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

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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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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.

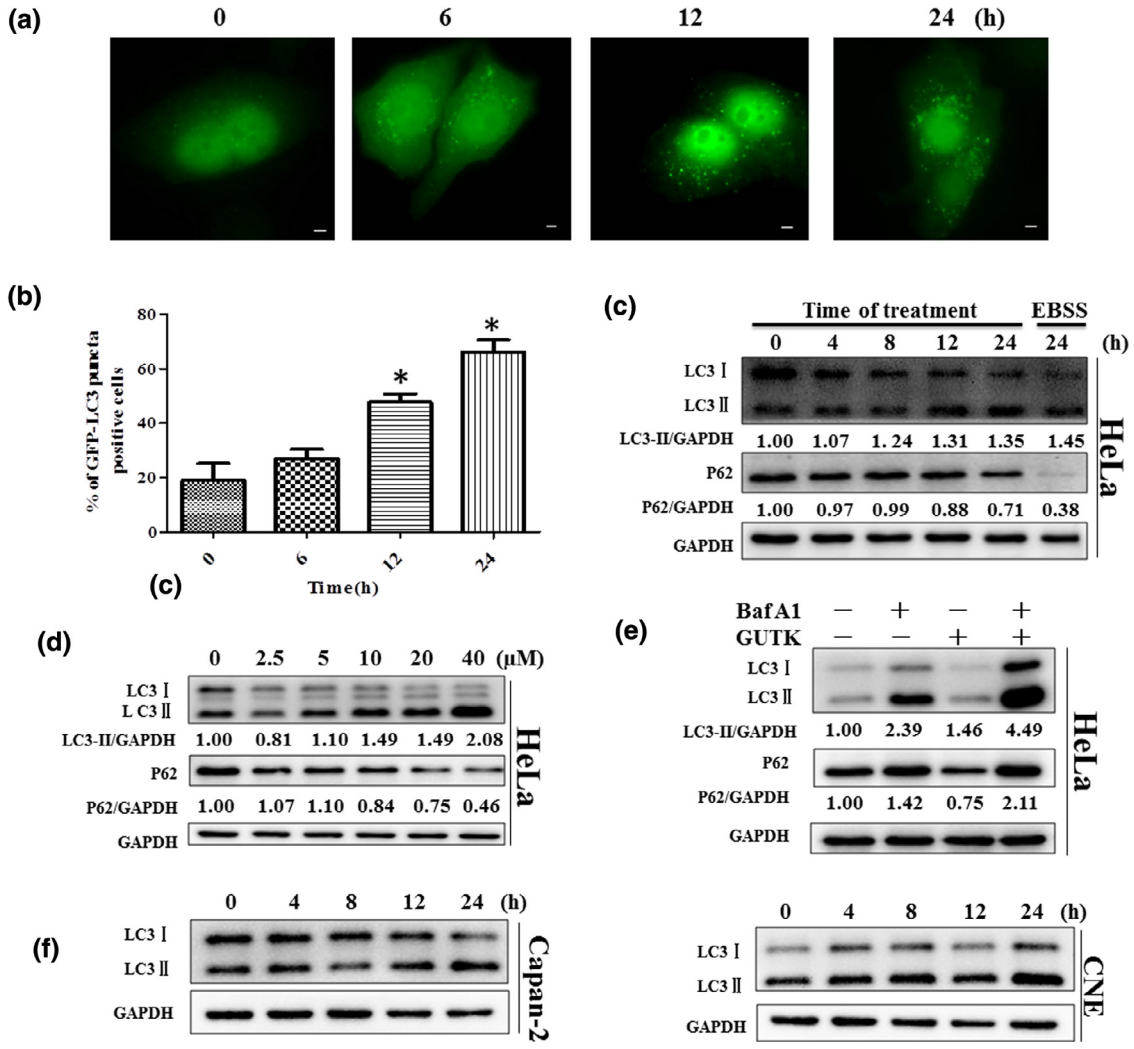

Fig. 1. GUTK induces autophagy in HeLa cells. (a) Accumulation of GFP-LC3 puncta in HeLa cells treated with GUTK (20 μM) over time (0, 6, 12 and 24 h). The distribution of GFP-LC3 was examined by Olympus IX 83 microscopy. Scale bar, 2 μm. (**b**) Quantification of GFP-LC3 puncta-positive cells over time (0, 6, 12 and 24 h). Each sample was analyzed using a threshold of > 10 dots/cell. (**c**) GUTK regulates the levels of LC3-II and p62 in a time-dependent manner. HeLa cells were treated with 20 μM GUTK over a certain time course, and samples were analyzed by western blotting for LC3, p62 and GAPDH. (**d**) GUTK regulates the amount of LC3-II and p62 protein in a dose-dependent manner. HeLa cells treated with GUTK (2.5, 5, 10, 20 and 40 μM) for 24 h were analyzed by western blotting for endogenous LC3 and p62, and GAPDH was used as a loading control. (**e**) HeLa cells were treated with DMSO or GUTK (20 μM) for 2 h in the presence or absence of 10 nM BAFA1 as indicated. A western blot analysis was performed to analyze the levels of LC3, p62 and GAPDH. (**f**) GUTK increases the amount of LC3-II in a time-dependent manner. Capan-2 and CNE cells were treated with GUTK (20 μM) for 4, 8, 12 and 24 h, and the LC3 level was assessed by western blot analysis. $^{*}P < 0.05$ compared with the control.

