RESEARCH ARTICLE

Tetrahydrocurcumin, a major metabolite of curcumin, induced autophagic cell death through coordinative modulation of PI3K/Akt-mTOR and MAPK signaling pathways in human leukemia HL-60 cells

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Scope: Autophagy (type II programmed cell death) is crucial for maintaining cellular homeostasis. Several autophagy-deficient or knockout studies indicate that autophagy is a tumor suppressor. Tetrahydrocurcumin (THC), a major metabolite of curcumin, has been demonstrated with anti-colon carcinogenesis and antioxidation in vivo.

Methods and results: In the present study, we found that treatment with THC induced autophagic cell death in human HL-60 promyelocytic leukemia cells by increasing autophage marker acidic vascular organelle (AVO) formation. Flow cytometry also confirmed that THC treatment did not increase sub-G1 cell population whereas curcumin did with strong apoptosis-inducing activity. At the molecular levels, the results from Western blot analysis showed that THC significantly down-regulated phosphatidylinositol 3-kinase/protein kinase B and mitogen-activated protein kinase signalings including decreasing the phosphorylation of mammalian target of rapamycin, glycogen synthase kinase 3β and p70 ribosomal protein S6 kinase. Further molecular analysis exhibited that the pretreatment of 3-methyladenine (an autophagy inhibitor) also significantly reduced acidic vascular organelle production in THC-treated cells.

Conclusion: Taken together, these results demonstrated the anticancer efficacy of THC by inducing autophagy as well as provided a potential application for the prevention of human leukemia.

Keywords:
Autophagy / Curcumin / Tetrahydrocurcumin

1 Introduction

Tetrahydrocurcumin (THC) is one of the major metabolites of curcumin (CUR) (extracted from the roots of the *Curcuma longa* Linn) [1]. It was identified both in intestinal and hepatic cytosol of humans and rats [2, 3] and has been reported with potent bioactivity in in vivo study. Recently, attention has focused on THC, because this compound appears to exert greater antioxidant activity in both in vitro and in vivo systems [4, 5]. Previous studies showed that

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Abbreviations: **Akt/PKB,** protein kinase B; **AVOs,** acidic vascular organelles; **CUR,** curcumin; **ERK,** extracellular signal-regulated kinase; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **GSK-3β,** glycogen synthase kinase 3β; **LC3,** light chain 3; **3-MA,** 3-methyladenine; **MAPKs,** mitogen-activated protein kinases; **mTOR,** mammalian target of rapamycin; **MTT,** 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide; **PDK1,** 3-phosphoinositide-dependent protein kinase 1; **PI3K,** phosphatidylinositol 3-kinase; **p70S6K,** p70 ribosomal protein S6 kinase; **PTEN,** phosphatase and tensin homolog; **THC,** tetrahydrocurcumin

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treatment with THC inhibited cellular migration and invasion by down-regulation of matrix metalloproteinase 9 [6]. Oral administration of THC also reduced 1,2-dimethylhydrazine-induced colonic carcinogenesis in mice [7]. However, studies also showed that THC with lower activities of anti-inflammation and inducing apoptosis than curcuminoids [8] implying other mechanisms might be involved in its bioactivities.

Autophagy is a response of eukaryotic cells to various microenvironment stresses, including starvation, pathogen infestation and chemotherapy [9–13]. Furthermore, a number of studies have established that autophagy is related to several human diseases, including neurodegenerative disease, cardiovascular disease and bacterial or viral infections. Moreover, several reports exhibit that autophagy induction appears to facilitate successful therapy-induced killing of tumor cells [14–16], and pro-autophagic drugs such as temozolomide are promising candidates for selective killing of apoptosis-resistant glioblastomas [15].

Autophagy, also known as type II programmed cell death, is characterized by the formation of a double-membrane or isolation membrane, which is derived from a part of the endoplasmic reticulum (ER) [17] or from the cytolplasmic lipid pool [18]. The double-membrane forms autophagosome by sequester portions of the cytoplasm and intracellular organelles [19].

The autophagosomes undergo acidification after maturation to become acidic vesicular organelles (AVOs) [20]. Finally, these authophagosomes fuse with lysosomes to mature into autophagolysosomes and their components are digested by lysosomal hydrolases. In addition, it has been demonstrated that beclin-1 and LC3 (light chain 3) are essential for autophagy and associated to the autophagosome membranes after processing. When autophagy is induced, Beclin-1 and LC3 distribute to the membrane of autophagosomes that is correlated to the extent of autophagosome formation [21].

