

Novel curcumin analogue IHCH exhibits potent anti-proliferative effects by inducing autophagy in A549 lung cancer cells

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Received October 19, 2013; Accepted March 24, 2014

DOI: 10.3892/mmr.2014.2183

Abstract. Curcumin is a natural polyphenolic compound that exhibits strong antioxidant and anticancer activities; however, low bioavailability has restricted its application in chemotherapeutic trials. The present study aimed to investigate the anticancer effect of the novel curcumin derivative 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone (IHCH) on A549 lung cancer cells. Cells were treated with IHCH at different concentrations (1-40 μ M) for different time periods (1-36 h). Microscopic analysis revealed that IHCH inhibited A549 cell growth and induced the formation of characteristic autophagolysosomes in a dose- and time-dependent manner. Furthermore, the inhibitory rate of IHCH (40 μ M) on A549 cell viability was 77.34% after 36 h of treatment. Acridine orange staining revealed an increase in autophagic vacuoles in the IHCH-treated A549 cells. Monodansylcadaverine staining was used to analyze autophagy rate. Immunocytochemistry revealed an increase in light chain (LC) 3 protein expression in the IHCH-treated cells and western blot analysis detected the conversion of LC3-I to LC3-II, as well as the recruitment of LC3 to autophagosomes in the cytoplasmic compartment, suggesting the occurrence of autophagy. These findings show that IHCH induced autophagy in A549 cells, which is a novel cell death mechanism induced by curcumin derivatives.

Introduction

Autophagy is an evolutionarily conserved biological process in which under certain types of cellular stress, damaged organelles and long-lived proteins are encapsidated and

directed to the lysosome for degradation (1,2). The cytoplasmic content is sequestered in autophagosomes, which are double-membraned vesicles that fuse with lysosomes to form autophagolysosomes, in which the cellular material is degraded by acidic lysosomal hydrolases. In addition to its physiological role in the elimination of aged or damaged cell components, autophagy acts as an important mechanism for cellular homeostasis and a survival mechanism for cells undergoing nutrient deprivation or other stresses (3). Furthermore, an increasing number of studies have shown that autophagy is involved in programmed cell death and may lead to cell death or have a cytoprotective function (4,5). As an important mediator of pathological responses, autophagy has attracted increasing attention, particularly in cancer research (6,7).

Curcumin is a yellow, dietary polyphenol derived from the rhizomes of *Curcuma longa*. Numerous studies have demonstrated that curcumin has anti-inflammatory, -oxidative and -carcinogenic effects in various types of tumor cells (8-11). However, based on its instability under certain physiological conditions, poor bioavailability and rapid metabolism, the application of curcumin in anticancer therapy has been limited (12). The generation of synthetic curcumin analogs may overcome the limitations associated with curcumin. Several studies have generated novel synthetic analogs or derivatives of curcumin in order to enhance the anti-proliferative activity of curcumin (13-15); however, such analogs have generally led to cancer cell death through apoptosis.

In the present study, a novel derivative of curcumin was chemically synthesized, termed 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone (IHCH) (Fig. 1). The present study aimed to investigate the cell death pathway induced by IHCH in A549 cells. IHCH was found to have an anti-proliferative effect in A549 cells by inducing autophagy. Acridine orange staining and monodansylcadaverine (MDC) fluorescence analysis were used to monitor autophagolysosomes and autophagic vacuoles, respectively. Immunocytochemistry of light chain (LC) 3 localization detected recruitment of LC3 to autophagic vesicles. Furthermore, western blot analysis was used to assess the conversion of LC3-I to LC3-II in the IHCH treated A549 cells. The present study identified that autophagy is an important process involved in IHCH-induced cell death.

