Garcinone E Blocks Autophagy Through Lysosomal Functional Destruction in Ovarian Cancer Cells

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Abstract

Background: High proliferative rate of cancer cells requires autophagy to maintain nutrient supply and intracellular homeostasis. As a result, impairing autophagic flux could be a novel strategy of cancer therapy. Aims and Objectives: In this study, the mechanism of a xanthone derivative isolated from Garcinia mangostana, garcinone E (GE), was investigated. Materials and Methods: Fluorescence assay was used to observe the accumulation and location of autophagosome and lysosome. Flow cytometry with Lyso-tracker red, MDC, and AO staining were applied to evaluate the lysosome accumulation and cellular acidity. Western blot and RT-qPCR were performed to evaluate the protein and mRNA levels, respectively. Results: GE could cause enhancement of LC3II and p62 and the accumulation of autophagosome and lysosome. Meanwhile, it limited the protein level of Rab7, increased lysosomal pH, and inhibited the maturation of lysosomal hydrolases such as Cathespins L, therefore blockaded the fusion of autophagosome and lysosome. Moreover, GE acted as a TFEB modulator by downregulating its protein level, which might contribute to autophagy dysfunction in ovarian cancer cells. Conclusions: GE interfered autophagosome–lysosome fusion in cancer cells, which demonstrated its application as an autophagy regulator and a potential therapeutic agent.

Keywords: Anticancer, autophagy, garcinone E, lysosome, TFEB

INTRODUCTION

Autophagy is a widespread mechanism in most of eukaryotic cells. It is a catabolic process that degrades their own organelles and cytoplasmic components of the cells to maintain the material recirculation and energy supply for intracellular homeostasis. During the process, the pending proteins or organelles are wrapped into autophagosome, a double membrane structure. Then, the whole autophagosome is shipped toward lysosome and fuses with it that forms autolysosome. Subsequently, the proteins and organelles are degraded by the hydrolases within the autolysosome, and the nutrient is reused to sustain cell survival, especially during nutrient deprivation or other stresses. Autophagy is usually induced by stimulation such as nutrient deprivation and hypoxia to tolerate hostile environment and maintain basic cellular activities. Ischemia and hypoxia occur inside the tumor due to its rapid growth. Therefore, autophagy is required to supply material and energy for rapid cell proliferation.

Despite the continuous development of medicine, chemoresistance remains one of the major problems of conquering cancer. Evidences have been shown that chemoresistance is closely related to the appearance of autophagy. By activating autophagy, cancer cells trigger survival-related signaling pathways and generate chemoresistant mechanisms against the anticancer drugs. Another high risk of malignant cancer is metastasis. Moreover, autophagy is also been proved crosstalking with epithelial–mesenchymal transition progress and improving tumor metastasis. Not only promotes cell migration and invasion, autophagy also enables cancer cells to maintain stemness, accelerate extracellular matrix degradation, and avoid anoikis. As a result, developing autophagy inhibitors as anticancer agent is a prospective strategy in the fight against cancer.

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The species of *Garcinia* L. have been used in traditional Chinese medicine application for a long time. Xanthones, such as gambogic acid, are considered the major pharmacological ingredients.\(^9\) Garcinone E [GE, Figure 1a] is a xanthone derivative that first isolated from pericarps of *Garcinia mangostana*, which spread in tropical Asian area including Hainan Province in China.\(^{10}\) Its anticancer effect had been tested on hepatocellular, breast, colorectal, and oral cancer...
cell lines. Recently, we found GE-exerted remarkable anticancer properties in ovarian cancer cells. It triggered endoplasmic reticulum (ER) stress and induced apoptosis and inhibited cell migration and invasion by suppressing the activities of Rho GTPases and matrix metalloproteinases. In the meantime, we also spotted GE affecting autophagy process in cancer cells. Therefore, in this study, we would further investigate and discuss the autophagy-interfering mechanisms of GE.