The initiating signal for autophagy formation is poorly understood, whereas it has been established that several molecules and signaling pathways are implicated in regulating autophagy, such as phosphatidylinositol 3-kinase/PKB/mammalian target of rapamycin (PI3K-Akt-mTOR), mitogen-activated protein kinases (MAPKs), Raf-1-MEK1/2-ERK1/2 and PTEN (phosphatase and tensin homolog) pathways [9, 22–24]. PTEN and Akt are upstream regulators of the mTOR pathway, which acts as an inducer or inhibitor of autophage, respectively [22, 23]. Akt inhibits autophage by regulating downstream molecular 4 elongation-bind protein 1, p70 ribosomal protein S6 kinase (p70S6K), which result in promoting mRNA translation. It has also been reported that phosphorylation of glycogen syntheses kinase 3β (GSK-3β) by Akt could affect various biological processes such as cell proliferation [25–27].

Previous studies also reported that the treatment with anticancer drugs and phytochemicals such as pterostilbene, 6-shogaol and irradiation combined with arsenic trioxide, induced autophagy in various human cancer cells [10, 28–30]. In the present study, we investigated the anticancer effect of CUR and THC, the major metabolite of CUR, in human acute myelogenous leukemia HL-60 cells in vitro. We found that THC induced cell death through autophagy but not apoptosis. This study demonstrated that treatment with THC decreased phosphorylation of PI3K, Akt and mTOR and modulated phosphorylation of MAPKs in HL-60 cells, as well as the protein expression of LC3 and Beclin-1, followed by AVOs formation. Taken together, these results indicated the anticancer efficacy of THC by inducing autophagy that provides a potential application for the prevention of human leukemia.

2 Materials and methods

2.1 Cell culture

The human leukemia cell lines (HL-60) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

The purity of CUR and THC was determined by HPLC as higher than 98.0%. THC is stable under cell culture condition.

2.2 Cell proliferation assay

Inhibition of cell proliferation by CUR or THC was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay. HL-60 cells were subcultured at 2×10^4 cells/well in 96-well culture plate and incubated at 37°C. After 12 h incubated, the cells were treated with vehicle alone (0.1% dimethyl sulfoxide) and various concentrations of CUR or THC for 48 h. At the end of treatment, 10 μL of MTT was added, and cells were incubated for another 3 h. Then, 100 μL of dimethyl sulfoxide was added to each well after removing the medium. Cell proliferation inhibition was obtained by scanning with an enzyme-linked immunosorbent assay (ELISA) reader with a 570 nm filter. All experiments were performed in triplicate.

2.3 Apoptotic ratio assay

Cell apoptotic ratio was analyzed by flow cytometry as follows. At each time point, cells were harvested, washed with phosphate-buffered saline, resuspended in 100 μL of phosphate-buffered saline, and fixed in 800 μL of 95% cold-ethanol at −20°C for 12 h. At the end of fixing, the cell pellets were collected by centrifugation at 5000 *g* for 10 min at 4°C, resuspended in 0.5 L of hypotonic buffer (0.5% Triton X-100 in phosphate-buffered saline and 0.5 μg/mL

RNase) and incubated at 37°C for 15 min. After incubation, the cells were stained with 10 μL of propidium iodide (1 mg/mL) and incubated at 37°C for 15 min. The stained cells were analyzed by FACScan laser flow cytometer (Becton Dickinson, San Jose, CA).

2.4 DNA extraction and electrophoresis analysis

HL-60 cells were subcultured at 2×10^5 cells/mL in 100 mm Petri dishes and incubated for various times. The cells were harvested, washed with phosphate-buffered saline, and lysed with digestion buffer (0.5% sarkosyl, 0.5 mg/mL proteinase K, 50 mM Tris (hydroxymethyl) aminomethane (pH 8.0) and 10 mM EDTA) at 56°C for 12 h and were treated with RNase A (0.5 μg/mL) at 50°C for 3 h. The DNA was extracted by PCI (phenol:chloroform:isoamyl alcohol = 25:24:1) before loading and analyzed by 2% agarose gel electrophoresis. Approximately 20 μg DNA were loaded in each well and the agarose gel was run at 50 V for 120 min in Tris-borate/EDTA electrophoresis buffer (TBE). After electrophoresis, the agarose gel was visualized under UV light and photographed.