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Key words: autophagolysosomes, light chain 3, programmed cell death, synthetic analogs

Materials and methods

Chemicals and cell culture. IHCH was obtained from the College of Chemistry and Chemical Engineering, Henan University of Technology (Zhengzhou, China) and was dissolved in dimethylsulfoxide (DMSO; stock solution, 0.1 M). Wortmannin was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). MDC and acridine orange were purchased from Sigma-Aldrich (St. Louis, MO, USA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies were purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA). A549 cells were maintained at Henan University of Technology (Zhengzhou, China) in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) containing 1% penicillin and 10% (v/v) fetal bovine serum (Hyclone, Rockford, IL, USA) in a humidified atmosphere containing 5% CO₂ at 37°C.

Cell proliferation assays. A549 cells were plated in 24-well culture plates (Corning Inc., Corning, NY, USA) and treated with IHCH at different concentrations (1, 5, 10, 20 and 40 mM) for different time periods (1, 3, 6, 12, 24 and 36 h). Cell morphology was analyzed using an inverted microscope (Nikon Eclipse TS100; Nikon Corporation, Tokyo, Japan). All experiments were repeated at least three times.

2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone (MTT) analysis. Cells were seeded on 96-well plates and treated with IHCH at 1, 5, 10, 20 or 40 mM for 6, 12, 24 or 48 h. MTT (5 mg/ml) was filter sterilized and added to each well (20 μl). Plates were kept in the dark for 4 h at 37°C until a purple precipitate was visible. DMSO (100 μl/well) was then added and the absorbance was read using an ELISA reader (BioTek, Winooski, VT, USA) at 490 nm. The percentage of cell viability was assessed using the following formula: Cell viability (%) = $(100 - A_m/A_n) \times 100$. A_m and A_n represent the absorbance of the test substances and solvent control, respectively.

Acridine orange staining. Acridine orange staining was used to analyze autophagic vacuoles (16). A549 cells were treated with IHCH at 1, 5, 10, 20 or 40 μM for 3, 6, 12 or 24 h. Cells were then washed twice in phosphate-buffered saline (PBS) and stained with 5 μg/ml acridine orange for 40 sec at room temperature. Cell micrographs were analyzed using a fluorescence microscope (Nikon Eclipse TE2000-U; Nikon Corporation).

MDC labeling assay. Cells were seeded on 24-well flat bottomed plates over night followed by treatment with different concentrations of IHCH for 3 h. Subsequent to treatment with hydrazinobenzoylcurcumin (IHCH) at identical concentrations and time points, cells were incubated for 10 min with 50 μM MDC at 37°C and observed using fluorescence microscopy with a 380 nm excitation filter.

Immunocytochemistry analysis. Cells were seeded on coverslips in six-well plates and treated with IHCH for 3 h. Subsequent to washing with PBS and fixing in 4%

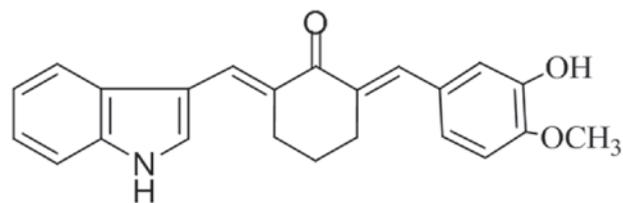


Figure 1. Chemical structure of IHCH. The molecular formula of IHCH is C₂₃H₂₁NO₃ and it has a molecular weight of 359.15 g/mol. IHCH, 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone.

paraformaldehyde for 15 min at room temperature, A549 cells were blocked using 5 mg/ml bovine serum albumin for 30 min. Cells were then incubated with anti-LC3 antibodies (Beyotime Institute of Biotechnology) diluted 1:500 in PBS, followed by FITC-conjugated goat anti-rabbit antibodies diluted 1:100 in PBS for 2 h. Slides were then analyzed using a fluorescence microscope (Nikon TE2000-U; Nikon Corporation).

