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Curcumin potentiates the galbanic acid-induced anti-tumor effect in non-small cell lung cancer cells through inhibiting Akt/mTOR signaling pathway

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ABSTRACT

Background: Galbanic acid (GBA), which is known as a sesquiterpene coumarin, has been reported to have various anti-tumor activities in different cells. Our study intended to investigate whether curcumin potentiates GBA-induced anti-tumor effect in non-small cell lung cancer cells.

Materials and methods: The combined effect of GBA and curcumin on cell viability was examined by MTT analysis. Cellular apoptosis was evaluated by flow cytometry analysis. Autophagy was defined by autophagosome observed by confocal microscopy after infected with GFP-LC3 adenovirus. In addition, the expression of marker proteins involved in cell apoptosis, autophagy, and Akt/mTOR signaling pathway were estimated by qRT-PCR and Western Blotting assay.

Results: 15 μM curcumin combined with 40 μM GBA could obtain better synergistic repressive efficacy on cell viability and notably induced cell apoptosis in A549 cells. Besides, curcumin in alliance with GBA could significantly inhibit cell migration and invasion. GFP-LC3 infection experiments elaborated that curcumin could potentiate GBA induced cell autophagy and restrain the phosphorylation of Akt/mTOR/P70s6k signaling pathway. What's more, the reaction of migration, apoptosis, and autophagy induced by curcumin and GBA treatment could be reversed by mTOR inhibitor rapamycin and AKT activator insulin.

1. Introduction

In recent years, lung cancer has become the most frequent malignancy along with the leading cause of cancer-related death in worldwide. On the other hand, among lung cancer cases, data shows that approximately 85% are non-small cell lung cancer (NSCLC), which is characterized by high morbidity and death rates [1]. In spite of rapid advancements in clinical diagnosis and resection therapy for NSCLC patients during the last decades, the 5-year survival rates of NSCLC are still less than 15% because of tumor recurrence, distant metastasis and resistance to chemotherapy [2,3]. Therefore, it is imminently needed to investigate more safe and effective administration therapies as well as their therapeutic molecular mechanisms for the treatment of NSCLC.

Recently, more and more herbal medicines were used alone or combined with classical anti-cancer agents to cancer prevention and even treatment because of its low toxicity and wide anti-cancer spectrum. Galbanic acid (GBA), a natural active product isolated from *Ferula assafoetida* [4], has been reported to have various anti-tumor activity in different cells including anti-angiogenic and anti-proliferative actions in human umbilical vein endothelial cells (HUVECs) [5], C26 colon carcinoma [6], cytotoxic effect against prostate cancer cells [7] and apoptotic effect against H460 NSCLC cells [8]. Nevertheless, drug cocktail therapy may be an effective method to increase the efficacy and reduce the toxicity of both drugs for the treatment of cancers [9]. Curcumin, a primary bioactive extractive from turmeric rhizome, have been extensively studied on its anti-tumor effect. More recently, numerous trials have reported the significant anti-tumor effects of curcumin alone or combined with other chemotherapeutic drugs on cancer therapy. For example, curcumin enhanced the anti-tumor effects of GBA in treatment of esophageal squamous carcinoma [10] and gastric cancer [11] *in vitro* and *in vivo*; curcumin potentiated the sensitivity of cisplatin against human NSCLC cell lines [12]. Because

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of its high safety and tolerability, curcumin acts as an ideal agent to synergize the function of GBA during the treatment of NSCLC.

Curcumin has been reported to modulate various cellular signal pathways, such as protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) molecular signaling pathway [13], which is a vital process involved in various physiological and pathological processes. However, whether curcumin potentiates galbanic acid-induced antitumor effect via Akt/mTOR was not clear yet. The human adenocarcinoma alveolar basal epithelial cells (A549 cells) [14,15]were enriched in proteins related to cellular respiration, ubiquitination, apoptosis, and response to drug/hypoxia/oxidative stress. The functional profile of human A549 cells was widely studied in NSCLC research [16]. Therefore, our current study aims to evaluate the response of the A549 human lung cancer cells taking into account an *in vitro* model of lung cancer together to the antineoplastic mechanisms of curcumin alone or combined with galbanic acid.

