

# Tetrahydrocurcumin-induced autophagy via suppression of PI3K/Akt/mTOR in non-small cell lung carcinoma cells

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**Abstract.** Lung carcinoma is the leading cause of mortality due to cancer worldwide. Autophagy has a significant role in the development and progression of non-small cell lung carcinoma (NSCLC). A previous study has revealed that tetrahydrocurcumin (THC), a traditional Chinese medicine isolated from *Curcuma wenyujin* (Chen & Ling, 1981), induces autophagy in human A549 NSCLC cells. The present study evaluated THC-induced autophagy in A549 cells using various assays, including the Cell Counting Kit-8, acridine orange staining, flow cytometry, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and western blot analysis of the markers of autophagy. THC inhibited the growth and proliferation of A549 cells ( $P<0.05$ ). Acridine orange staining and flow cytometry revealed that THC treatment significantly enhanced autophagic cell proliferation inhibition ( $P<0.05$ ). The RT-qPCR analysis revealed that THC treatment increased Beclin-1 expression level and compared with the control group ( $P<0.05$ ). The light chain 3 (LC3)-II/LC3-I ratio was reduced in THC-treated cells when compared with the control group ( $P<0.05$ ). Protein expression of various markers of autophagy, including p62, phosphorylated (p)-mechanistic target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K), p-PI3K, protein kinase B (Akt), and p-Akt was significantly reduced in THC-treated cells ( $P<0.05$ ). In conclusion, the present study revealed the underlying mechanisms associated with THC-induced autophagy.

A promising method of enhancing the therapeutic efficacy of THC against NSCLC cells may include inducing autophagy via inhibition of the PI3K/Akt/mTOR signaling pathway.

## Introduction

Lung carcinoma is the leading cancer mortality worldwide (1). The most common lung cancer is non-small cell lung carcinoma (NSCLC), which differs from small cell lung carcinoma (SCLC) in its histological and cytological features (2). At present, the treatment of NSCLC is very limited, including chemotherapy, radiation therapy, surgical resection and targeted therapy (3). The prognosis for lung cancer is unfavorable, and novel therapeutic strategies to improve the outcome of patients with NSCLC are required. Traditional Chinese medicine is a source of novel compounds with antineoplastic activity, including isocryptotanshinone, mangiferin and xanthatin (4-6). Tetrahydrocurcumin (THC) is a traditional Chinese medicine isolated from *Curcuma wenyujin* (Chen & Ling, 1981). THC has a  $\beta$ -diketone structure and two phenolic groups (Fig. 1) and has anti-inflammatory, antioxidative and antitumor properties (7-12); treatment of NSCLC via THC requires further investigation.

Autophagy is important for cellular functioning and has a key role in various physiological and pathological processes (13). In carcinoma, processes with both promotional and suppression properties are considered a double-edged sword (14). The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) molecular signaling pathway is important for autophagy. Activation of PI3K and mTOR leads to an increase in the level of phosphorylated (p)-Akt, which can integrate upstream signals active via the PI3K/Akt signaling pathway and phosphorylation, suppressing autophagy (15,16). In order to evaluate the clinical use of THC, the present study investigated the antineoplastic mechanisms of THC in A549 cells. The findings indicate that the PI3K/Akt/mTOR molecular signaling pathway is involved in THC-induced autophagy.