Western blot analysis. A549 cells were cultured in 60 mm round dishes in the presence of 1, 10, 20 or 40 μM IHCH for 3 h, and harvested and lysed in cold lysis buffer (150 mM NaCl, 1% NP-40, 20 mM Tris-HCl, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 1 mM orthovanadate and 100 mM phenylmethanesulfonyl fluoride, pH 7.4). The cell lysates were electrophoresed using 15% SDS-PAGE and transferred to nitrocellulose membranes (Immobilon™; Millipore, Billerica, MA). Membranes were blocked with 8% non-fat dry milk in Tris-buffered saline containing Tween-20 at room temperature for 1 h, then incubated with rabbit anti-LC3B antibodies diluted 1:1,000 in PBS, followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:1,000 in PBS at 4°C for 18 h. Tubulin was used as an internal control. Membranes were washed and subsequently incubated with substrate solution containing nitroblue tetrazolium and bromo-4-chloro-3-indoxyl-phosphate (Boster Biological Technology, Ltd., Wuhan, China). Image J (National Institutes of Health, Bethesda, MA, USA) was used to quantify the intensity of each protein band. Band intensity values were presented as the fold increase or decrease with respect to the control bands.

Statistical analysis. Experiments were performed three times and data are presented as the mean ± standard deviation. Differences between mean values were analyzed using Student's t-test. P<0.05 or <0.01 were considered to indicate statistically significant differences.

Results

IHCH inhibits A549 cell proliferation. A549 cells were treated with different concentrations of IHCH (1-40 μM) for 36 h. DMSO was used as a negative control. After 3 h, the IHCH-treated cells exhibited vacuole-like structures. Furthermore, significant changes were observed in cell morphology after 6 h of IHCH treatment (Fig. 2A). MTT was