2.5 Detection and quantification of acidic vesicular organelles during autophagy formation

The autophagy level caused by THC was measured by the previously described method [31]. HL-60 cell staining with acridine orange (Sigma Chemical) was performed according to published procedures adding a final concentration of 1 mg/mL for a period of 20 min. Photographs were obtained with a fluorescence microscope (Axioscop, Carl Zeiss, Thomwood, NY) equipped with a mercury 100-W lamp, 490-nm band-pass blue excitation filters, a 500-nm dichroic mirror and a 515-nm long-pass barrier filter. Flow cytometric analysis is another technique to detect AVOs, which are a characteristic of autophagy. To quantify the development of AVOs, HL-60 cells were stained with acridine orange (1 μg/mL) for 15 min and were analyzed by FACScan laser flow cytometer and and CellQuest software.

2.6 Western blot analysis

Total cellular protein lysates were prepared by harvesting cells in protein extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at $10\,000 \times g$ for 30 min at 4°C. Equal amounts of total cellular proteins (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride membranes with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine and 20% methanol, and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL), The densities of the bands were quantified with a computer densitometer (AlphaImagerTM 2200 System Alpha densitometer. Innotech Corporation, San Leandro, CA). The expression of glyceraldehyde 3-phosphate dehydrogenase GAPDH was used as the control for protein loading. The antibodies to PI3K, phospho-PI3K, Akt, phospho-Akt, p38 MAPK, phospho-p38MAPK, JNK1/2, phospho-JNK1/2, phospho-p70S6K at Thr389 or Ser379, total p70S6K, extracellular signal-regulated kinase (ERK), phospho-ERK, phospho-PDK1 (PDK1, 3-phosphoinositide-dependent protein kinase 1), phospho-GSK3β, GAPDH and phospho-PTEN were obtained from Cell Signalling Technology (New England Biolabs, Ipswich, MA); LC3 was obtained from Abgent (San Diego, CA).

2.7 Statistical analysis

The statistical significance of differences between the means was evaluated using Student's test. A different with a p value less than 0.05 was considered statistically significant.

3 Results

3.1 Inhibition of cell proliferation in CUR- and THC-treated HL-60 cells

To examine the effect of CUR and THC on cell proliferation, we treated HL-60 cells with different concentrations of CUR and THC for 48 h and measured cell viability using the MTT assay. The IC_{50} after 48 h of CUR and THC treatment is 25.7 ± 2.8 and 88.7 ± 3.6 μM, respectively (Fig. 1). The results of the present study indicate that the conjugated double bonds of the central seven-carbon chain of CUR may play an important role in its antiproliferative activity. To understand the molecular mechanisms of action of conjugated moiety in inhibiting cell proliferation, we compared the inhibitory effects of CUR and THC in HL-60 cells using DNA fragmentation and flow cytometry analyses. As shown in Fig. 2, significant DNA ladders and sub-G1, hallmarks of apoptosis, were observed in HL-60 cells treated with 100 μM CUR and this effect was dose dependent. In contrast, no evidence of apoptotic induction was observed with THC.

3.2 THC shows stronger inducing effects on autophagy than CUR in HL-60 cells

Interestingly, THC inhibits the growth of HL-60 cells but does not primarily display features typical of apoptosis.

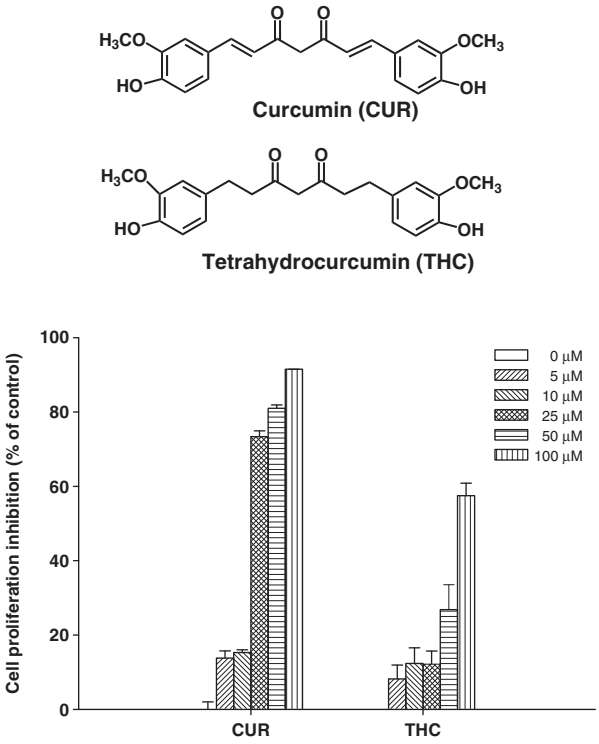

Figure 1. Inhibitory effect of CUR and THC on cell proliferation in HL-60 cells. Cells were treated with different concentrations of CUR or THC for 48 h and subjected to MTT assay. The inhibition of cell proliferation was calculated using the following formula: inhibition% = [100 - (ODt/ODs) × 100] %, where ODt and ODs are the optical density of the test substances and solvent control, respectively. The data are expressed as mean ± SD of triplicate experiments.

