Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

One novel curcumin derivative ZYX01 induces autophagy of human non-small lung cancer cells A549 through AMPK/ULK1/Beclin-1 signaling pathway

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Received November 10, 2018; **Accepted** February 2, 2019; **Published** February 28, 2019

Doi: http://dx.doi.org/10.14715/cmb/2019.65.2.1

Abstract: Presently, curcumin derivatives had been paid more attention in view of their high bioavailability or water solubility, which herein possibly replaced the curcumin for their functional applications in future. Here, one novel chemically synthesized curcumin derivative, ZYX01, was used to identify anti-proliferation activity of human non-small lung cancer cells A549 and its anti-proliferative mechanism. Our study showed that ZYX01 could induce autophagic death of A549 cells by morphological observation, MTT assay, acridine orange staining and MDC assay, which possess a dose-and time-dependent manner. ZYX01-treated A549 cells possessed an increase in LC3-II/LC3-I ratio, upregulation of beclin-1 and downregulation of p62 expression. We further confirmed the cellular AMPK/ULK1/Beclin-1 signaling pathway in A549 cells after ZYX01 treatment. The anti-migration effect of ZYX01 in A549 cells was also explored by wound healing assay and transwell experiment. Current results had confirmed that ZYX01 induced A549 cells autophagy through AMPK/ULK1/Beclin-1 pathway and shed light on the future study on the anti-cancer molecular mechanism.

Key words: Human non-small lung cancer cells; Autophagy; Anti-proliferation; Anti-migration.

Introduction

In recent years, human lung cancer, especially the incidence of non-small lung cancer has become one of the most prevalent and threatening malignant tumors (1-4). As the most effective treatment method for non-small lung cancer, poor prognosis and low success rate of current surgery should not be ignored. The cancer cell proliferation and metastasis are the main reasons for treatment failure (5-7). Curcumin and its analogues have been frequently reported to possess anti-proliferative effects in different cancer cells (3, 8, 9), and we also have been doing related research and screened several curcumin derivatives with the activities of anti-human non-small lung cancer cells A549 proliferation (10-12). But obviously, it's not enough to establish a huge potential anti-cancer molecule candidate library for future possible in vivo screening and identification research. In current study, we explored the anti-proliferation effect and its preliminary molecular mechanism of one novel chemically synthesized curcumin derivative ZYX01 (Figure. 1).

As a form of type-II programmed cell death, autophagy could lead to cell death through lysosome-related degradation (1, 13), which associated with tumorigenesis and cancer cell metabolism in recent literatures (14, 15). Inducing cancer cell autophagy could be as a potential strategy to evoke cell death. Here, based on the above anti-proliferative effect of ZYX01 on A549 cells, we continued to identify its specific molecular mechanism and found that it could induce A549 cells autophagy.

As one important serine-threonine kinase that plays a major role in maintaining metabolic homeostasis, AMP-activated protein kinase (AMPK) has been confirmed to be a critical role in the autophagical process (16). Activation of AMPK could promote autophagy through Unc-51-like kinase 1 (ULK1), which is the mammalian homologue of autophagy-related protein 1 (Atg1) and responsible for the initiation of autophagy (17). AMPK could directly activate ULK1 through phosphorylation and further promote the expression of the beclin-1, the mammalian homologue of Atg6 (18, 19). The protein complex of the ULK1 and Beclin-1 localizes to the site of autophagosome initiation and helps to the activation of autophagy components (20). In this study, we further examined the expression changes of the AMPK-

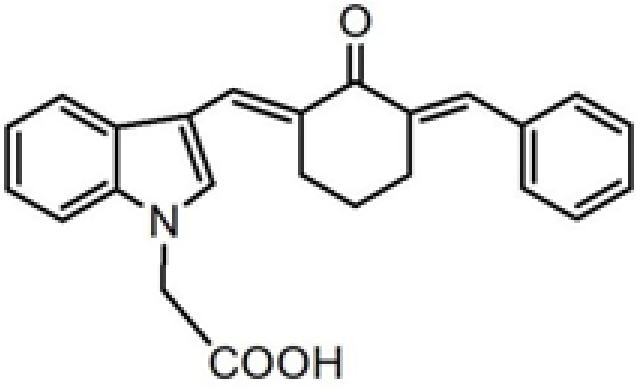

Figure 1. The chemical structure of ZYX01 with a molecular weight of 371 g/mol.

related proteins after ZYX01 treatment in A549 cells and confirmed the autophagic pathway via the AMPK-ULK1-Beclin-1 signaling pathway, which contributed to the autophagy induction of ZYX01 in A549 cells.