Results

Guttiferone K induces autophagy in cancer cells. Autophagy is closely associated with tumorigenesis and plays an important role in cancer therapy. Therefore, targeting autophagy is a potential therapeutic strategy in adjuvant chemotherapy (Maycotte et al., 2012). To identify novel autophagy regulators, we performed a functional screen using a cell-based assay with HeLa cells stably expressing GFP-LC3B. Autophagosome accumulation can be detected with a fluorescence microscope. We previously identified several polycyclic polyprenylated acylphloroglucinols (PPAPs) with activity on GFP-LC3 puncta formation, including Guttiferone K (GUTK) and Oblongifolin C (OC) (Supplementary Fig. 1a and b) (Lao et al., 2014). In this study, we investigated the functions and mechanisms of GUTK on the autophagy signaling pathway. First, we examined the effect of GUTK on GFP-LC3. As shown in Fig. 1a, GUTK significantly induced GFP-LC3 puncta formation. We then quantified the number of GFP-LC3 puncta in the cells after GUTK treatment. The statistical analysis showed that GUTK increased the number of GFP-LC3 puncta in a time-dependent manner (Fig. 1b). During autophagy, the cytoplasmic form LC3-I (18 kD) is processed and recruited to the autophagosomes, where LC3-II (16 kD) is generated by site-specific proteolysis and lipidation near the C-terminus. Thus, the amount of LC3-II is positively correlated with the number of autophagosomes. SQSTM1/p62 serves as a link between LC3 and ubiquitinated substrates. The induction of autophagy correlates with decreased levels of p62 (Bartlett et al., 2011). We then examined the effect of GUTK on LC3 conversion and p62 degradation. In HeLa cells, GUTK caused a marked increase in LC3-II and a decrease in the p62 protein level in a time- and dose-dependent manner (Figs. 1c and d), suggesting that GUTK induces autophagy. In addition, to distinguish whether autophagosome accumulation is due to autophagy induction or blockage of the downstream steps, we performed an autophagic flux assay. Bafilomycin A1 (BAFA1), a vacuolar H^{+}-ATPase inhibitor, significantly increased the amount of LC3-II and p62 in HeLa cells. The GUTK-induced accumulation of LC3-II and decrease of p62 were significantly changed in the presence of BAFA1 (Fig. 1e), indicating that GUTK increased the degradation of the autophagic contents. Consistently, increased