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## Materials and methods

**Chemicals and reagents.** THC (Hangzhou Haoxin Biotech, Co., Ltd., Hangzhou, China) was dissolved at a concentration

of 200  $\mu\text{M}$  in absolute dimethyl sulfoxide (DMSO) as a stock solution, which was stored at 4°C and was diluted with DMSO for the subsequent experiments. DMSO, tetramethylethylenediamine, ammonium persulfate, and acridine orange were purchased from Sigma-Aldrich, Merck Millipore (Darmstadt, Germany). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin antibiotics, and phosphate-buffered saline (PBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Tris-HCl and 4xTris-HCl were purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Additionally, 30% acrylamide/sis solution (30%) and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad Laboratories, Inc. (Shanghai, China). Tris and Glycine were purchased from Amresco, LLC (Solon, OH, USA). Tween-20, methanol, and sodium chloride were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). SDS-polyacrylamide gel electrophoresis (PAGE) protein loading buffer (5X), cell lysis buffer for western blotting and immunoprecipitation, phenylmethanesulfonyl fluoride, an ECL Plus Luminescence kit, and a BCA Protein Assay kit were purchased from Beyotime Institute of Biotechnology (Nantong, China). The Cell Counting Kit-8 (CCK-8), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, MAB5465), goat anti-rabbit immunoglobulin G (IgG; GAR0072), and goat anti-mouse IgG (GAM007) secondary antibodies were purchased from MultiSciences Biotech Co., Ltd. (Shanghai, China). A high-purity total RNA rapid extraction kit was purchased from Genaray Biotech Co., Ltd. (Shanghai, China). A PrimeScript™ RT reagent kit was purchased from TaKaRa (Dalian, China). SuperReal PreMix Color (SYBR® Green) was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). Akt (cat. no. 4691S), p-Akt (cat. no. 4060S), mTOR (cat. no. 2983S), p-mTOR (cat. no. 5536S), light chain (LC)3 I/II (cat. no. 4108S), p62 (cat. no. 13121S), PI3K (cat. no. 4249P), and p-PI3K (cat. no. 4228S) antibodies were purchased from Cell Signaling Technology, Inc. (Shanghai, China).

**Cell lines and cell culture.** The A549 human lung adenocarcinoma epithelial cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 supplemented with 10% FBS, penicillin G (100 U) and streptomycin (100  $\mu\text{g}/\text{ml}$ ). Cells were incubated in a humidified atmosphere containing 5%  $\text{CO}_2$  at 37°C.

**Cell viability assay.** The CCK-8 assay was used to quantify the effect of THC on the cell viability of A549 cells. Cells were seeded in 96-well plates ( $5 \times 10^3$  cells/well) in 90  $\mu\text{l}$  medium for 24 h. The cells were subsequently treated cells with 10  $\mu\text{l}$  of 0, 30, 70, 100 or 130  $\mu\text{M}$  THC for 12, 24, 48 and 72 h. Following the THC treatment, 10  $\mu\text{l}$  CCK-8 was added to each well and incubated for an additional 2 h at 37°C. The present study quantified cell viability at 490 nm using a plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

**Flow cytometry.** To verify the effect of THC on autophagy in A549 cells, the present study quantified cellular autophagy using flow cytometry. A549 cells were seeded in 6-well plates ( $3 \times 10^5$  cells/well) for 24 h and treated with 0, 10, 30, 70, 100 or 130  $\mu\text{M}$  THC for 12, 24, 48, and 72 h. Cells were harvested,

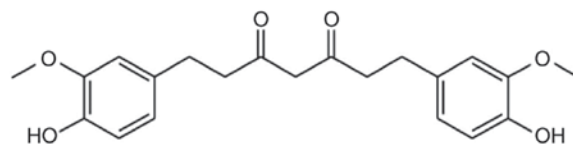


Figure 1. Chemical structure of tetrahydrocurcumin.

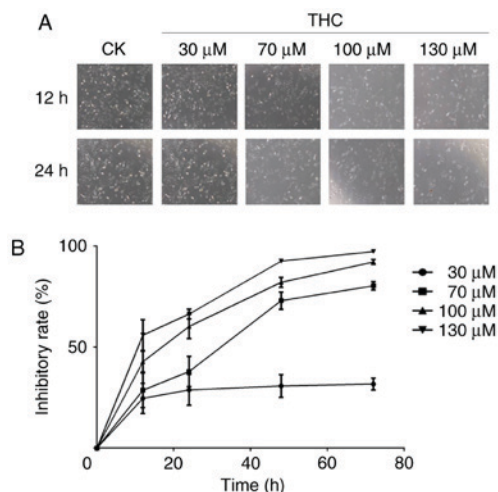


Figure 2. Effect of tetrahydrocurcumin on A549 cell proliferation determined by (A) micrograph (magnification, x40) and (B) Cell Counting Kit-8 assay. Data are presented as the mean  $\pm$  standard deviation. THC 130  $\mu\text{M}$  was concentration that had the highest inhibitory effect on A549 cell proliferation, whereas THC 30  $\mu\text{M}$  has the lowest inhibitory effect, showing the dose-dependent effect of THC treatment. CK, control group; THC, tetrahydrocurcumin.