According to a previous study, nonapoptotic programmed cell death is principally attributed to autophagy [11]. Autophagy is characterized by the formation of numerous acidic vesicles that are called AVOs [20]. We next assessed whether CUR and THC also induce autophagy in HL-60. As shown in Fig. 3, THC treatment resulted in marked appearance of AVOs than CUR when cells were stained with acridine orange after 48 h treatment. To quantify the incidence of THC-induced autophagy, cells with AVOs showed enhanced red fluorescence analyzed by flow, which significantly increased after treatment with THC in a time-dependent manner (Fig. 3B). However, CUR treatment resulted in a less effect. In addition, we used 3-methyladenine (3-MA), an inhibitor of autophagy, to determine whether inhibition of autophagy suppresses THC-induced cytotoxicity. As showed in Fig. 4, the results indicated that treatment with 3-MA reveled a significant decrease of THC-induced acridine orange$^+$ cells in a dose-dependent manner. These results corroborate the observation that THC treatment induces autophagic cell death in HL-60 cells.

3.3 THC inhibits the mTOR/p70S6K and GSK-3β pathway in HL-60 cells

To confirm the occurrence of autophagy induced by THC, we examined the processing of LC3B and beclin-1, hallmarks of autophagy, using immunoblot to detect cell-extracted lysates from HL-60 cells treated with or without THC. As shown in Figs. 5A and B, we observed that the amounts of LC3B I/II proteins increased after treatment with THC. Similar to LC3B/I/II, the expression of beclin-1 decreased following the treatment by THC in HL-60 cells. Thus, these data confirmed that THC induced an autophagy in HL-60 cells. To further understand the molecular mechanisms of action of THC-induced autophagy in HL-60 cells, we examined the phosphorylation of mTOR and its downstream effectors, p70S6K. The results showed that phosphorylation of mTOR and p70S6K (Thr389) markedly decreased in cells treated with THC. Moreover, treatment with THC also inhibited the phosphorylation of p70S6K in a time-dependent manner (Fig. 5C). Previous reports have studied the effects of GSK-3β on apoptosis. However, the role of GSK-3β in autophagy remains elusive. As shown in Fig. 5C, treatment with THC also decreased phosphorylated GSK-3β gradually for 3–24 h in HL-60 cells; however, the total protein of GSK-3β slightly increased in THC-treated cells. These results indicated that THC induces autophagic cell death in HL-60 cells by suppression of mTOR/p70S6K and GSK-3β signaling pathways. Collectively, these findings suggest that THC-induced autophagy in HL-60 cells is mediated by the mTOR/p70S6K and GSK-3β signaling pathways.

3.4 PI3K/Akt and MAPK signaling pathways are involved in THC-induced autophagy in HL-60 cells

Accumulating evidence supports that PI3K/Akt and ERK1/2 pathways are involved in regulating autophagy [32, 33]; however, the functions of p38 and JNK1/2 MAPKs in this pathway remain to be clarified. Therefore, we investigated how these signaling pathways functioned in inducing autophagy by THC in HL-60 cells. As shown in Fig. 6A, the phosphorylation of PI3K, Akt and PDK1 decreased in cells treated with THC in a time-dependent manner. On the contrary, treatment with THC increased the phosphorylated ERK1/2 and JNK1/2 effectively for a period of 1–3 h, then gradually decreased the phosphorylation of ERK1/2 and JNK1/2 for 6–24 h compared with total ERK1/2 and JNK1/2 protein in HL-60 cells (Fig. 6B). Furthermore, the degree of phosphorylation of p38 MAPK was decreased after 1 h of 100 μM THC treatment compared with total p38 MAPK protein. Taken together, these results indicated that THC inhibits the PI3K/Akt/mTOR/p70S6K, GSK-3β and p38MAPK pathways and activates the ERK1/2, JNK1/2 MAPK pathways and suggests that these changes mediate THC-induced autophagy in HL-60 cells.