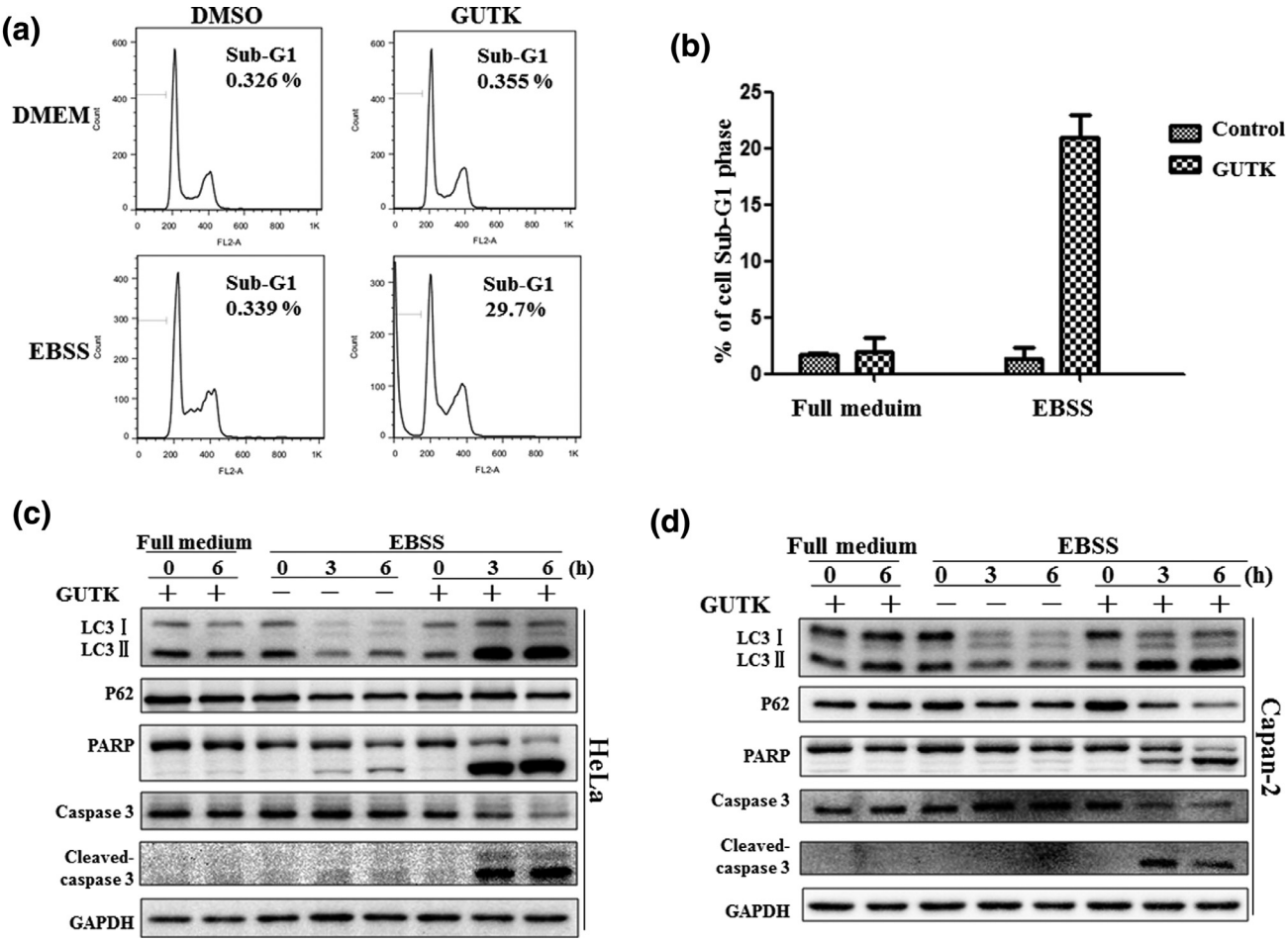

Fig. 2. GUTK sensitizes cells to cell death under nutrient starvation conditions. (a–b) Flow cytometry analysis of the sub-G1 population in GUTK-treated cells. HeLa cells were treated with 5 μM GUTK for 6 h and cultured in either complete medium (DMEM with serum) or nutrient-deprived medium (EBSS without serum) for 6 h. The cells were fixed and stained with propidium iodide (PI). (**c**) HeLa or (**d**) Capan-2 cells were treated with 5 μM GUTK for 6 h and cultured in either DMEM with serum or EBSS for 6 h. The samples were analyzed by western blotting for cleaved caspase 3, LC3, p62 and PARP. GAPDH was used as a loading control.

LC3-II conversion was also detected in Capan-2 and CNE cells after GUTK treatment (Fig. 1f). Taken together, the results provide evidence that GUTK is an autophagy inducer and that GUTK-induced autophagosome accumulation is due to an increase in autophagic flux.

Guttiferone K sensitizes cells to cell death under nutrient starvation. Cancer cells show an inherent ability to tolerate extreme conditions, such as low nutrient and oxygen supplies, by modulating their energy metabolism. Thus, targeting nutrient-deprived cancer cells may be a novel strategy in anticancer drug development because interfering with autophagy may sensitize cancer cells to nutrient starvation-induced cell death. We then investigated whether GUTK plays a role in Earle's balanced salt solution (EBSS)-induced cell death in HeLa cells. Interestingly, we found that GUTK can rapidly induce cell death in nutrient-deprived conditions. As shown in Figs. 2a and b, HeLa cells were largely resistant to GUTK in full medium, whereas GUTK increased the Sub-G1 fraction when the cells were cultured in EBSS. In fact, the statistical analysis revealed a significant increase in the Sub-G1 population after 6 h of treatment, whereas the cells grown in full medium were resistant to treatment with 5 μM GUTK. We further confirmed the cell death under a microscope and through a cell viability assay. From the phase-contrast and PI-permeable images, we found that HeLa cells underwent cell death after combine treatment with GUTK and nutrient starvation (Supplementary Fig. 2a). The cell viability assay indicated that a GUTK concentration of 10 μM killed more than 80% of the cells subjected to nutrient deprivation (Supplementary Fig. 2b). To examine whether the cells undergo apoptosis, we detected the cleavage of caspase 3 and PARP, two major proteins involved in apoptosis. The western blotting results showed that GUTK induced caspase-3 and PARP activation in HeLa cells (Fig. 2c). Consistent with our flow cytometry observations, GUTK treatment in full medium did not cause the activation of caspase-3 and PARP. The treatment of GUTK combined with EBSS showed strong apoptotic induction after 3 and 6 h. We also detected changes in the autophagy markers LC3-II and p62 under the same conditions. Under the EBSS condition, GUTK accelerated the LC3-II conversion and decreases the p62 protein level in HeLa cells. In Capan-2 cells, GUTK exhibited strong activity to efficiently induce caspase-3 and PAPR cleavage, LC3-II conversion and p62 degradation only in EBSS, as detected by western blot (Fig. 2d). These results suggested that GUTK sensitizes cancer cells to cell death under nutrient starvation conditions. In addition, GUTK increased the autophagy process under EBSS, as indicated by the observed changes in LC3-II and p62.