washed twice with cold PBS, and stained for 15 min at 4°C with acridine orange. The fluorescence intensity of the cells was detected using channels FL1 and FL3 of the flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The analyses were repeated three times. The autophagy rate (%) was calculated as follows: Autophagy rate (%) = (FL3/FL1)  $\times$  100.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Using TRIzol reagent, the present study treated total mRNA from the A549 cells with 0, 30, 70, 100 and 130  $\mu\text{mol}/\text{l}$  THC for 24 h at 37°C. Following the THC treatment, the mRNA expression levels in A549 cells were determined using qPCR assay on a CFX Connect Real-Time PCR system (Bio-Rad Laboratories, Inc.). The present study extracted total RNA using a high-purity total RNA rapid extraction kit and synthesized the cDNA using a PrimeScript™ RT reagent kit; in an ice bath the RT reaction was mixed and then reacted at 37°C for 15 min and then at 85°C for 5 sec. The reaction was then stopped and stored at 4°C until use. Each sample was run in triplicate in a final volume of 20.0  $\mu\text{l}$  containing 10  $\mu\text{l}$  2X SuperReal Color PreMix, 1  $\mu\text{l}$  forward primer (10  $\mu\text{M}$ ), 1  $\mu\text{l}$  reverse primer (10  $\mu\text{M}$ ) and 8  $\mu\text{l}$  cDNA ( $\beta$ -actin forward, TGACGTGGACATCCGCAAAG, reverse, CTGGAAGGTGGACAGCGAGG; Beclin-1 forward, AAACAGATGCGTTATGCCCC and reverse, GCGACC CAGCCTGAAGTTAT). Cycling conditions for the qPCR were as follows: 1 cycle at 95°C for 15 min followed by 40 cycles at 95°C for 10 sec, 57°C for 15 sec, and 72°C for 15 sec. At the end of each reaction, a melting curve analysis was performed.

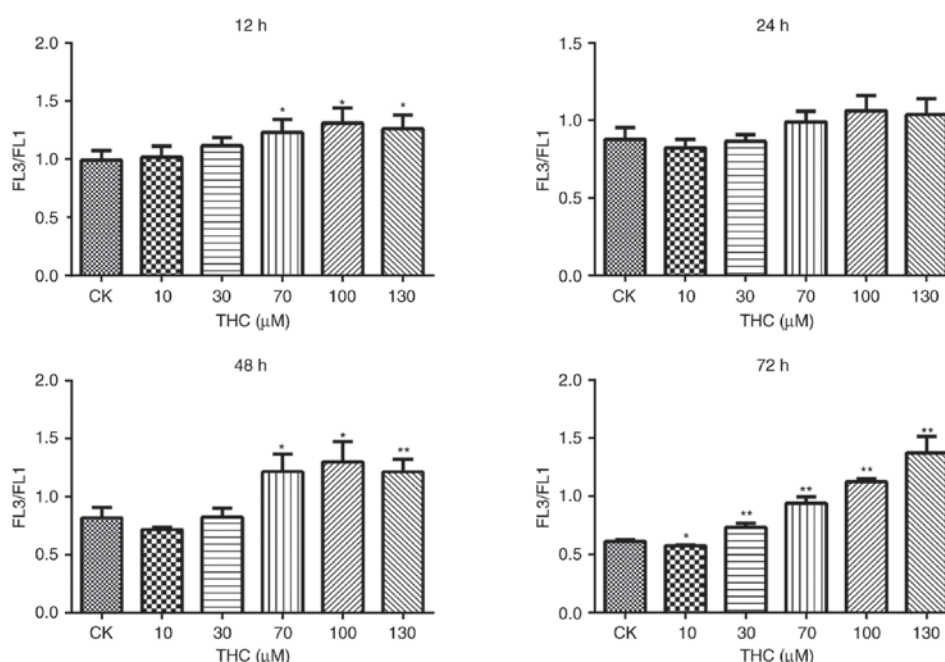


Figure 3. Autophagy rate was quantified using flow cytometry. Autophagy was evident at 12 h and peaked at 72 h. \* $P < 0.05$ , \*\* $P < 0.01$  vs. CK group. CK, control group; FL3/FL1, the ratio of the fluorescence intensity of the FL3 channel to the FL1 channel of the flow cytometer; THC, tetrahydrocannabinol.

$\beta$ -actin was used as the reference gene, and the  $2^{-\Delta\Delta C_q}$  method was used to determine the relative expression of each gene (17).