Materials and Methods

Chemicals

ZYX01 was a kind gift from Dr. Gangchun Sun (College of Chemistry and Chemical Engineering, Henan University of Technology, China), which is dissolved in dimethyl sulfoxide (DMSO) for stocking. During this study, DMSO (0.015% (v/v)) was used to treat cells as negative control.

The recombinant plasmid GFP-LC3 was kept in our laboratory (21). Chloroquine (CQ) and wortmannin (two autophagy inhibitors), LC3 and p62 antibodies were all purchased from Sigma Aldrich, while AMPK, p-AMPK and p-ULK1 (ser555) antibodies were obtained from Cell Signaling Technology. Anti-beclin-1 antibody, alkaline phosphatase (AP) or peroxidase conjugated secondary antibodies were purchased from Proteintech.

Cells culture

Human normal fetal lung fibroblast cells 2BS were cultured in DMEM media (Beijing Solarbio Science and technology Co., Ltd) with 10% fetal bovine serum (FBS) (Zhejiang Tianhang biotechnology Co., Ltd). Human non-small lung cancer cells A549 were cultured in RPMI-1640 fluid (Gibco, Carlsbad, CA, USA) with 10% FBS. The two cell lines were both kept in a humidified atmosphere at 37°C incubator with 5% CO_2.

MTT assay

MTT assay was used to detect the cell viability after chemical treatment. Firstly, 5×10^3 cells/well were inoculated in a 96-well plate (Corning incorporated, Costar) and cultured for 24 h, and then treated with different concentrations of ZYX01 for 12 h, 24 h and 48 h, respectively. MTT (5 mg/ml stocked in PBS solution and filter sterilized, Genview) was added into each well and the plate was incubated for another 4 h. Finally, DMSO (100 μl/well) was added to each well and the absorbances of wells at 590 nm were measured with an ELISA reader (BioTek, Winooski, VT).

Acridine Orange (AO) staining

A549 cells were seeded into a 24-well plate and incubated for 24 h. ZYX01 (15 μM) was added to each well of the plate for 6 h, 12 h and 24 h, respectively. After washing three times with PBS solution, acridine orange (5 μg/ml) was then added into each well for 40 s. PBS was applied to rinse the wells and to remove floating color. The morphology of cells in each well was observed under a fluorescence microscope (Nikon Eclipse TS200-U, Tokyo, Japan). DMSO was also added to the control group wells for above procedures.

MDC assay

MDC assay was used to detect autophagic vesicles after treatment with ZYX01 in A549 cells. The protocol of treatment was accorded to previous literature (10). Briefly, after washing the cultured cells with PBS, MDC (50 μM) was added into each well for 15 minutes. Changes of autophagic vesicles in ZYX01-treated A549 cells were detected by the fluorescence microscopy.

Confocal laser microscope detection of LC3

A549 cells were firstly seeded in 6-well plates for 24 h. After coming to 50-60% confluence, cells were transfected with GFP-LC3 recombinant DNA for 24 h using lipofectamine^{2000} according to the manufacturer's protocols (Invitrogen, CA). Then the transfected cells were treated with ZYX01 (15 μM) for 12 h. One lysosomal fluorescent probe, LysoTracker Red (1 mM, Invitrogen, Carlsbad, CA) was used to stain the cells for 30 minutes. After washing several times with PBS, the fluorescent signals of ZYX01-treated A549 cells were examined with a confocol laser scanning microscope (Olympus FV3000, Japan).

Western blotting

Western blotting analysis was carried out according to previous literature (12). Briefly, the treated A549 cells were collected and proteins were extracted for SDS-PAGE analysis to identify the loading concentrations. After SDS-PAGE, the proteins were transferred to PVDF membrane. The membranes were then incubated with different primary antibodies overnight at 4°C and secondary antibodies for 4 h at room temperature, respectively. The Enhanced chemiluminescence

Substrate (ECL) kit for detecting horseradish peroxidase or NBT/BCIP solution for alkaline phosphatase were used to show protein bands of the membranes. The densitometry protein bands were analyzed with ImageJ software.