Hydroxychloroquine (HCQ) inhibits GUTK-induced cell death in nutrient starvation conditions. We previously reported that GUTK induces cell cycle arrest and apoptosis in colon cancer in full medium (Kan et al., 2013). In this study, we aimed to characterize the functional role of GUTK in cell death under nutrient deprivation. We applied the autophagy inhibitor hydroxychloroquine (HCQ) and the apoptosis inhibitor z-VAD-fmk to GUTK treated cells and assessed the resulting cell death through the Annexin V-FITC/PI staining assay. HCQ is an autophagic inhibitor that can prevent endosomal acidification and block autophagosome–lysosome fusion. As shown in Fig. 3a, HeLa cells in

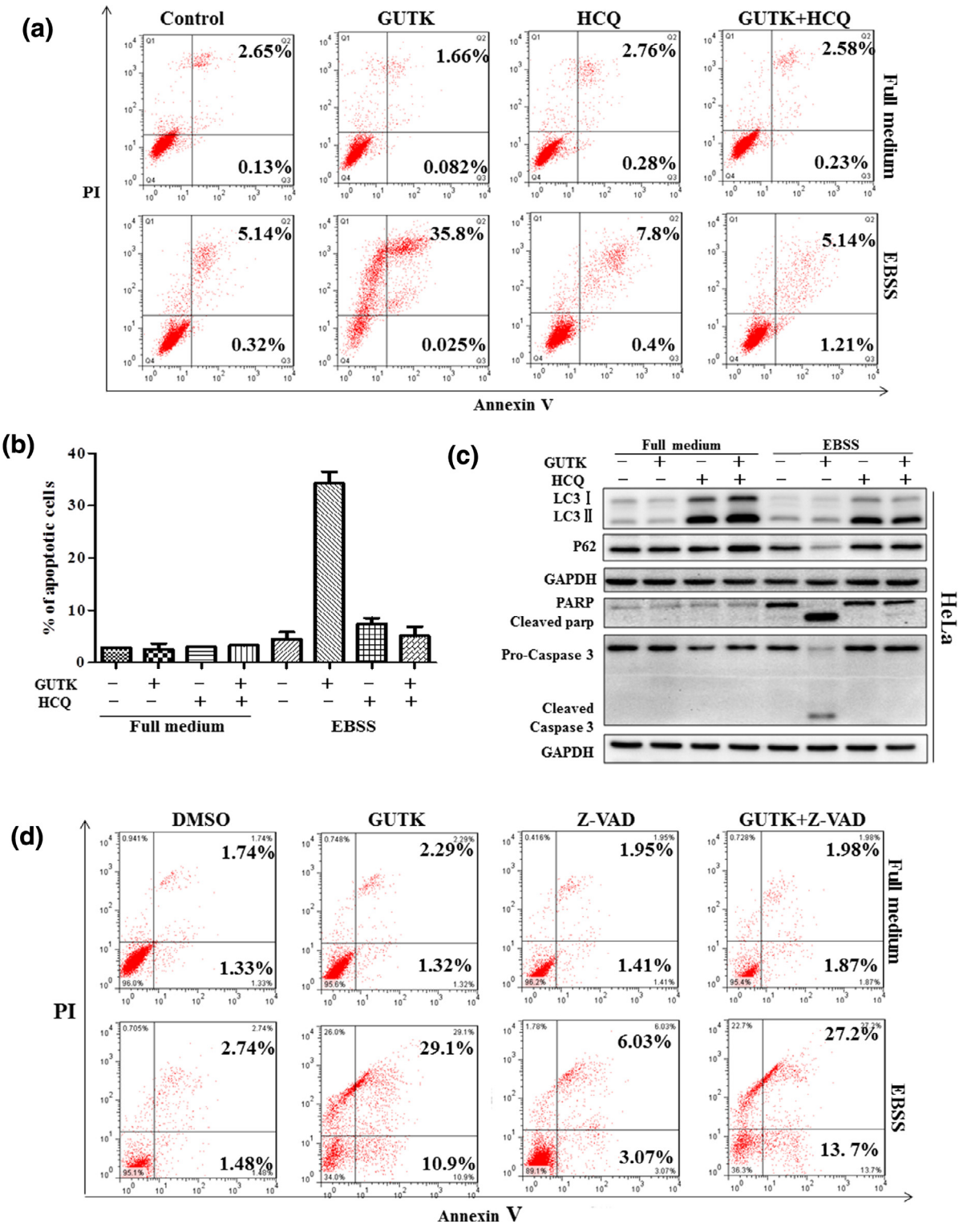

Fig. 3. HCQ inhibits GUTK-induced cell death under nutrient starvation conditions. (a–b) Annexin V/PI flow cytometry analysis of GUTK HeLa cells. HeLa cells were treated with GUTK (5 μM) in the absence or presence of HCQ for 2 h and then incubated in EBSS or full medium for 6 h. The cells were then collected and double-stained with FITC-conjugated Annexin V and propidium iodide (PI). The analyses were performed on a flow cytometer. (**c**) HeLa cells were treated with GUTK (5 μM) in the absence or presence of HCQ for 2 h and then incubated in EBSS or full medium for 6 h. The levels of LC3, P62, PARP and caspase 3 were determined by western blot analysis. (**d**) HeLa cells were treated with GUTK (5 μM) in the absence or presence of Z-VAD-fmk for 2 h and then incubated in EBSS or full medium for 6 h. The cells were collected and double-stained with FITC-conjugated Annexin V and propidium iodide (PI). The analyses were performed on a flow cytometer.