2. Materials and methods

2.1. Reagents and antibodies

Curcumin and galbanic acid (GBA) were purchased from Sigma Company (Sigma Chemical Co., St. Louis, MO, USA), MTT, Hoechst 33258 staining, and Annexin V-FITC Apoptosis Detection Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The rapamycin and insulin were acquired from Selleck Chemicals (HOU Houston, Texas). Antibodies against BAX, Bcl-2, cleaved-caspase 3, cleaved-caspase 8, cleaved-caspase 9, GAPDH, p-AKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, p-p70S6K (Thr421/Ser424), and HRP-linked anti-rabbit IgG were all purchased from Cell Signaling Technology (Danvers, MA, USA). LC3-II and Beclin-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell lines and cell culture

The A549 cells were obtained from Shanghai Institutes of Biological Sciences Cell Bank and cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA) containing with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA) and 1% penicillin/streptomycin at 37 °C in a humidified incubator of 5% CO2.

2.3. Cell viability analysis

The cell viability analysis of human NSCLC cells A549 was performed by MTT assay. 4×10^3 cells/well were seeded in 96-well plates overnight and treated with curcumin alone or combined with GBA at different concentrations for 24 h, 48 h, and 72 h. Then 10 μl 5 mg/ml MTT was added into each well and incubated for an additional 4 h at 37 °C. Following 100 μl DMSO dissolved formazan product, the cell viability was measured at 490 nm using a microplate (Molecular Devices, CA, USA).

2.4. Cell migration and invasion

For the migration and invasion assay, cells were conducted in 8 μm micro-pores transwell chambers (6.5-mm diameter, Corning Costar, Manassas, United States) placed above in 24-well plates without or with Matrigel (1 mg/ml, BD Biosciences) on the upper membrane. Briefly, drug-treated cells were harvested, and then suspended in 200 μl serum-free medium. 1×10^5 cells were seeded on the upper membrane, which was then placed into the lower chamber with 600 μl culture medium containing 10% FBS. After incubating for 24 h at 37 °C, the chambers were fixed with 4% paraformaldehyde for 15 min and then stained with 0.1% crystal violet dye (Sigma-Aldrich, Shanghai, China) for 30 min. After rinsing with PBS twice, the migratory and invasive cells were counted and imaged under an inverted microscope (Olympus, Tokyo, Japan).

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from cell lines was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were measured by NanoDrop ND-1000 (NanoDrop Technologies, DE). 100 ng RNA was reverse transcribed into cDNA using PrimeScript RT reagent kit (Takara Bio, Inc., Japan) according to the manufacturer protocol. The qPCR was performed using SYBR Green PCR Master Mix (Takara Bio, Inc., Japan) on Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, CA, USA). GAPDH acted as an internal control, and the expression of target gene was calculated using the $2^{-\Delta\Delta}$Ct method. The primer sequences (5′–3′) used in this experiment are as follows:

GAPDH forward primer: GAAGGTCGGAGTCAACGGATTT, reverse primer: ATGGGTGGAATCATATTGGAAC;

uPA forward primer: CACACTGCTTCATTGATTACCCA; reverse primer: AAGGCAATGTCGTTGTGGTG;

MMP2 forward primer: TTGACGGTAAGGACGGACTC; reverse primer TCTCAAAGTTGTAGGTGGTGGA.

2.6. Autophagosome detection

A549 cells infected with GFP-LC3 fluorescence autophagy indicator system (Hanbio, Shanghai, China) were used to label and monitor the changes of LC3 and autophagy flow for 24 h. Then the cells were treated with 40 μM GBA, curcumin, or both for another 24 h. Next, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with Hoechst 33258. The cells with GFP dots were quantified under a confocal microscope.

2.7. Cell apoptosis analysis

1×10^5 cells/well were seeded in six-well plates overnight and treated with 25 μM curcumin, 50 μM GBA, or both for 48 h. The cells were harvested and washed with phosphate buffered saline (PBS) twice. Then cells were resuspended in 100-μl binding buffer with 5 μl Annexin V-FITC and 10 μl propidium iodide (PI) (20 μg/ml) and incubated for 15 min in dark conditions following the instructions. The number of apoptotic cells was examined by flow cytometry (BD Biosciences, San Diego, CA, USA).