Wound healing assay

A549 cells were seeded in a 6-well plate for 24 h and then scratched on the cell plate with a small pipette tip (10 μL). After washing, ZYX01 (15 μM, final concentration) were added to each well and cultured for another different hours (6 h, 12 h and 24 h), respectively. The treated cells were observed and under the microscope and pictures were taken by a photographer (Canon, Japan).

Transwell experiment

A549 cells were cultured with serum-free medium and starved for 12 h. After digestion, the cell suspension (1×10^4 cells/ml) was added to the upper chamber of a Cepada transwell 24-well plate with a certain concentration of ZYX01, and complete medium was added to the lower chamber to induce cell migration. After incubating for different hours, the upper chamber was stained with 1% crystal violet for 15 minutes, and then the upper chamber was cleaned and migrated cells were stained purple and observed under the microscope. 75% ethanol (600 μL) was added into the lower chamber to dissolve the crystal violet at the bottom of the upper chamber. The dissolved solution was placed in a 96-well plate, and the absorbances at 450 nm were measured with the microplate reader.

Statistical analysis

Different experiments were carried out at least three replicates and paired-samples T-test was applied to analyze the data by SPSS software (version 22.0; IBM

Corp, Armonk, NY, USA) (mean standard error (S.E.)). Significant differences were expressed as P <0.05 (*) and P <0.01 (**).

Results

Effect of ZYX01 on the proliferation of A549 cells

As a novel chemically synthesized curcumin analogue, here we firstly applied ZYX01 (15 μM) to treat normal human embryonic lung fibroblast cells 2BS and found that there's almost no apparent change in the 2BS cells monolayer as the time went on (Figure. 2), which suggested that ZYX01 possessed weak even no identical cytotoxicity to the normal 2BS cells. Then, we further identified its anti-proliferation activity of A549 lung cancer cells. After treatment with ZYX01 (50 μM) for 24 h, microscope observation showed that the A549 cells were all mostly damaged to dying. So we further reduced the ZYX01 concentrations and set several concentration gradients. As shown in Figure. 3A and 3B, ZYX01 inhibited the proliferation of A549 cells in a time- and dose-dependent manner. The damaged cells became round and detached from the monolayer. Our further MTT assay of cells viability showed that ZYX01 with higher concentration could lead to bigger cell death ratio and the activity of cells gradually declined when the treatment concentration increased (Figure. 3C). Besides that, longer incubation also reduced the cell viabilities of treated A549 cells.

Detection of acidic vesicles in ZYX01-treated A549 cells

A549 cells were treated with ZYX01 (15 μM) for different hours and stained with MDC. The changes of intracellular acidic vesicle of treated cells were detected by the fluorescence microscope and the results showed that much more obvious punctate aggregation of acidic autophagic vesicles was observed by MDC staining as the times became longer (Figure. 4A). Besides that, the acridine orange staining analysis was also carried out after ZYX01 treatment in A549 cells and the characteristic autophagic vesicles were observed compared to the control group (Figure. 4B).

The Figure. 4C and Figure. 4D showed the statistical analysis data of acidic vesicles of ZYX01-incubated A549 cells for different hours. It is suggested that the number of positive signals in treated cells both increased in a dose-dependent manner with current MDC or acridine orange staining experiment, respectively.

LC3 identification of ZYX01-treated A549 cells

As a characteristic autophagic marker protein, subcellular localization and expression level change of LC3 protein in ZYX01-treated A549 cells were studied. Confocal laser immuofluorescence microscope detection exhibited that ZYX01 treatment led to the fluorescent puncta aggregation of LC3 protein, while LC3 protein showed a dispersed distribution in GFP-LC3 recombinant vector-transfected A549 cells with no ZYX01 incubation (Figure. 5A).

lapidated LC3-II colocalize to the autophagosome of autophagic cells, and then the autophagosome could fuse with lysosomes to form the autolysosomes, which helps to fulfill the autophagic flux. Here, lysoTracker,