**Western blot analysis.** A549 cells ( $2 \times 10^5$  cells/plate) were treated with 0, 30, 70, 100, and 130  $\mu\text{mol/l}$  THC for 24 h at  $37^\circ\text{C}$  and lysed in cell lysis buffer for western blotting and immunoprecipitation. Proteins were quantified using the BCA Protein Assay kit. Protein (30  $\mu\text{g/lane}$ ) were subjected to 12% SDS-PAGE and transferred to a polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk for 1 h, followed by overnight incubation at  $4^\circ\text{C}$  with the following primary antibodies: Akt, p-Akt, LC3 I/II, mTOR, p-mTOR, p62, p-PI3K, and PI3K (all 1:1,000). Subsequently, the membranes were incubated with the corresponding horseradish-peroxidase secondary antibody, goat anti-rabbit IgG, and goat anti-mouse IgG (all 1:5,000) for 1 h at room temperature. The blots were developed and visualized using the ECL Plus Luminescence kit and ChemiDoc XRS+ System (Bio-Rad Laboratories).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. Student's t-test and one-way analysis of variance followed by a Tukey's post hoc test were used to analyze the data. Differences between experimental groups were assessed using SPSS version 19.0 (IBM Corporation, Armonk, NY, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**THC promotes cell proliferation inhibition.** To evaluate the effects of THC on cell proliferation, A549 cells were treated with 0, 30, 70, 100, and 130  $\mu\text{M}$  THC for 12, 24, 48 and 72 h. Cell viability was determined using a CCK-8 assay. A representative micrograph is presented in Fig. 2A (magnification,  $\times 40$ ). The inhibitory effect increased with

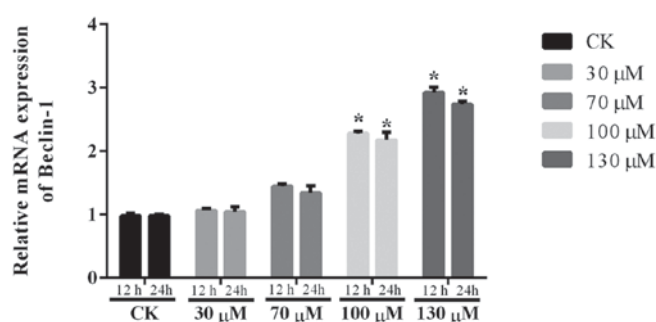


Figure 4. Relative normalized expression of Beclin-1. Beclin-1 expression levels were detected using reverse transcription-quantitative polymerase chain reaction at 12 and 24 h of THC treatment. The expression of Beclin-1 in the 100 and 130  $\mu\text{M}$  THC groups was significantly higher when compared with the CK group. \* $P < 0.05$  vs. 24 h CK group. CK, control group; THC, tetrahydrocannabinol.

higher THC concentration at 12 and 24 h. THC inhibited cell growth in a dose-dependent manner at each time point (Fig. 2B).

**THC induces autophagy in A549 cells.** The aforementioned findings suggested that THC induced an autophagic response in A549 cells. Flow cytometry determined that THC-induced autophagy in A549 cells (Fig. 3).

**THC increases Beclin-1 expression.** Beclin-1 is associated with genes involved in the formation of autophagosomes and may influence the rate of autophagy. Following THC treatment in A549 cells, a dose-dependent increase in Beclin-1 expression was observed ( $P < 0.05$ ; Table I, Fig. 4).

**THC-induced autophagy involves inhibition of the PI3K/Akt/mTOR signaling pathway.** The PI3K/Akt/mTOR



Table I. Expression data for Beclin-1 at 12 and 24 h (n=5, mean  $\pm$  standard deviation).

Time	THC treatment				
	CK	30 $\mu$ M	70 $\mu$ M	100 $\mu$ M	130 $\mu$ M
12 h	0.98 $\pm$ 0.04	1.06 $\pm$ 0.03	1.45 $\pm$ 0.04	2.28 $\pm$ 0.03 <sup>a</sup>	2.92 $\pm$ 0.08 <sup>a</sup>
24 h	0.98 $\pm$ 0.03	1.04 $\pm$ 0.08	1.34 $\pm$ 0.11	2.17 $\pm$ 0.13 <sup>a</sup>	2.74 $\pm$ 0.05 <sup>a</sup>