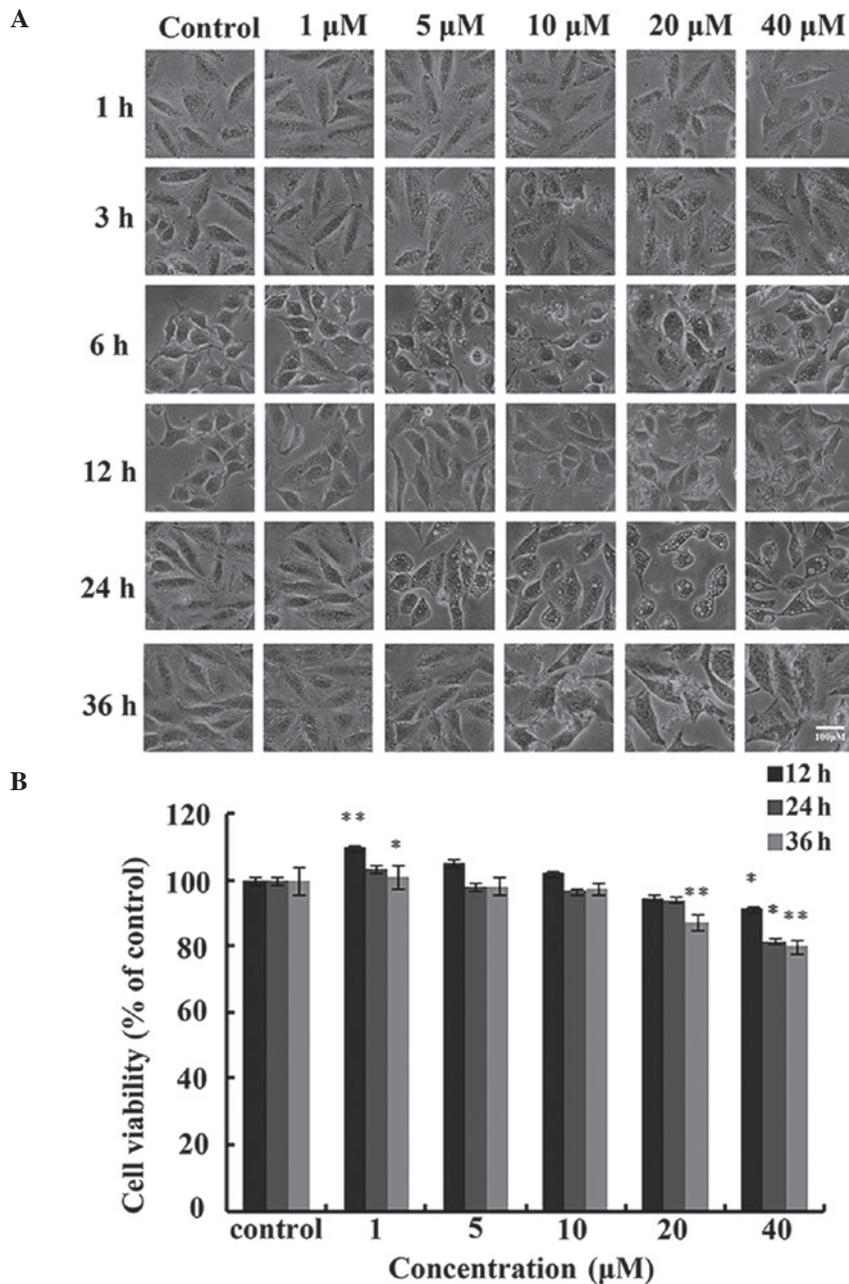


Figure 2. Effect of IHCH treatment on A549 cell morphology. (A) IHCH-treated A549 cells at different concentrations and time points. (B) Proliferative activity of A549 cells assessed using an 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone assay. IHCH (40 μM) significantly inhibited the proliferation of A549 cells. Values are presented as the mean ± standard error of at least three independent experiments. *P<0.05 and **P<0.01 vs. control (n=5). IHCH, 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone.

used to assess the effect of IHCH on A549 cell viability and showed that cell viability was reduced to 77.34% after 36 h of treatment with 40 μM IHCH (Fig. 2B).

IHCH induces the formation of acidic vesicular organelles (AVOs) in A549 cells. Autophagolysosomes generate an acidic compartment, which may be fluorescently stained red or green using acridine orange (17). To determine whether IHCH induced autophagy in A549 cells, acridine orange staining was performed in IHCH-treated A549 cells. Autophagy is associated with an increase in acridine orange positive AVOs. Red AVOs were observed following 3 h of IHCH treatment (1-40 μM) in the A549 cells (Fig. 3A), suggesting AVO

formation. Furthermore, the percentage of cells exhibiting AVOs was analyzed among 100 IHCH-treated cells. After 3 h of IHCH treatment, the percentage of cells with AVOs was found to increase in a concentration-dependent manner (1-20 μM) (Fig. 3B).

Wortmannin inhibits IHCH-induced A549 cell autophagy. As a specific dye for autophagosomes, MDC accumulates in mature autophagic vacuoles (AVs) and autophagolysosomes, but not in the early endosome compartment. In the present study, MDC-stained AVs appeared as distinct dot-like structures distributed within the cytoplasm or localized in the perinuclear region. Furthermore, the highest number