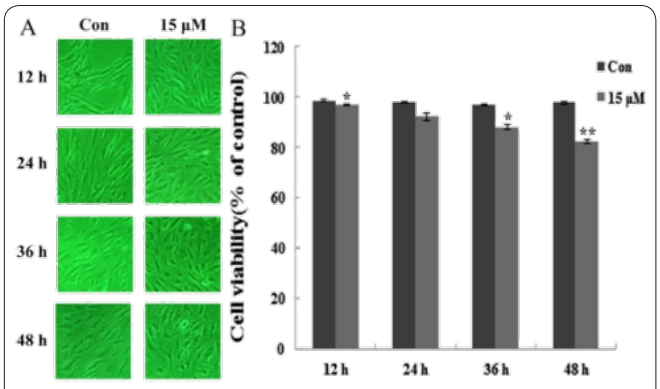

Figure 2. Effect of ZYX01 (15 μM) on normal human embryonic lung fibroblast cells 2BS. There's no apparent morphological change in the 2BS cells after ZYX01 treatment (A). MTT assay also showed no statistical difference in the experiment (B).

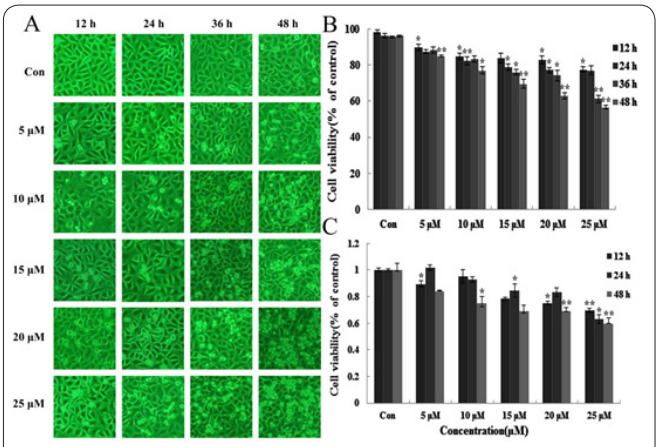

Figure 3. Anti-proliferation effect of ZYX01 in A549 cells. A. Morphological change of A549 cells after treatment with ZYX01 at various concentrations for different hours. B. Statistical analysis of the damaged cells after ZYX01 treatment for different hours. C. MTT assay of ZYX01-treated A549 cells.

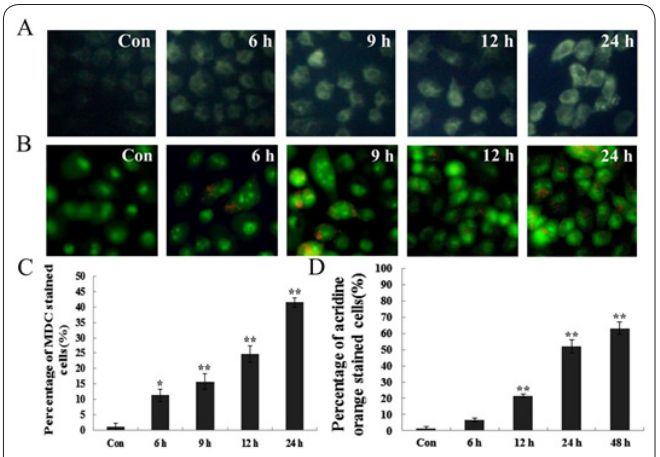

Figure 4. Detection of acidic vesicles in ZYX01-treated A549 cells. Immunofluorescence microscope detection of ZYX01-treated A549 cells after MDC (A) or AO (B) staining, respectively (×400). C and D showed the statistical analysis of autophagosomes punctuates signals by MDC (C) or AO (D).

the lysosome immunofluencent probe was used to stain the cells and the results showed that the stronger red signals could be detected in the ZYX01-treated A549 cells, which colocalized to the GFP-LC3 protein (Figure. 5A). The control groups didn't possess the characteristic staining.

We also carried out the western blotting analysis of LC3 protein of ZYX01-treated A549 cells for different

hours. The ratio of LC3-II/LC3-I exhibited a time-dependent manner before 48 h in current experiment (Figure. 5B and 5C). Oppositely, the specific autophagic inhibitor, wortmannin could inhibit the production of LC3-II after ZYX01 incubation at 24 h (Figure. 5D and 5E). These outcomes further confirmed the ZYX01-induced A549 cells autophagy.