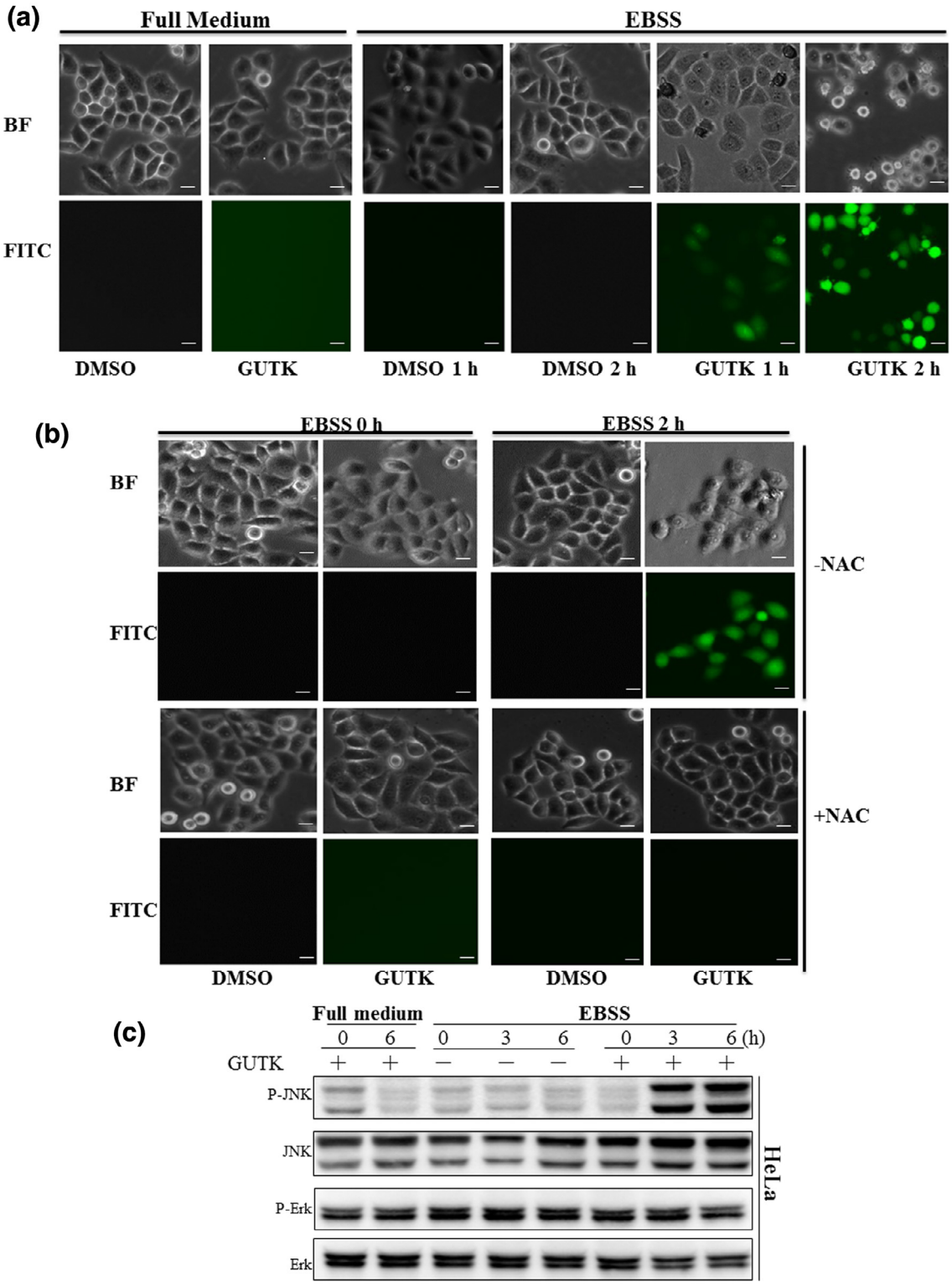

Fig. 4. GUTK activates ROS/JNK in nutrient starvation conditions. (a) HeLa cells were treated with GUTK (5 μM) or DMSO and then incubated in EBSS or full medium for 2 h. The cells were incubated with DCFH-DA and observed under a fluorescence microscope. (**b**) HeLa cells were pretreated with 10 mM NAC for 2 h and then incubated in DMSO or 5 μM GUTK for 2 h in EBSS or full medium. The cells were incubated with DCFH-DA and observed under a fluorescence microscope. (**c**) HeLa cells were treated with 5 μM GUTK or DMSO for a certain time period (3 and 6 h) in EBSS or full medium, and the *p*-JNK and *p*-Erk levels were then analyzed by western blot.

EBSS showed massive cell death when treated with 5 μM GUTK for 6 h, and pre-treatment with 50 μM HCQ for 2 h efficiently suppressed the observed cell death. The statistical analysis indicated that the Annexin-V/PI positive fraction was reduced from ~35% to less than 10%, suggesting that HCQ blocked the cell death pathway in GUTK-induced cells under EBSS (Fig. 3b). The protection effect of HCQ was also observed in the analysis of the cell morphology under a microscope (Supplementary Fig. 3). In addition, the immunoblotting analysis showed that pre-treatment with HCQ prevented the degradation of p62, cleaved caspase-3 and cleaved PARP upon GUTK treatment