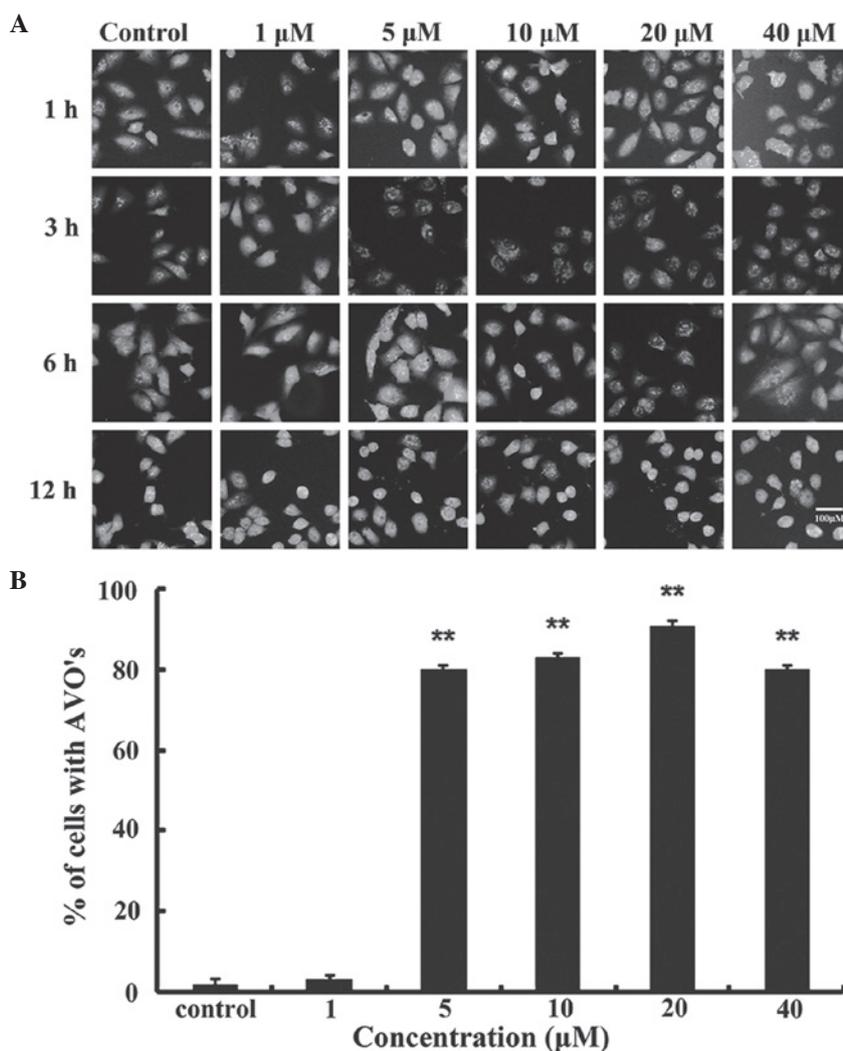


Figure 3. Detection of AVOs in IHCH-treated A549 cells using fluorescence microscopy. (A) Cells were incubated with various concentrations of IHCH for different durations and stained with acridine orange. (B) Percentage of cells exhibiting AVOs following treatment with different concentrations of IHCH. A total of 100 cells were counted in more than three, randomly selected fields of view. Data are presented as the mean \pm standard error of triplicate experiments. ** $P < 0.01$ vs. control. IHCH, 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone; AVOs, acidic vesicular organelles.

of dot-like structures were observed after 3 h of treatment with 20 μM IHCH (Fig. 4A). Wortmannin was also used to treat the IHCH-incubated A549 cells. As a highly specific inhibitor of phosphatidylinositol-4,5-bisphosphate 3-kinase, wortmannin is capable of inhibiting the Akt signaling pathway and cell autophagy (18). In the present study, wortmannin was found to decrease the intensity of the MDC fluorescence at the different concentrations of IHCH (Fig. 4A). Furthermore, the rate of cell autophagy following wortmannin treatment was observed to be lower than that in the untreated cells (Fig. 4B).

IHCH induces LC3 protein accumulation. The intracellular localization of the LC3 protein was analyzed using immunocytochemistry. IHCH-treated (20 μM ; 3 h) A549 cells were immunostained using anti-LC3 primary antibodies. Fluorescence microscopy revealed that the specific fluorescent signals were punctate, which is typical of the distribution of LC3-II within autophagosomes (Fig. 5). The DMSO-treated control cells showed no LC3 immunofluorescence.

IHCH induces the conversion of LC3-I to LC3-II. Western blot analysis was used to detect LC3-II and -I, with increases in the LC3-II/LC3-I ratio indicative of autophagy (19). Lysates of A549 cells treated with DMSO (40 μM) or IHCH (1-40 μM) for 3 h were subjected to western blot analysis. Fig. 6A shows the conversion of LC3-I to LC3-II. The level of autophagy was represented as the ratio of LC3-II expression to tubulin expression (Fig. 6B). LC3-II protein expression was observed to significantly increase in the IHCH-treated cells in a dose-dependent manner. This finding suggests IHCH induced autophagy in the A549 cells.