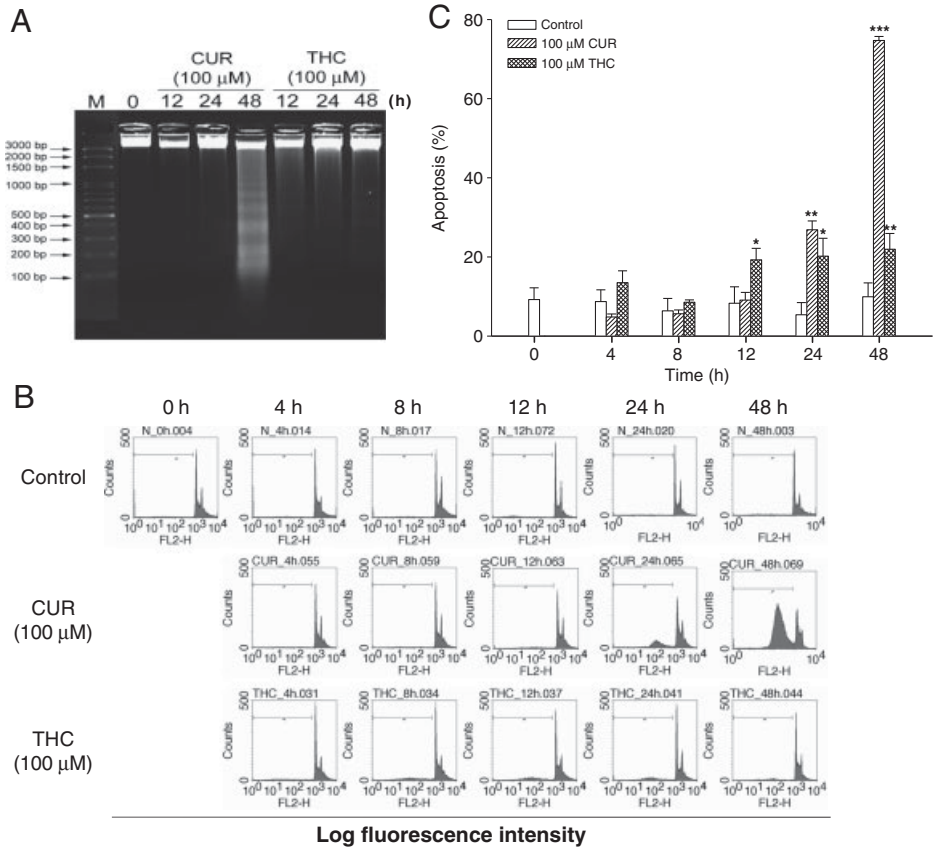

Figure 2. DNA fragmentation induced by CUR and THC in HL-60 cells. Cells were treated with 100 μM CUR or THC for 0, 12, 24, 48 h as indicated (A). Effect of CUR and THC on induction of apoptosis in HL-60 cells (B). Time-dependent effect of CUR or THC on cell cycle distribution in HL-60 cells. Quantification of sub-G1 phase in HL-60 cells. Cells were treated with 100 μM CUR or THC for 0–48 h. Data were presented as mean ± SD of triplicate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistically significant differences from the solvent control group.