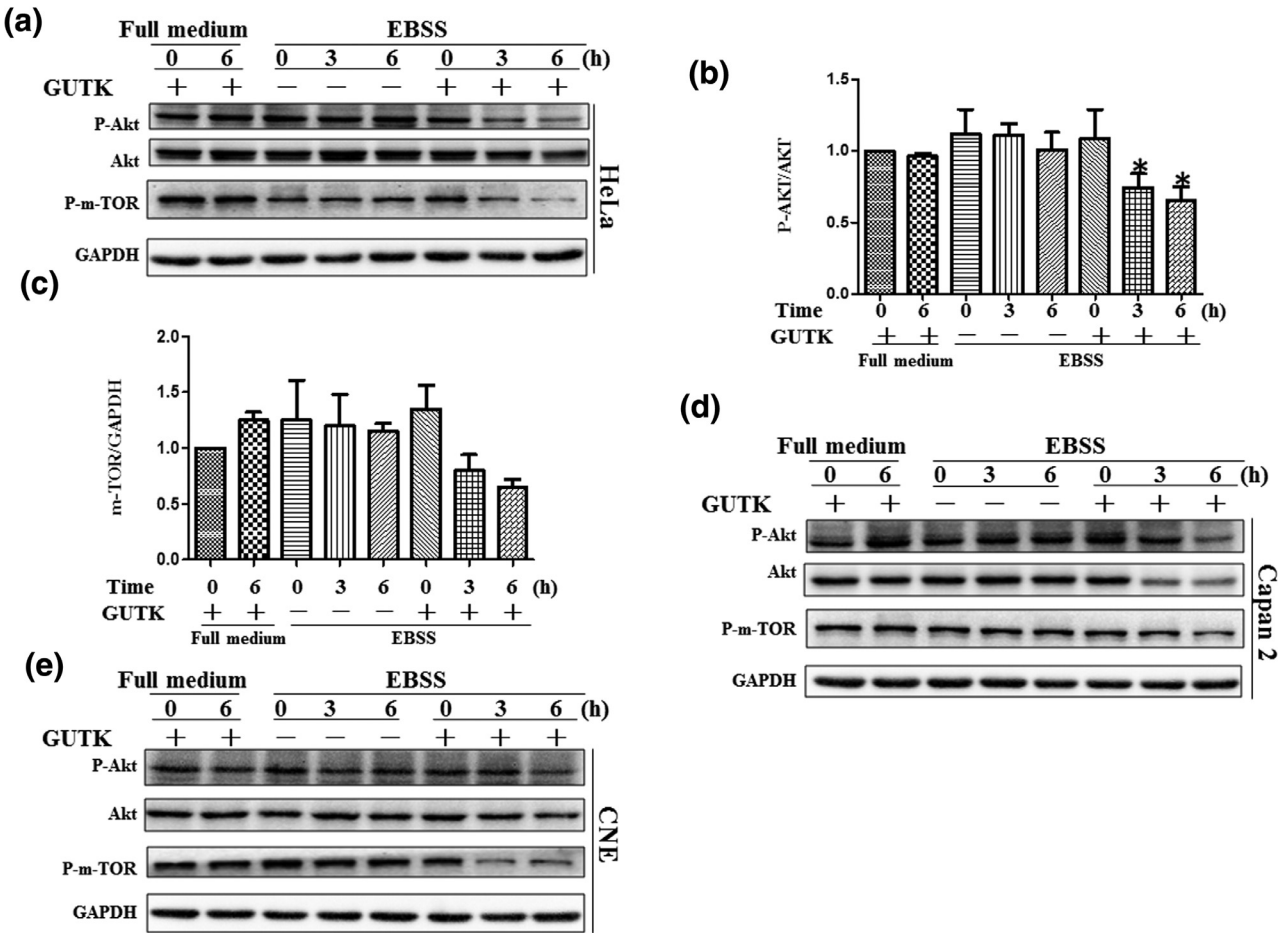

Fig. 5. GUTK inhibits the Akt-mTOR signaling pathway in nutrient starvation conditions. (a–c) HeLa cells cultured in DMEM or EBSS medium were treated with GUTK (5 μM) for a certain time period (3 and 6 h). The samples were analyzed by western blotting for p-Akt and p-mTOR. GAPDH was used as a loading control. (**d**) Capan-2 or (**e**) CNE cells cultured in DMEM or EBSS medium were treated with GUTK (5 μM) for a certain time period (3 and 6 h). The samples were analyzed by western blotting for p-Akt and p-mTOR. GAPDH was used as a loading control. $^{*}P < 0.05$ compared with the control.

(Fig. 3c). To further confirm the essential role of autophagy in GUTK-induced cell death, we examined the effect of GUTK in ATG7$^{-/-}$MEFs, an autophagy-deficient cell line, by PI staining. Consistently, ATG7$^{-/-}$ null MEFs exhibited resistance to GUTK under nutrient starvation conditions (Supplementary Fig. 4). In contrast, the pan-caspase inhibitor z-VAD-fmk only partially prevented the cell death caused by GUTK in EBSS (Fig. 3d). To confirm the effect of z-VAD-fmk on caspase inhibition, we examined the cleavage of caspase-3 and PARP by western blotting (Supplementary Fig. 5). Taken together, our results suggest that the GUTK-induced cell death during nutrient starvation is largely dependent on autophagy induction but not caspase 3 activation.

Guttiferone K activates ROS/JNK in nutrient starvation conditions. Because autophagy dominates the GUTK-induced cell death under nutrient deprivation, it was interesting to investigate the mechanism through which GUTK induces autophagy. Reactive oxygen species (ROS) plays an important role in regulating cellular metabolism and up-regulation of ROS is involved in autophagy induction (Li et al., 2012). We then tested whether GUTK induced ROS generation HeLa cells in full medium or in EBSS using intracellular ROS indicator DCFH-DA. As shown in Fig. 4a, we observed a significant increase in intracellular ROS after exposure to 5 μM GUTK in EBSS in for a short period (1–2 h), but not in full medium. The