Discussion

A number of bioactive compounds are phytochemicals, which have been found to demonstrate growth suppressive activity as well as chemopreventive properties against various types of cancer (20). One of the most widely characterized phytochemicals is curcumin, whose inhibitory effects on tumorigenesis and tumor growth have been confirmed *in vitro*

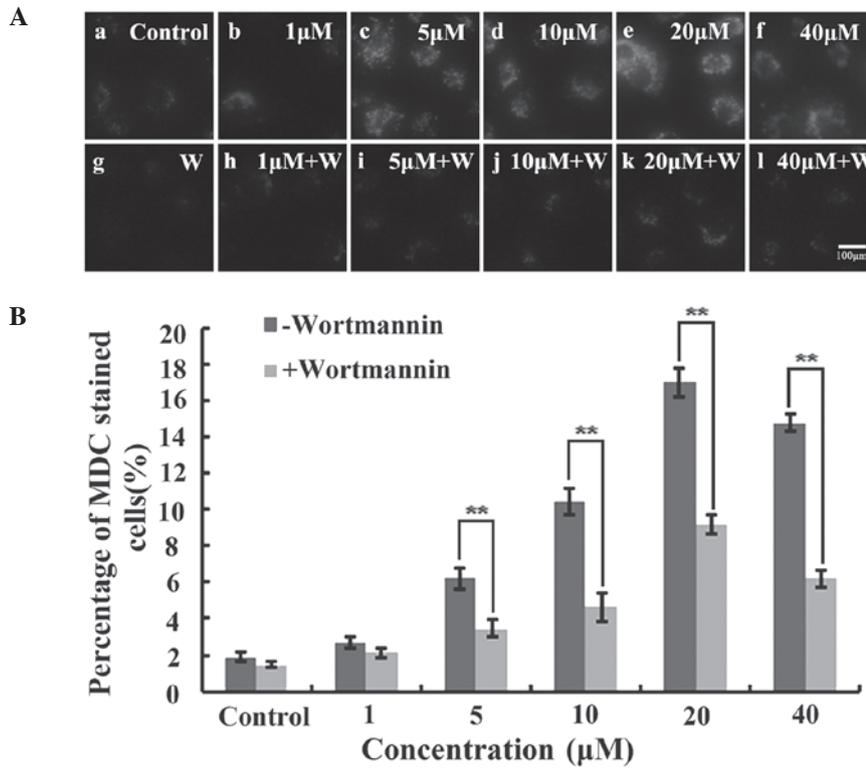


Figure 4. MDC labeling of IHCH-treated A549 cells. (Aa-f) Fluorescence microscopy of MDC-stained punctate autophagosomes; (Ag-l) wortmannin (100 nM) inhibited the formation of autophagosomes detected using MDC staining. (B) Statistical analysis of the rate of autophagy in A549 cells based on MDC staining. Data are presented as the mean \pm standard error of 10 fields of view. **P<0.01 vs. control. IHCH, 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone; MDC, monodansylcadaverine.

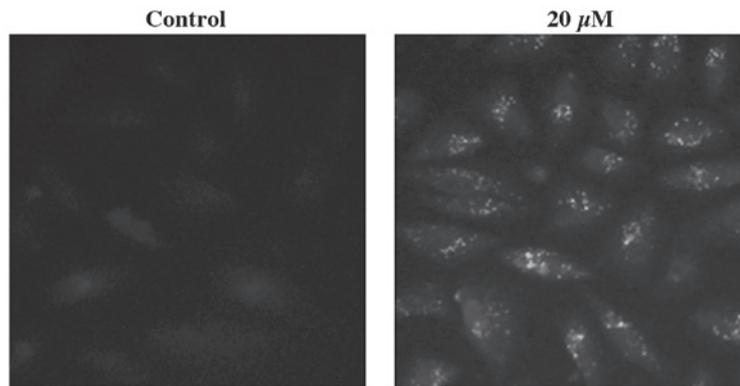


Figure 5. Immunofluorescence of LC3 in IHCH-treated A549 cells. Treatment with 20 μ M IHCH induced punctate LC3 expression. Control cells were treated with dimethylsulfoxide. Magnification, x400. IHCH, 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone; LC3, light chain 3.