ZYX01 induces autophagy through the AMPK/ULK1/Beclin-1 signaling pathway

In above experiments, we found that ZYX01 induced autophagy in A549 cells. As is known, Beclin-1 and p62 are two important autophagic marker proteins of cells (3, 22), whose expression exhibit Beclin-1 up-regulation and p62 down-regulation pattern, respectively. Here, we further explored the changes of Beclin-1 and p62 expression in ZYX01-treated A549 cells by western blotting analysis. The results exerted that the Beclin-1 expression increased and the expression level of p62 protein had an obvious reduction, respectively (Figure. 6A, 6B and 6C), which confirmed in depth that ZYX01 induced characteristic autophagy of A549 cells.

Besides that, as one important autophagic process pathway, AMPK-ULK1-Beclin-1 signaling pathway was also investigated in ZYX01-treated A549 cells. Western blotting analysis were performed to examine immunoreactivity against p-AMPK (Thr172) and p-ULK1 (Ser555), respectively, which exhibited significant increase of the proteins in a time-dependent manner (Figure. 6A, 6D and 6E). On the contrary, after treatment with CQ (the late stage autophagy inhibitor) in ZYX01-treated A549 cells, the cellular expression levels of p-AMPK, p-ULK1 and Beclin-1 were significantly lower than those of ZYX01-only group (Figure. 7). The above results both indicated that ZYX01 induced activation of AMPK in the A549 cells and the following AMPK-ULK1-Beclin-1 signaling pathway.

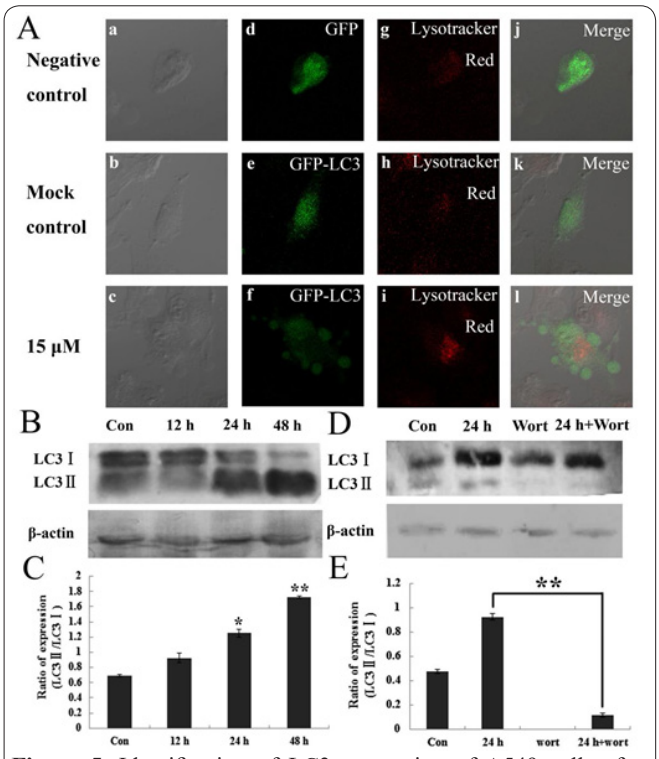

Figure 5. Identification of LC3 expression of A549 cells after ZYX01 treatment. A. Confocal laser microscopy analysis of GFP-LC3 protein in ZYX01-treated A549 cells (A, × 40, objective). The above panel (a, d, g and j) stands up the GFP blank vector-transfected A549 cells and Lysotracker was used to stain the lysosomes. The medium panel (b, e,h and k) represents the GFP-LC3 recombinant vector-transfected A549 cells. The below group (c, f, I and l) means the GFP-LC3 plasmid-transfected A549 cells for ZYX01 treatment. Western blotting was used to analyze the LC3 protein expression (B), and wortmannin (wort) could inhibit the conversion of LC3-I to LC3-II (D). Data were also statistically studied (C, E), respectively.