induction of ROS was significantly attenuated when the cells were pretreated with the antioxidant NAC (Fig. 4b). However, the inhibition of ROS by NAC could not attenuate the cytotoxicity of GUTK on cells. As shown in Supplementary Fig. 6A, the treatment of NAC did not reduce the sub-G1 fraction in flow cytometry assay. In addition, the cleavage of PARP and caspase-3 could not be prevented upon NAC treatment (Supplementary Fig. 6B). Because ROS is a potent regulator of MAP kinase family members, we assessed the involvement of two critical MAPK family members, namely JNK and ERK. Interestingly, GUTK increased the phosphorylation of JNK in a time-dependent manner but did not alter the phosphorylation of ERK (Fig. 4c). In summary, our results indicate that GUTK significantly induces ROS and the activation of JNK under nutrient starvation, which may contribute to the mechanism through which GUTK affects autophagy.

Guttiferone K inhibits the Akt-mTOR signaling pathway in nutrient starvation conditions. The Akt-mTOR signaling pathway is an important negative regulator of autophagy (O'Reilly et al., 2006; Yang et al., 2014; Zhang et al., 2014). We then examined the phosphorylation of Akt and mTOR in GUTK-treated cells by western blotting. As shown in Fig. 5a, in nutrient starvation conditions, treatment with 5 μM GUTK significantly decreased the phosphorylation of Akt and the mTOR level compared with the control cells. However, GUTK treatment in full medium and incubation with EBSS for 6 h did not alter the phosphorylation of Akt and mTOR, as shown in Figs. 5b and c, respectively. Consistent with the findings in HeLa cells, the down-regulation of the phosphorylation of Akt and mTOR was also observed in Capan-2 and CNE cells (Figs. 5d and e). The data demonstrate that GUTK may activate multiple signaling pathways, including ROS generation, JNK activation and Akt-mTOR inhibition, under nutrient starvation within a

short period of time. In other words, cells under nutrient stress are more sensitive to GUTK, which may indicate its potential for anticancer drug development.