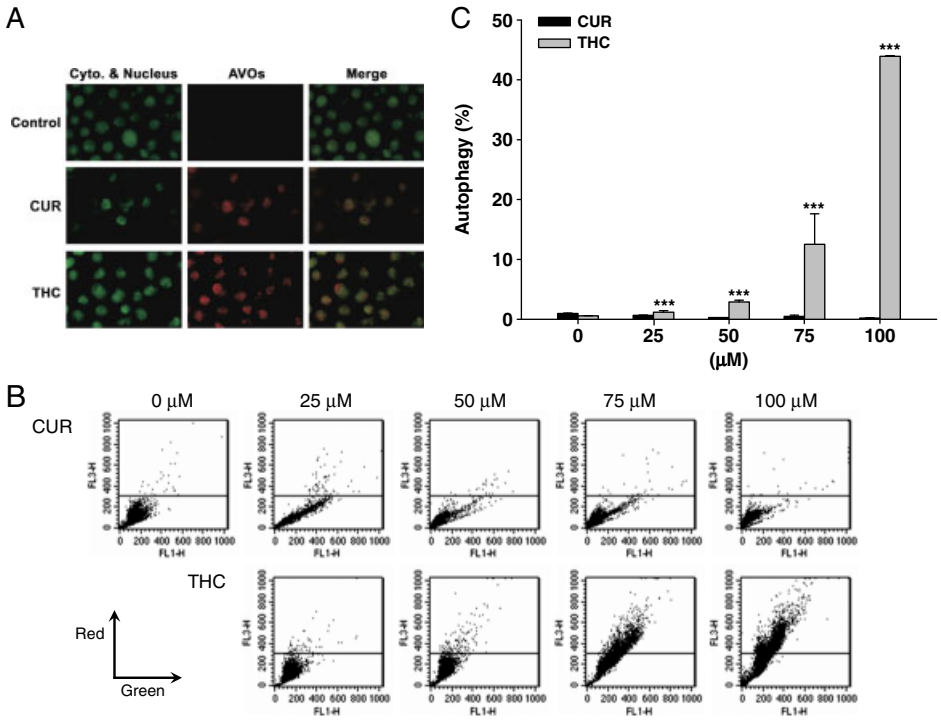

Figure 3. CUR and THC induced the formation of AVOs in HL-60 cells. Cells were treated with 100 μM CUR or THC for 48 h and stained with acridine orange. Green and red fluorescence in acridine orange-stained cells were observed under fluorescence microscope (A). Measurement of AVOs and quantification of autophagic flux in HL-60 cells (B). Development of AVOs in HL-60 cells. Cells were treated with different concentration of CUR or THC for 48 h and stained with acridine orange. Detection of green and red fluorescence in acridine orange-stained cells was performed using flow cytometry. Quantification of cells with enhanced red fluorescence using flow cytometry after acridine orange staining. Data were presented as mean ± SD of triplicate experiments. $^{***}p < 0.001$ indicate statistically significant differences from the solvent control group.

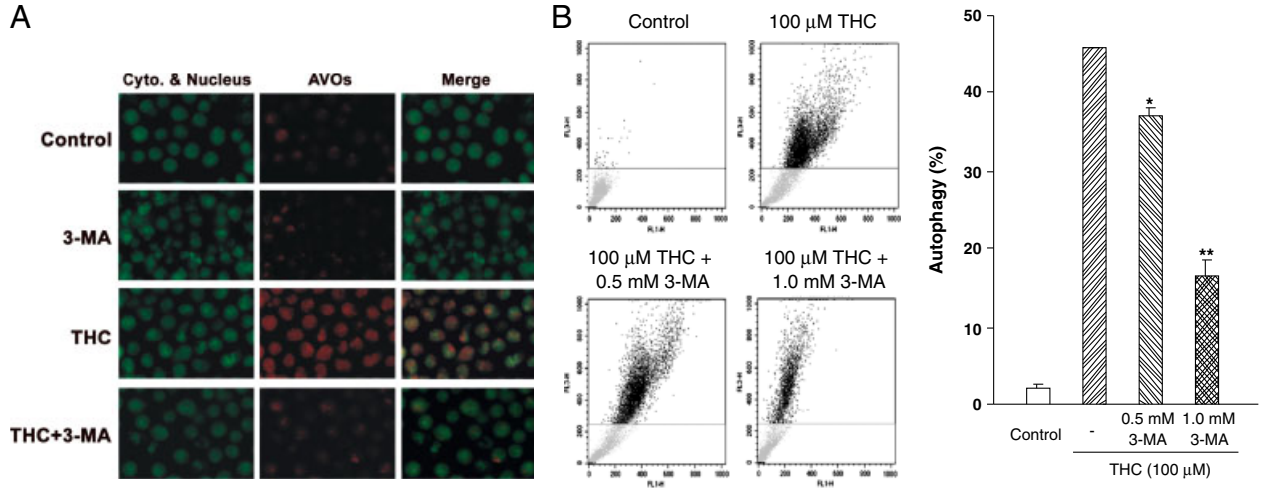

Figure 4. 3-MA inhibited THC induces autophagy in HL-60 cells. Cells were pretreatment with 1.0 mM 3-MA for 1 h before treatment with 100 μM THC for 48 h and stained with acridine orange (A). Cells were pretreatment with 0.5 or 1.0 mM 3-MA for 1 h before treatment with 100 μM THC for 48 h and stained with acridine orange. Detection of green and red fluorescence in acridine orange-stained cells was performed using flow cytometry (B). Quantification of acridine orange stained-cells with enhanced red fluorescence. Data were presented as mean ± SD of triplicate experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistically significant differences from the group treated with THC only.