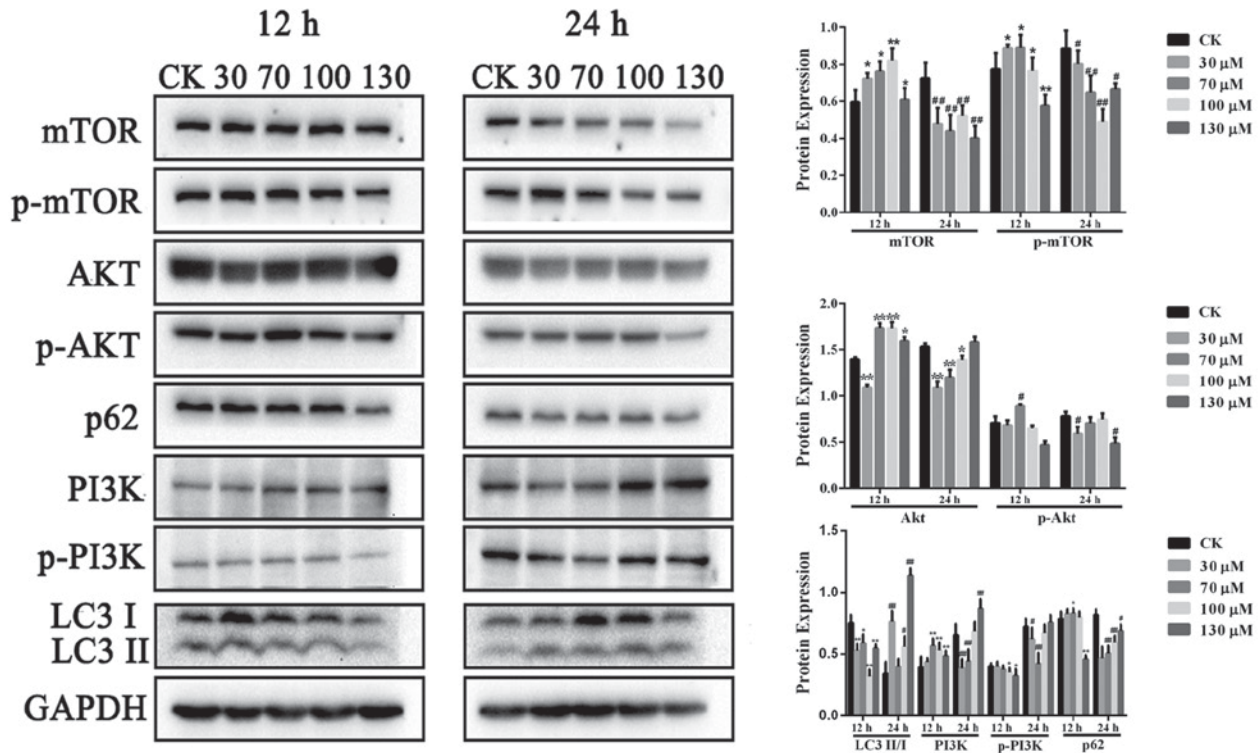
<sup>a</sup>P<0.05; THC, tetrahydrocurcumin; CK, control group.

Figure 5. Expression levels of p-mTOR, p-Akt, p-PI3K, LC3I/II and p62 in the THC 24 h treatment group were markedly reduced when compared with the 12 h. Treatment with THC for 24 h led to a significant reduction in levels of p-mTOR, p-Akt, and p62 compared to treatment for 12 h. \*P<0.05 vs. 24 h CK group, \*\*P<0.01 vs. 24 h CK group, #P<0.05 vs. 24 h CK group and ##P<0.01 vs. 24 h CK group; THC, tetrahydrocurcumin; p, phosphorylated; mTOR, mechanistic target of rapamycin; Akt, protein kinase B; PI3K, phosphoinositide 3-kinase; LC3, light chain 3I/II.

molecular signaling pathway is involved in autophagy regulation, and suppression of the PI3K/Akt/mTOR pathway promotes autophagy. To examine the role of this pathway in THC-induced autophagy, the present study quantified the expression of mTOR, p-mTOR, Akt, p-Akt, LC3-II/LC3-I, p62, PI3K, and p-PI3K using western blotting. Treatment with THC for 24 h led to a significant reduction in levels of p-mTOR, p-Akt and p62 compared to treatment for 12 h (Fig. 5). These findings indicate that the PI3K/Akt/mTOR pathway is involved in THC-induced autophagic cell proliferation inhibition via inhibition of Akt and mTOR phosphorylation.

## Discussion

THC has anti-inflammatory, antioxidative and antitumor effects (7-12). Previous studies have revealed that THC inhibits the invasion of breast cancer (10,18). The present study confirmed the suppressive effect of THC on A549 lung cancer

cells. THC inhibits the proliferation of A549 cells by inducing autophagy via the PI3K/Akt/mTOR signaling pathway (Fig. 6).

