulation may preserve cellular fitness and genome integrity to prevent cancer development and progression. Accelerating autophagy in apoptosis-resistant

Abbreviations: BAFA1, bafilomycin A1; DCFH-DA, dichlorofluorescin diacetate; DMEM, Dulbecco's modification of Eagle's medium; EBSS, Earle's balanced salt solution; GUTK, Guttiferone K; HCQ, hydroxychloroquine; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; NAC, N-acetyl-L-cysteine; OC, oblongifolin C; PI, propidium iodide; PPAPs, polycyclic polyprenylated acylphloroglucinols; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.phymed.2015.06.008

cancer cells would be an attractive alternative strategy in cancer therapy. Starvation is a unique biological situation in which the activation of autophagy and apoptosis occur simultaneously. Many cancer cells have the ability to tolerate nutrient deprivation before angiogenesis but have the ability to survive under extreme conditions, such as conditions with low nutrient and oxygen supplies. Troglitazone, LY294002 and an insulin sensitizer have been used for treatment of nutrient deprivation and have been demonstrated to kill cancer cells only under nutrient-deprivation conditions (Awale et al., 2006; Izuishi et al., 2000).

Reactive oxygen species (ROS) plays a key role in cancer cell death. Under starvation or stress conditions, ROS are increased and essential for the induction of autophagy (Chen et al., 2009). Many anticancer drugs induce ROS generation, which can serve as either a direct mechanism of cell death or an important factor to induce drug resistance. Moreover, in some cancer cells, the phosphorylation of JNK is a crucial factor for autophagic cell death (Shimizu et al., 2010; Sui et al., 2014). Above all, autophagy can be activated by ROS or the JNK pathway.

The Akt/mTOR signaling pathway plays a crucial regulatory role in cellular proliferation and survival, glucose metabolism and angiogenesis (O'Reilly et al., 2006). mTOR is phosphorylated at Ser2448 via the PI3-Kinase/Akt signaling pathway and autophosphorylated at Ser2481 (Rabinowitz et al., 2010). It has been reported that mTOR is frequently inappropriately activated in many cancer types, and the development of drugs that inhibit mTOR is an alluring therapeutic target in cancer therapy.

Garcinia species have shown various bioactivities, such as antitumor, anti-inflammatory, antiviral and neuroprotective effects. Guttiferone K was isolated from the Garcinia *yunnanensis* Hu (Xu et al., 2008). It has been found that Guttiferone K induces G0/G1 cell cycle arrest in HT-29 cells by down-regulating cyclin D1 and D3 and cyclin-dependent kinases 4 and 6 and thereby stimulates caspase-dependent apoptosis. In addition, Guttiferone K was observed to effectively decreased the tumor volume in a xenograft mouse model when used alone or in combination with 5-fluorouracil (Kan et al., 2013). In this study, we screened novel autophagic regulators from a library of natural compounds extracted from *Garcinia* species using HeLa cells stably expressing GFP-LC3. We further examined the molecular mechanisms underlying Guttiferone K-mediated cell death in nutrient starvation conditions. Our results show that Guttiferone K induces autophagy and sensitizes cancer cells to nutrient deprivation-induced cell death. In addition, GUTK was found to cause ROS generation, JNK activation and Akt/mTOR inhibition under nutrient deprivation conditions but did not affect full medium-cultured cells. Importantly, GUTK-induced cell death was observed to be inhibited by hydroxychloroquine (HCQ), an autophagy inhibitor, which suggests that the GUTK-induced cell death is largely dependent on autophagy induction. Our results suggest that GUTK is a promising anticancer compound that targets nutrient-stressed cells by modulating the autophagy signaling pathway.

Materials and methods

Materials

Guttiferone K (GUTK, purity > 98%) was isolated from the Garcinia *yunnanensis* Hu. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and penicillin–streptomycin were obtained from Gibco (Carlsbad, CA, USA). Earle's balanced salt solution (EBSS), propidium iodide (PI) and 2',7'-dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Primary antibodies of caspase-3, PARP, p-Akt, Akt, p-Erk, Erk and p-mTOR were purchased from cell signaling. LC3B antibody was from Sigma-Aldrich. SQSTM1/p62 antibody was from MBL. GAPDH antibody was from Abcam. Anti-mouse antibodies and anti-rabbit antibodies were purchased from KPL.

Cell culture

HeLa, Capan-2 and CNE cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were maintained in DMEM supplemented with 10% FBS and 10 U/ml penicillin–streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C. For nutrient starvation, the medium with serum was removed and washed by PBS for three times and EBSS was applied.

GFP-LC3 translocation and quantitative analyses

The GFP-LC3 translocation assay was performed as previously described (Lao et al., 2014; Yang et al., 2014). Briefly, the cells were transfected with GFP-LC3 plasmid using lipofectamine 2000. One day after transfection, the cells were treated with 20 μM GUTK for 24 h prior to fixation. Image acquisition was performed using a fluorescence microscope (Olympus IX 83, Tokyo, Japan). The number of GFP-LC3 dots was counted in at least 100 cells from randomly placed positions within each sample.

Propidium iodide staining for DNA content

The cells were fixed with 70% ethanol in PBS overnight. For cell cycle distribution analysis, cells were counterstained with PI, and their DNA contents were analyzed using a BD FACS Calibur flow cytometer (Becton & Dickinson Company, Franklin Lakes, NJ, USA). The data were analyzed with the FlowJo 7.6.1 software.

Annexin V-FITC/PI staining assay

The effect of GUTK on cell viability was assessed using flow cytometry by staining with Annexin V/PI (Yang et al., 2014). In brief, the cells were cultured for 6 h after GUTK treatment and washed twice with ice-cold phosphate-buffered saline. A total of 1×10^6 cells were resuspended in 400 μl of binding buffer, and 5 μl of 2 mg/ml Annexin V and 5 μl of 20 μg/ml PI were then added. After 15 min of incubation in the dark, flow cytometry was performed.

Western blotting analysis

The cell lysate containing 20 μg of protein was fractionated by SDS-PAGE, and then proteins were transferred to a polyvinylidene difluoride membrane. The membranes were incubated at 4 °C overnight with different primary antibodies diluted in 3% bovine serum albumin in washing buffer. Afterward, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Protein bands were visualized using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA).

Detection of ROS generation

The intracellular ROS production was measured as described previously (Cheng et al., 2013; Kim et al., 2013). The cells were stained with 100 μM DCFH-DA for 10 min at 37 °C. After rinsing with PBS, the cells were observed immediately under a fluorescence microscope.

Statistical analysis

All data were given as mean ± standard deviation (SD). The significance of difference between groups was estimated by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Values of $P < 0.05$ were considered to be significant.