2.8. Western blot analysis

Cells were harvested and lysed with RIPA buffer containing freshly dissolved protease inhibitor cocktail. The proteins were quantified by using the BCA protein assay kit (Beyotime, Shanghai, China). 30 μg protein samples were loaded and separated by 8–12% SDS polyacrylamide gels and then transferred to the polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Waltham, MA, United States). Following blocked with 5% nonfat milk in TBST for another 1 h, the membrane was incubated with primary antibodies overnight at 4 °C. After washing with TBST twice, the PVDF membrane was incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Finally, the proteins were visualized by enhanced chemiluminescence kit (Millipore, Bedford, MA) and detected by AmershamTM Imager 600 System (GE Healthcare Bio-Sciences, Pittsburgh, PA, United States).

2.9. Statistical analyses

All expressed data was calculated by SPSS 21.0 software (Chicago, IL, USA), and expressed as means ± standard deviation (SD). Each group was performed at least three independent experiments. $P < 0.05$ was considered a statistically significant difference.

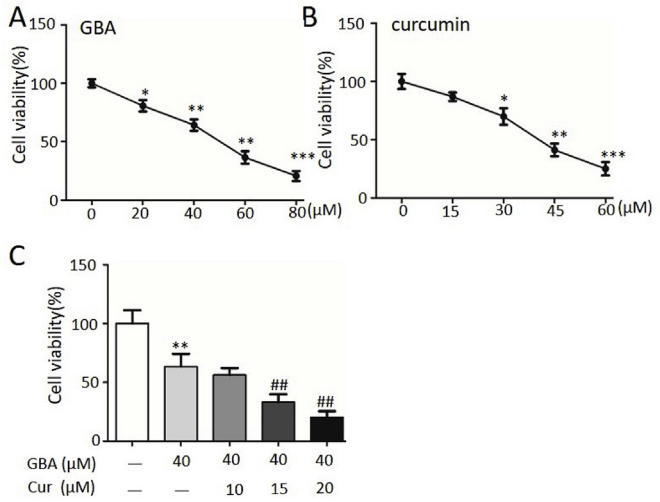

Fig. 1. Curcumin alone or combined with galbanic acid could inhibit the proliferation of A549 cells. (A) A549 cells were treated with GBA (0, 20, 40, 60, 80 μM) for 48 h and the cell viability was examined by MTT assay. (B) A549 cells were treated with curcumin (0, 15, 30, 45, 60 μM) for 48 h and the cell viability was examined by MTT assay (C) A549 cells were treated with GBA (40 μM) curcumin (10, 15, 20 μM) for 48 h and the cell viability was examined by MTT assay. *P < 0.05, **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group. The data are represented as mean ± SD, and obtained from three independent experiments.

3. Results

3.1. Curcumin alone or combined with galbanic acid could inhibit the proliferation of A549 cells

We first performed MTT assays to evaluate the cytotoxic effect of curcumin or galbanic acid with various concentrations on the NSCLC cell A549 at 48 h, respectively (Fig. 1A and B). As the data showed that curcumin or GBA treatment alone remarkably inhibited A549 cells growth in a time- and concentration-dependent manner and 40 μM GBA exhibited about 60% growth inhibition. Therefore, 40 μM GBA was utilized in combination with different concentrations of curcumin (10, 15, 20 μM) to prove a more potent inhibitory effect than curcumin or GBA alone (Fig. 1 C). According to the results, 15 μM curcumin and 40 μM GBA could obtain better synergistic repressive efficacy in A549 cells. This particular combined treatment was used for further studies.

3.2. Curcumin enhanced galbanic acid-induced apoptosis in A549 cells

Morphological alteration is an important characteristic of cell growth suppression or apoptosis. We firstly observed the visible morphological changes of A549 cells after treated with 15 μM curcumin with or without GBA presence for 48 h via phase contrast microscopy (Fig. 2A). The pictures showed that untreated cells with fat polygonal shape and strong refraction were significantly decreased when treated with GBA and further declined by the combination with curcumin treatment compared with the GBA group. (*P < 0.05, ##P < 0.01). This phenomenon suggested that combination treatment might cause premature senescence of A549 cells.