Discussion

The involvement of autophagy within tumorigenesis and cancer therapy is a double-edged sword. Therefore, novel autophagy regulators, including inducers and inhibitors, may contribute to clinical application. We applied HeLa cells stably expressing GFP-LC3 to screen autophagy regulators from a library of natural compounds (Lao et al., 2014). In this study, we identified GUTK as a potent autophagy inducer that could sensitize cancer cells to undergo autophagy-dependent cell death in nutrient starvation conditions. In this study, we focused on the effect of GUTK on EBSS medium, which simulates nutrient deprivation conditions. Our data demonstrated that GUTK induces the accumulation of LC3-II and the down-regulation of p62 in a time-dependent and concentration-dependent manner. Notably, the effect of GUTK at a concentration of 5 μM in EBSS was found to be highly significant within 6 h compared with that in the control cells grown in full medium. In addition, HCQ (autophagy inhibitor) could significantly block the GUTK-induced autophagy and result in the recovery of cell viability in nutrient starvation conditions. In contrast, other inhibitors, such as z-VAD-fmk (caspases inhibitor) and NAC (ROS inhibitor), were unable to rescue the cells from cell death when EBSS and GUTK were applied in combination. Together, these results importantly suggest that GUTK activates different signaling pathways in full medium and under nutrient starvation. Targeting cancer cells under nutrient deprived or hypoxia may be a promising strategy in anticancer drug development (Moyer, 2012). On the one hand, targeting nutrient-deprived cancer cells may contribute to drug delivery and reduce the side effects to normal tissues (Saggar et al., 2014). On the other hand, a compound that shows more potency to starved cells may be less toxic to normal cells because these organs are found in an environment with a good nutrient supply. It will be interesting to further investigate the *in vivo* effects of GUTK combined with a calorie restriction diet model.

Compounds from natural plants are important resources for drugs against a wide variety of diseases, including cancer. *Garcinia* species have been studied for more than 7 decades, and many bioactive compounds with anticancer potential have been identified (Han et al., 2009). Polycyclic polyprenylated acylphloroglucinols (PPAPs) are one of the main chemicals found in *Garcinia* plants (Richard et al., 2012). GUTK and OC are two typical PPAPs extracted from *Garcinia yunnanensis* Hu with similar structures (Supplementary Fig. S1). However, these two PPAPs exhibit opposite effects on the autophagy signaling pathway. OC inhibits autophagic flux by blocking autophagosome–lysosome fusion, increasing the lysosomal pH and inhibiting lysosomal proteolytic activity. In this study, we found that GUTK enhances autophagic flux and that this effect can be attenuated by HCQ. The mechanism of action of GUTK may involve ROS generation, JNK activation and Akt/mTOR inhibition. However, nutrient starvation enhances the effect of GUTK on cell death, which can be blocked by HCQ but no other inhibitors, such as z-VAD-fmk and NAC. These data suggest that GUTK activates multiple cell death pathways in nutrient starvation conditions and that autophagy may play a dominant role in regulating this process. However, the detailed mechanism through which GUTK activates autophagy and how autophagy is involved in the resulting cell death remain unclear. Moreover, further studies are needed to determine why GUTK and OC, which have similar structure, show different effects on autophagy.

The Akt/mTOR signaling pathways play vital roles in regulating cancer cell proliferation, cell death, and metastasis. Some natural compounds were found to inhibit Akt/mTOR in recent reports. For instance, curcumin modulated Akt/mTOR in multiple cancer cells (Johnson et al., 2009; Beevers et al., 2006; Yu et al., 2008). Gambogic acid was found to inhibit PI3K/mTOR pathway in myeloma cells. Most studies investigated the effects of natural compounds in full medium. In our study, we found that GUTK treatment did not affect Akt/mTOR signals in full medium. In addition, EBSS starvation for 6 h did not alter Akt/mTOR in our tested cancer cells (Fig. 5). However, the combination of GUTK and EBSS starvation boosted the suppression of Akt/mTOR in a very short time, following dramatic cell death. Here, the inhibition of Akt/mTOR was possibly mediated by the ROS activated autophagy, since we found that HCQ could revert the effect of GUTK in HeLa cells (data not shown). Therefore, our data suggested that the combination of GUTK and EBSS formed a positive feedback to inhibit the Akt/mTOR, resulting in the acceleration of cell death. The changes of upstream signaling such as VEGFR may need to be further explored.

Taken together, our present study shows that GUTK, a natural compound isolated from *Garcinia yunnanensis* Hu, induces autophagy in cancer cells. In addition, our findings suggest that GUTK efficiently accelerates cell death under nutrient starvation and that this cell death is largely dependent on autophagy. Furthermore, we provide evidence that GUTK activates multiple autophagy-related signaling pathways, including ROS, JNK phosphorylation and Akt/mTOR inhibition. Our study indicates that GUTK may be a promising compound to be further developed for cancer therapy.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2015.06.008.

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