**Materials and Methods**

**Cell culture**
The human ovarian cancer HEY cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), and the human ovarian cancer A2780 cell line was obtained from KeyGEN Biotech (Nanjing, Jiangsu, China). All cell lines were cultured in DMEM (GIBCO, Carlsbad, CA, USA) and added with 10% FBS (GIBCO), 100 U/mL penicillin, and 100 ng/mL streptomycin (GIBCO) and cultured in a humidified incubator containing 5% CO₂ at 37°C. The morphological characteristics of the cells were observed and imaged using an Olympus IX73 microscope (Olympus, Tokyo, Japan).

**Fluorescence assay**
GFP-LC3-HeLa cells were seeded in 96-well plates. After 24 h of GE treatment, the supernatants were removed, and the cells were rinsed with phosphate-buffered saline (PBS) and fixed by 4% PFA for 15 min before they were stained with DAPI for 10 min at 25°C. Afterward, the cells were imaged using an InCell Analyzer 2000 system (GE Healthcare, Uppsala, Sweden).

**Lysotracker-red staining assay**
HEY cells were seeded in 12-well plates. After 24 h of GE treatment, the supernatants were removed, and the cells were rinsed with PBS and incubated at 37°C for 30 min with lysotracker red dye at a dilution of 1:15,000 (Beyotime, Nantong, Jiangsu, China) and medium containing 0.5% FBS. Then, the cells were trypsinized, collected, and analyzed by a BD FACS Canto™ flow cytometer (BD Biosciences, San Jose, CA, USA).

**Monodansylcadaverine staining assay**
HEY cells were seeded in 12-well plates. After 24 h of GE treatment, the supernatants were removed, and the cells were rinsed with PBS and incubated at 37°C for 30 min with medium containing 0.5% FBS and monodansylcadaverine (MDC) (Sigma) at a dilution of 1:1000. Then, the cells were trypsinized, collected, and analyzed by a BD FACS Canto™ flow cytometer.

**AO staining assay**
HEY cells were seeded in 12-well plates. After 24 h of GE treatment, the supernatants were removed, and the cells were rinsed with PBS and incubated at 37°C for 30 min with medium containing 0.5% FBS and AO at a dilution of 1:1000. Then, the cells were trypsinized, collected, and analyzed by a BD FACS Canto™ flow cytometer.

**Real-time-polymerase chain reaction analysis**
The methods of RNA extraction and reverse transcription, and real-time polymerase chain reaction (PCR) were described as previous publication. All the 2ΔΔCt values were normalized with the reference gene (GAPDH). The primers (Invitrogen) used in this study were presented as follows.

**Western blot analysis**
The methods of protein extraction and Western blotting were described as previous publication. The polyvinylidene fluoride membranes were probed with specific primary antibodies against p62, LC3, TFEB, CTSL, CTSD, CTSB, Rab7, and GAPDH (Cell Signaling Technology, Beverly, MA, USA). GAPDH antibody was used to verify the equal protein loading. The images were visualized by ChemiDoc MP Imaging System. Protein bands were quantified using Image Lab 5.1.

**Statistical analysis**
All data were presented as mean values and standard deviation. Significance was analyzed by GraphPad Prism (Demo, Version 5) with ANOVA and Tukey’s multiple comparison test. P value of less than 0.05 was considered as statistical significance (P<0.05, *), and the one less than 0.01 was considered as extremely distinct statistical significance (P<0.01, **).