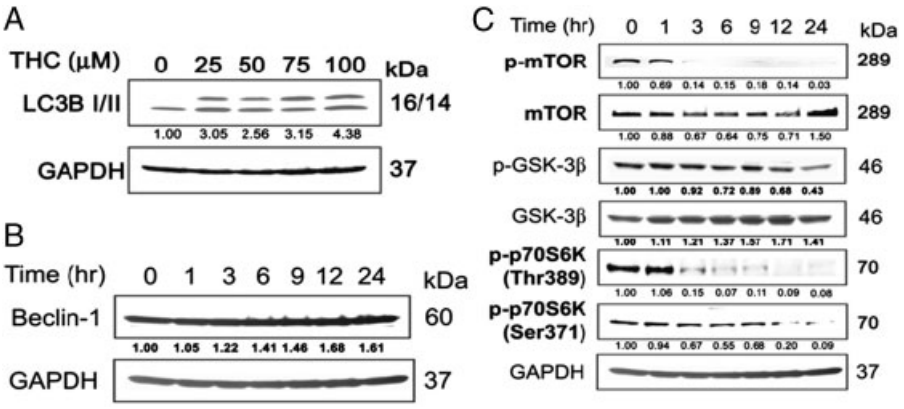

Figure 5. The effect of THC on mTOR/70S6K and GSK-3β pathway. Cells were treated with different concentrations of THC for 48 h (A). The cells were treated 100 μM THC at different time (B). Cell lysates were prepared, subjected to Western blot analysis for p-mTOR, p-GSK-3β, p-70S6K (C). The GAPDH was used as an internal control to normalize the amount of proteins applied to each lane. The values under each lane indicate relative density of the band normalized to GAPDH using a densitometer. Data are representative of three independent experiments.

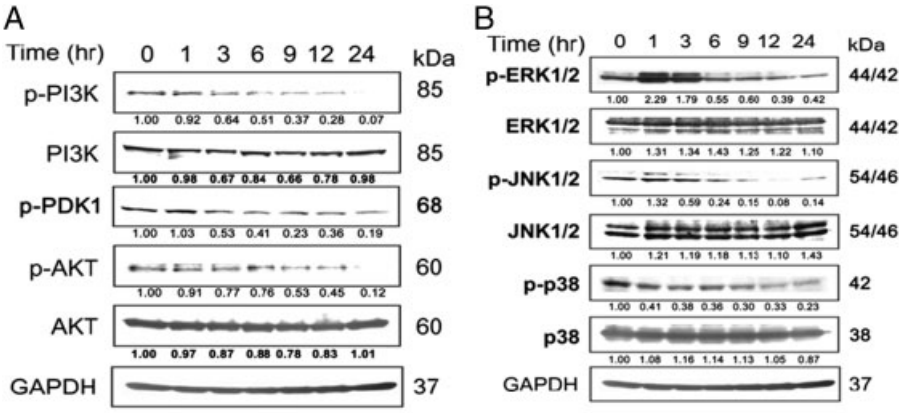

Figure 6. THC inhibits the PI3K/PDK1/Akt and prior to activate the ERK1/2 and JNK1/2 MAPK. Western blot analysis for PI3K/Akt and MAPKs pathway. HL-60 cells were treated with 100 μM THC at different times. Cell lyates p-PI3K, PI3K, p-PDK1, p-Akt and Akt (A) and p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, p-p38 and p38 (B) Western blotting analysis. Western blot is a representative of at least three independent experiments. The values under each lane indicate relative density of the band normalized to GAPDH using a densitometer.

4 Discussion

CUR (diferuloylmethane) is a major naturally occurring polyphenol of Curcuma species, which is commonly used as a yellow coloring and flavoring agent in foods. Recently, CUR has been considered a potentially important chemopreventive agent against cancer [34]. We previously reported that most of the CUR administered is reduced by an endogenous reductase system in a stepwise manner and subsequently glucuronidated by Uridine 5'-diphosphoglucuronosyltransferase [35]. THC is one of the major metabolites of CUR. Although CUR has shown anticarcinogenic activity in animal models, the serum concentration of taking 8000 mg CUR was 1.77 μM and urinary excretion of CUR was undetectable in human [36]. THC has been reported recently to be a less effective chemopreventive agent in mouse skin than CUR [33]. In contrast to the case of skin carcinogenesis, mice fed 0.5% THC in the diet showed a stronger inhibition of 1,2-dimethylhydrazine-induced mouse colon carcinogenesis than mice fed CUR [7]. We suggested that CUR could exert its bioactivity through its metabolites in vivo. These conflicting findings prompted us to determine the efficacies of CUR and THC in inhibiting the proliferation of human leukemia HL-60 cells.