ZYX01 inhibited migration in A549 cells

Whether possessing the anti-migration activity or not is an important facet for potential anti-cancer candidate molecule. In view of pernicious progression of lung cancer cells *in vivo*, anti-migration activity of ZYX01 in A549 cells were investigated. As shown in the Figure. 8A, in the wound healing experiment, the

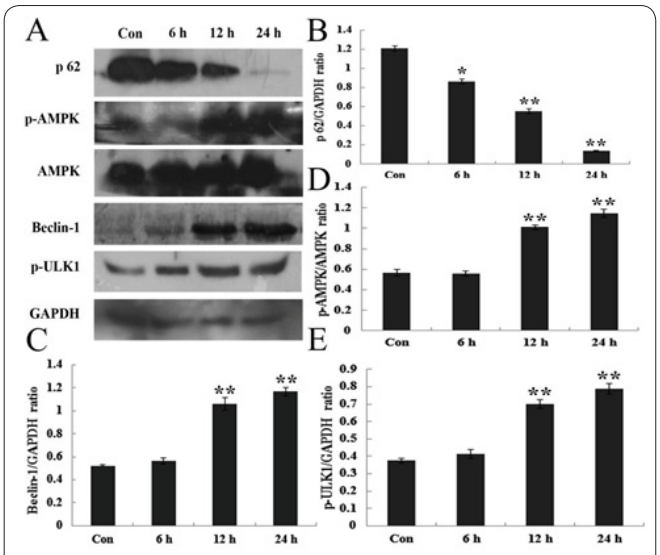

Figure 6. Confirmation the activation of AMPK-ULK1-Beclin-1 signal pathway in ZYX01-treated A549 cells. Western blotting analysis of p62, p-AMPK, AMPK, Beclin-1 and p-ULK1 expression and GAPDH was as control (A). The above immunoblotting data were also statistically analyzed by ImageJ software, respectively (B, C, D, E).

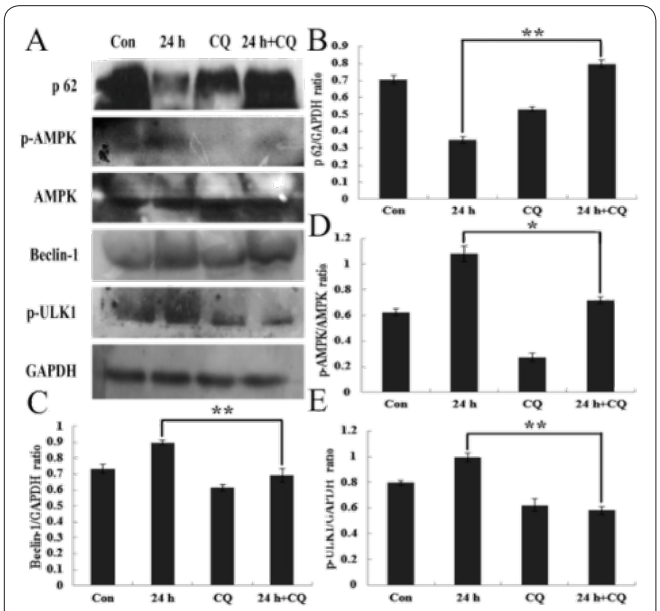

Figure 7. Western blotting analysis of ZYX01-treated A549 cells after chloroquine incubation. p62, p-AMPK, AMPK, Beclin-1 and p-ULK1 expression were analyzed (A), and the statistical data of immunoblotting were also showed, respectively (B, C, D, E).

numbers of migrated cell in the ZYX01-treated groups (24 h and 48h) were significantly lower than that' in the control group, which indicated that ZYX01 can inhibit cell migration of A549 (Figure. 8B).

In addition, the transwell experiment was further used to confirm the anti-migration effect of ZYX01 in A549 cells. There are some polycarbonate membranes with a certain size of pores in the bottom of the upper chamber. Migration capacity was determined by the number of cells that migrated to the other side of the membrane. In Figure. 8C, we could see that the migrated cells were stained with crystal violet and the number of migrated cells in the experimental group was significantly less than that in the control group at different hours. The Figure. 8D showed the absorbance values of the migrated cells dissolved in ethanol firstly in the upper chamber, which were smaller than those' of control groups.

Discussion

At present, to remove the tumor surgically is obviously the most effective method for treating cancer, although the recurrence and metastasis of the tumor is still a great obstacle to treatment (7). In this regard, it is necessary to find new chemotherapeutic drugs against cancer. Previous literatures have demonstrated that curcumin and its curcumin analogues could inhibit proliferation of different cancer cells and activate various cellular death pathways (1, 3, 8). These studies provided a potential molecule library of curcuminoid candidate anti-cancer drugs, although much more molecules need to be explored and characterized for their pharmacological effects.