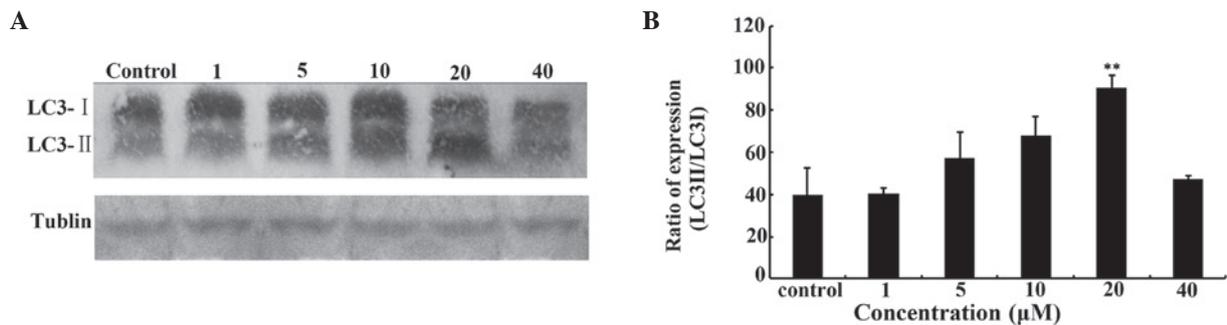


Figure 6. (A) Conversion of LC3-I to LC3-II in IHCH-treated A549 cells. A549 cells were exposed to IHCH (1-40 μ M) for 3 h. Tubulin was used as a loading control. (B) Ratio of LC3-II/LC3-I expression in IHCH-treated A549 cells (n=3). **P<0.01 vs. control. IHCH, 2E,6E-2-(1H-indol-3-yl) methylene)-6-(4-hydroxy-3-methoxy benzylidene)-cyclohexanone; LC3, light chain 3.

and *in vivo* (21). Due to the low bioavailability of curcumin, curcumin alternatives have been investigated. In our previous study, an analog of curcumin, HBC, was chemically synthesized and its inhibitory effect on A549 lung cancer cells was identified (22).

In the present study, an MTT assay revealed that 40 μ M IHCH inhibited cell proliferation after 36 h. In order to investigate the cell death mechanism associated with this IHCH-induced decrease in cell proliferation, IHCH-treated A549 cells were subjected to acridine orange and MDC staining, as well as immunofluorescent and western blot analyses. Acridine orange staining revealed a concentration-dependent increase in red fluorescent structures after 3 h of IHCH treatment. However, the red fluorescence in the A549 cells was found to decrease with increasing IHCH treatment duration. Thus, all subsequent experiments were performed using A549 cells treated with IHCH for 3 h. MDC staining revealed a concentration-dependent increase in fluorescent structures in the IHCH-treated A549 cells, which was inhibited upon pretreatment with wortmannin (100 nM). Wortmannin is a typical inhibitor of autophagy (18). These findings suggest that IHCH may lead to A549 cell death through inducing autophagy.

As a key marker of cell autophagy, LC3 is associated with autophagosome expression. In order to investigate whether IHCH induces A549 cell autophagy, immunofluorescence and western blot analyses of LC3 protein expression were performed. Immunofluorescence revealed punctate accumulation of LC3 in the cytoplasm of A549 cells in response to IHCH. Furthermore, western blot analysis showed a dose-dependent increase of LC3-II expression in IHCH-treated A549 cells. These findings show that IHCH induces A549 cell death through autophagy; however, the specific autophagy pathway involved requires further investigation. The present study has shown that IHCH may have potential as a therapeutic, anti-proliferative agent in cancer.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (grant nos. 31101931 and 81172240), the High-Level Talents Fund from Henan University of Technology (grant no. 2010BS016) and the Plan for Scientific Innovation Talent of Henan University of Technology (grant no. 11CXRC13).

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