**Results**
**Garcinone E regulated autophagy in cancer cells**
LC3 protein is often used as the autophagosomal marker to monitor the autophagic activity. During autophagy, the cytoplasmic form LC3-I is recruited to autophagosome membranes and lipidated as LC3-II, and the conversion from LC3-I to LC3-II relates to activation of autophagy. Garcinone E increased lysosome accumulation in cancer cells was found remarkably enhanced in a concentration-dependent manner after GE treatment, and the puncta were accumulated and agglomerated. p62 is a ubiquitously expressed cellular protein that serves as a link between LC3 and ubiquitinated substrates. In Figure 1c-e, the protein level of LC3-II and p62 in ovarian cancer cell lines was found significantly increased in concentration- and time-dependent manner after GE treatment, indicating the progress of autophagy in cancer cells might be affected after GE treatment.
correlated with the progress of autophagy. These acidic vesicles contain more than sixty hydrolases for the degradation and recycling of the nutrients within autophagosome to support cell metabolism. Lyso-tracker-red is an alkaeleascent probe that specifically enters and be detained in lysosomes; thus, we used lyso-tracker-red to label the lysosome in HEY cells and detected the fluorescence intensity by flow cytometry. As shown in Figure 2a and b, after 24 h of GE treatment, the fluorescence was remarkably ascended as the enhancement of GE concentration, indicating lysosome in the cells might increase and accumulate after the treatment.

**Garcinone E blockaded autophagic flux by interfering autophagosome–lysosome fusion**

To further investigate the fusion process of autophagosome and lysosome after GE treatment, we evaluated the level of Rab7, a family member of Ras-like GTPase that modulates lysosome biogenesis and governs docking and fusion events. As shown in Figure 2c and d, the levels of Rab7 were reduced after GE treatment in HEY and A2780 cells, suggesting GE might block autophagosome–lysosome fusion, at least partially, by decreasing the expression of Rab7.

**Garcinone E impaired the function of lysosome**

Besides transportation, Rab7 also regulates organelles pH by administrating assembly and function of the V-ATPase on lysosome and late endosome with its effector. Therefore, the pH level of HEY cells was further evaluated by assays using two dyes, MDC and AO. MDC can accumulate in autophagic vacuole and other acidic vesicular organelles and emit green fluorescence that roughly indicates intracellular pH. AO emits green fluorescence in cytoplasm, and the fluorescence turns red when it enters lysosome and be protonated that mediated by the V-ATPase. Thus, its ratio of red/green fluorescence can reflect the function of lysosomal H⁺-ATPase. The results were shown in Figure 3a-d. Contrary to the results of lyso-tracker-red assay, the fluorescence of MDC in HEY cells treated with GE was remarkably reduced compared to the ones in the control group, indicating the acidity of total organelles was neutralized despite the accumulation of lysosome. Moreover, in AO staining assay, the ratio of red/green fluorescence intensity of HEY cells was declined in the treatment group in a concentration-dependent manner as well. These results preliminary confirmed that the pH level of ovarian cancer cells was enhanced after GE treatment, suggesting the function of the acidic organelles, especially lysosome, might be impaired.

The maturation and function of the hydrolases within lysosome is controlled by the pH inside it. As a result, the main group of hydrolases that involve in lysosomal protein degradation, cathepsins (CTSs), were examined. As shown in Figure 3e and f, no significant changes were observed on the mature CTSB and CTSD, whereas the mature CTSL was dramatically decreased in the two cell lines. As the cleavage and maturation (which demonstrates its endopeptidase activity) of CTSL was known relevant to acidic environment inside lysosome, this evidence also supported that GE treatment caused lysosome dysfunction and upregulated its pH.

![Figure 2: Garcinone E blockaded autophagosome–lysosome fusion. (a and b) After 24 h of garcinone E treatment, lysosome in HEY cells was stained with lyso-tracker red and the fluorescence was detected using a flow cytometry. (c and d) The protein levels of Rab7 in HEY and A2780 cells were evaluated by Western blot after 24 h of garcinone E treatment. Quantitation and statistics of the results were obtained. *P < 0.05.](image-url)
Garcinone E diminished the protein level of TFEB

The recent study demonstrated a transcription factor, TFEB, controls the expression of multiple autophagic and lysosomal genes and is highly relevant with lysosome biogenesis.\(^{[24]}\) As shown in Figure 4a-c, we found the protein level of TFEB in ovarian cancer cells remarkably decreased after GE treatment in concentration- and time-dependent manner by Western blot. The expression of Beclin 1, one downstream gene of TFEB that involved in activation of autophagy, also diminished as well, suggesting GE not only diminishing the amount of intracellular TFEB but also might impair its function in modulating autophagy. Meanwhile, quantitative PCR demonstrated that the mRNA level of TFEB was not significantly affected by GE treatment [Figure 4d and e], indicating GE might eliminate TFEB in posttranscription phase. These results suggested that GE-induced TFEB decline might also contribute as a factor on its way of autophagy inhibition.