The present study revealed a potential novel molecular mechanism for the antitumor effects of THC in A549 cells. Treatment of A549 cells with THC led to increased autophagy. Concurrently, activation of the PI3K/Akt/mTOR molecular pathway was inhibited by THC, which indicates that activation of the PI3K/Akt/mTOR pathway may be associated with autophagy. This is consistent with the previously reported mechanism of action of THC in the literature (19). Wu *et al* determined that THC induced autophagic cell death via the PI3K/Akt/mTOR and MAPK molecular signaling pathways in HL-60 human leukemia cells (19). Therefore, it may be concluded that THC-induced autophagic cell proliferation inhibition is the predominant mechanism underlying inhibition of A549 cell proliferation. This conclusion may contribute to the future development and clinical application of curcumin antitumor drugs.

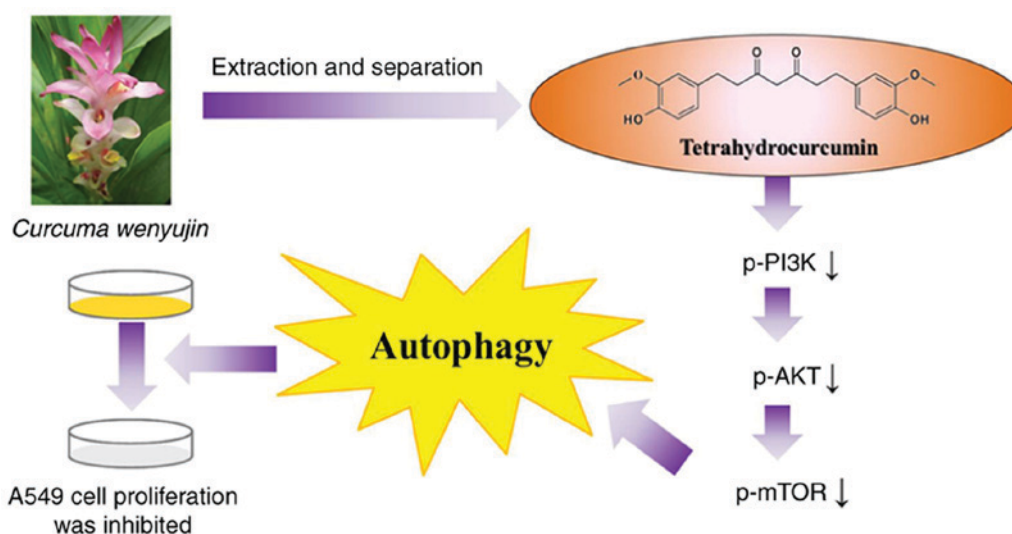


Figure 6. Tetrahydrocurcumin inhibits the proliferation of A549 cells by PI3K/Akt/mTOR pathway-induced tumor cell autophagy. mTOR, mechanistic target of rapamycin; Akt, protein kinase B; PI3K, phosphoinositide 3-kinase.

LC3 has been previously used as a specific marker to monitor autophagy and LC3-II (the conjugated form of LC3) is highly correlated with autophagosome number (20,21). p62 is a selective autophagy substrate (22). The present study determined that THC induction activated the levels of the autophagy-associated protein LC3II/I, reduction in autophagy for the decreasing p62 expression. This suggested that THC inhibition of A549 lung cancer cells may be achieved through the autophagy pathway.

The PI3K/AKT/mTOR molecular signaling cascade regulates cell autophagy. As an important member of the PI3K-associated kinase family, mTOR is associated with cell proliferation and metabolism. Previous studies suggested that suppression of Akt and downstream target protein mTOR activation may induce autophagy (23-25). The PI3K/Akt/mTOR pathway has a key role in the pathogenesis of NSCLC. Therefore, inhibition of PI3K/Akt/mTOR pathway activation may be a suitable therapeutic target for future lung cancer treatments (26,27). The findings of the present study revealed that THC may induce the antitumor effects of autophagy in A549 cells by reducing activation of the PI3K/Akt/mTOR pathway, which suggests that THC is a potential treatment for lung cancer.

In conclusion, THC induced autophagic cell proliferation inhibition in NSCLC cells via suppression of the PI3K/Akt/mTOR molecular signaling pathway. To the best of our knowledge the current findings present a novel anticancer mechanism of THC.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

GS, HL and BL conceived and designed the experiments and acquired reagents, materials, and/or analysis tools. HL, FC, YW, WF and WS performed the experiments. HL and BL analyzed the data. GS and BL produced the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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