In this study, for the first time, we compare the cancer cell growth inhibitory effect of CUR and THC in human leukemia HL-60 cells (Fig. 1). The results of this study showed that different from CUR, which induced apoptosis, THC induced autophagy in HL-60 cells. Regarding signal pathways, THC inhibited the PI3K/Akt/mTOR/p70S6K and activated the ERK1/2 and JNK1/2 in early stage, resulting in autophagy (Fig. 7). Therefore, THC has a potent anticancer effect and is a promising new therapeutic strategy. Autophagy is genetically programmed; promoters of autophagy have the potential for clinical benefit in the setting of cancer prevention. As autophagy is required for the effective management of metabolic stress, promoting autophagy through mTOR pathway inhibition might be expected to limit tumor progression [37]. Moreover, in a setting of chronic inflammation, the persistent tissue damage and cell proliferation, as well as the enrichment of reactive oxygen species and reactive carbonyl species, contribute to a cancer prone microenvironment [38]. Therefore, the possibility of activating autophagy to promote autophagic cell death or limit genome damage and progression in the setting of chemoprevention is intriguing [37].

In autophagy, unlike apoptosis, caspases are not activated, and neither is DNA fragmentation apparent. Most of cellular systems where autophagy has been proven to contribute to cell death have involved defects in the apoptosis signaling pathway [39]. Our results indicate that THC inhibits cell proliferation by autophagy in dose-dependent manner, but CUR via apoptosis (Figs. 2 and 3).

It was thought that autophagy induced by THC in HL-60 cells could be suppressed under the condition of 3-MA treatment, and the inhibitory effect of 3-MA is dose-dependent, which was consistent with the general opinion that 3-MA is a specific inhibitor of the autophagic pathway (Fig. 4). Our results also showed that after incubation with THC, the LC3 and beclin 1 expressions were remarkably increased in HL-60 cells (Fig. 5). The PI3K/Akt and mTOR/p70S6K pathways are main pathways that negatively regulate autophagy [32]. mTOR activates the downstream p70S6 Ser/Thr kinase that phosphorylates ribosomal protein S6, required for biosynthesis of the cell's translational apparatus, a critical component of cell growth and proliferation [40]. This study clearly demonstrated that THC inhibits the PI3K/Akt/mTOR/p70S6K and transit activates ERK1/2 pathway to promote autophagy (Figs. 5 and 6).

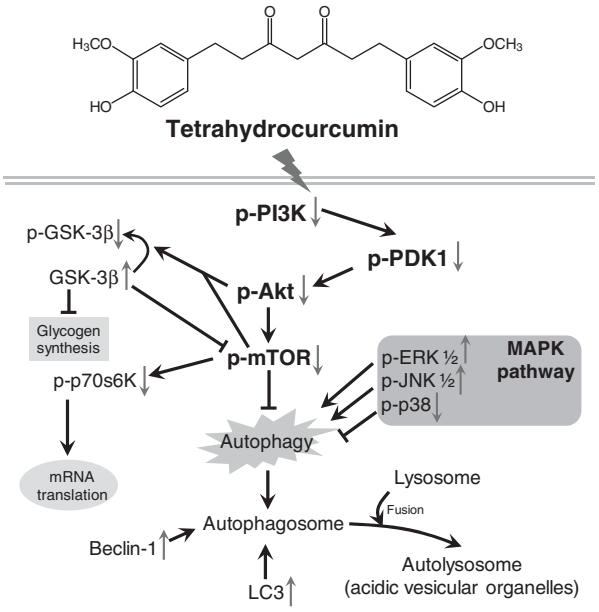

Figure 7. Schematic of mechanisms of action of THC-induced autophagy in HL-60 cells.

In summary, we have shown for the first time that THC induces autophagy in human leukemia HL-60 cells. Our results suggest that effect of autophagy on cell death may be dependent on its regulatory pathways. We recommend that the use of THC as a new anticancer agent for human leukemia should be pursued further because of its prominent effect and its new anticancer mechanism of inducing autophagy.

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The authors have declared no conflict of interest.

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