**DISCUSSION**

Autophagy is a high conserved mechanism that is essential to maintain intercellular homeostasis. The role autophagy plays in cancer evolution and progression has been well described, and the possibility of autophagic regulator for
In the past few years, we have been focused on the mechanism study of autophagy regulator candidates screened from natural products, and we have found autophagy inducers including platycodin D, licochalcone A, baicalein, and glycerrhetinic acid and autophagy inhibitors including dauricine, daurisoline, cepharanthine, and fangchinoline. Lately, we found another autophagy inhibitor, GE, that exhibits promising anticancer effects, which worth deeper investigation. GE blocked the fusion of autophagosome and lysosome, thereby causing their massive accumulation and cellular homeostasis dysregulation. ER stress is a cellular mechanism in response to inter- or extracellular stimulation, which regulates autophagy throughout IRE-1α, PERK, and ATF6 pathways at different stages. For example, the dissociation of BiP and IRE-1α triggers UPR and activates JNK, which promotes autophagy. Moreover, activation of XBP-1 and CHOP would transcriptionally induce autophagy-associated proteins. Our previous study demonstrated that GE could induce ER stress and activate IRE-1α signaling pathway in cancer cells, which might act as a preservative mechanism against the stimulation caused by GE. In this study, the enhancement of autophagosomal biomarker LC3-II and autophagic adaptor protein p62, and the aggregation of autophagosome and lysosome suggested the autophagy process in cancer cells might be affected under the stress caused by GE. Dephosphorylate and translocated into nuclear of TFEB leads to transcription of autophagic-related genes such as ATG9B, BCL2, and BECN1. In this study, we also found the protein level of TFEB in ovarian cancer cells was significantly reduced after GE treatment, which might cause the downregulation of crucial autophagy-associated genes such as BECN1 and impair biogenesis and function of autophagosome and lysosome. GE treatment also results in acidic vesicle damage and lysosomal dysfunction, and blockade of autophagosome–lysosome fusion, which might further sensitized ER stress and cell death. However, the results of this study have not yet thoroughly investigate the role of TFEB played under GE treatment, and overexpression of TFEB in ovarian cancer cells would be involved in our next step study.
CTSs, such as CTSL, are lysosomal peptidases that play important roles in maintaining intercellular homeostasis and vital activity. Not only degrade proteins within cells, but also they contribute to extracellular matrix that accelerate tumor progression and invasion and mediate drug resistance.[36,37] As they are mainly located in lysosome, most of the CTSs are matured and remain active by the acidic environment. As a result, activities of some CTSs were considered related to TFEB.[38,39] In this study, we tested the mature form of three of the most abundant CTSs, i.e., CTSB, CTSD, and CTSL, and the results demonstrated only CTSL was significantly downregulated after GE treatment. It is speculated that the reduction of TFEB caused by GE treatment might result in downregulation of lysosomal-related gene transcription, which disrupts lysosomal function and impacts its acidic environment, and therefore, impairs the activation of CTSL. Yet, we are still trying to test this theory of how the reduction of TFEB is related to the inhibition of CTSL maturation process.

Taken together, GE impaired the autophagy process in ovarian cancer cells, with the accumulation of autophagosome and lysosome. Meanwhile, it blocked autophagosome–lysosome fusion and caused lysosome dysfunction. Moreover, GE might further suppress transcription of relevant autophagic genes, partially due to downregulation of TFEB. Considering its notable antiproliferative and anti-invasive effects that are reported in our previous research, GE has been proved its anticancer potential, which needed to be evaluated further to unveil its mechanism.

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Conflicts of interest
There are no conflicts of interest.

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