Our current study showed that the novel curcumin derivative, ZYX01 could inhibit proliferation of A549 cells in a time- and dose-dependent manner by MTT assay. Also, we used the AO staining and MDC assay to detect the ZYX01-induced A549 cells autophagy, which could be further confirmed by immunoblotting analysis of LC3 and p62 proteins, the two representative markers of autophgic process.

It has been reported that starvation-induced cells can cause the phosphorylation of Unc-51-like kinase 1 (ULK1) mediated by Adenosine monophosphate-activated protein kinase (AMPK), which in turn triggers the autophagy induced by the downstream beclin-1 and Atg13 complexes (19, 23).

AMPK phosphorylates the ser317, 355, 777 locus of ULK1 to promote autophagy (24-26), which can be used as a target for the treatment of cancer to induce autophagic death of cancer cells. However, this signaling pathway is not much reported in inducing autophagy in A549 cells. Recent studies have shown that knocking out AMPKα1 can eliminate the effect of curcumin on LC3II/LC3I in A549 cells, suggesting that AMPK protein is important in regulating autophagy (9). In our current study, we detected the expression changes of AMPK, ULK1 and Beclin-1 after the ZYX01 treatment in A549 cells and found that ZYX01 could induce autophagy in A549 cells by activating AMPK. Activated AMPK further phosphorylated ULK1, which helps to initiate the auotphagy and following ULK1-Beclin-1 complex contributes to the activation of autophagy components

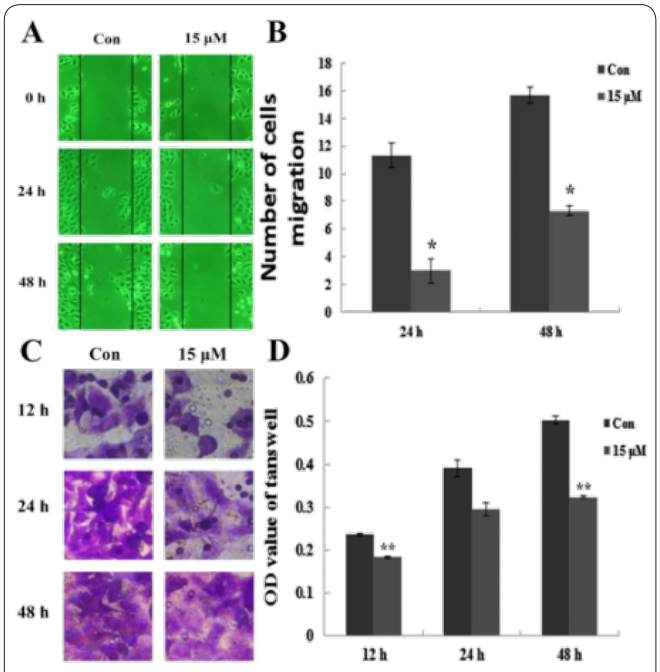

Figure 8. Anti-migration activity of ZYX01 in A549 cells. Wound healing experiment were firstly used to detect the anti-migration effect (A, B), and then transwell assay was applied to confirm the anti-migration activity of ZYX01 (C, D).

(27). With the increase of treatment time of ZYX01, AMPK and ULK1 phosphorylation, and Beclin-1 protein expression of A549 cells also got enhanced, which suggested that ZYX01 induced autophagy of A549 cells through AMPK/ULK1/Beclin-1 signaling pathway.

As a malignant lung tumor, lung cancer is characterized by uncontrolled cell growth in tissues of the lung and this growth could spread beyond the lung by the process of migration and metastasis into nearby tissues or other parts of the body (28). Here, we applied wound healing experiment and transwell assay to examine the anti-migration activity of ZYX01, which indicated that it could block the A549 cells migration remarkably, although the specific mechanism still need to be studied. But, there's no doubt that it will provide a potential candidate molecule for following in vivo screening of anti-lung cancer pharmacological drugs.

Conflict of interest

The authors declare that they have no financial/commercial conflicts of interest concerning this article.

Acknowledgements

The present study was supported, in part, by the Fundamental Research Funds for the Henan Provincial Colleges and Universities in Henan University of Technology (No.2018RCJH17), the National Natural Science Foundation of China (No.U1704112), Foundation for University Key Teacher by the Henan Province, China (No.2015GGJS-041), the Training Program for Youth Backbone Teacher of Henan University of Technology (No.21420004) and Science and Technology Planning Project of Henan Province, China (No.172102410082).

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