Then we calculated whether curcumin enhances GBA -induced apoptosis in A549 cells. 15 μM curcumin alone or combined with 40 μM GBA were added to A549 cells for 48 h (Fig. 2B). Flow cytometry assay with double staining with Annexin-V FITC/PI was performed to estimate the percentage of apoptotic cells (Fig. 2C). As shown in Fig. 2D, the number of late apoptotic cells triggered by 40 μM GBA was significantly increased, while 15 μM curcumin had a very limited effect on cell apoptosis compared with the control group. However, combined utilization of curcumin and GBA was notably augmented the cell cytotoxicity compared with GBA alone and exhibited a synergetic pro-apoptotic effect on A549 cells (P < 0.05).

To further exploding those findings, downstream apoptotic proteins such as pro-apoptotic proteins Bax and cleaved-caspase-3, -9, as well as anti-apoptotic member Bcl-2, were examined by immunoblotting analysis to further elucidate the underlying mechanism of synergetic pro-apoptotic effect on A549 cells (Fig. 2E and F). The data indicated that the combined use of curcumin and GBA enhanced the expression of cleaved-caspase-3, -9 and Bax and diminished the activation of Bcl-2, compared with GBA alone or control group. Thus, these consistent findings illustrated that curcumin could enhance galbanic acid-induced apoptosis in A549 cells.

3.3. Curcumin combined with galbanic acid could inhibit cell migration and invasion in A549 cells

Epithelial-mesenchymal transition (EMT), an essential process associated with tumor metastasis, is related to a variety of cellular and molecular biomarkers, including the loss of epithelial markers E-cadherin and high expression of mesenchymal markers, such as vimentin, β-catenin, and snail [13]. To further investigate the influence of curcumin and GBA over cell migration and invasion, transwell analysis was initially carried out to evaluate the migration ability of the cells. Transwell assay results showed that 15 μM curcumin did not affect cell migration, but 30 μM GBA could notably reduce the number of migrated cells (**p < 0.01). While using curcumin combined with GBA was further significantly decreased the migrated cells (##P < 0.01) compared with GBA treatment (Fig. 3A and B). Moreover, the western blot results also demonstrated that the combination of curcumin and GBA at proper concentration could effectively enhance the expression of E-cadherin and reduce the expression levels of vimentin and snail in A549 cells (Fig. 3C and D).

Subsequently, we investigated the inhibitory effect of curcumin in the presence or absence of GBA on A549 cells via matrix-gel invasion assay, which is a three-dimensional model that can be used to evaluate the invasive potency of cells. As shown in Fig. 3E and F, a synergetic inhibition effect of curcumin and GBA on cell invasion was remarkably enhanced when compared with GBA treatment (##P < 0.01). Similar results were obtained with the outstandingly downregulated expression of uPA and MMP2, which belong to the matrix metalloproteinase family of gelatinases and can promote cell invasion by stimulating ECM degradation (Fig. 3G and H). Taken together, these results suggested that the combination of curcumin and GBA inhibited cell migration, invasion, and EMT of A549 cells.

3.4. Curcumin potentiated galbanic acid induced-cell autophagy in A549 cells

Autophagy acted as type II cell death pathway that exists in many cancers. Several studies have reported that curcumin performed anticancer effect was closely associated with autophagy. To explore whether curcumin alone or combined with GBA induced cell autophagy in A549 cells, GFP-LC3 (Microtubule associated protein 1 light chain 3) adenovirus infection was performed to characterize the increased formation of autophagosomes (Fig. 4A). The statistical results in Fig. 4B indicated that curcumin co-treated with GBA could significantly increase the puncta formation of autophagy marked with green fluorescence intensity of LC3 than GBA treatment alone in A549 cells (Fig. 4B, ##P < 0.01). Then the level of the autophagy marker proteins, including LC3-II and Beclin-1, were further explored to verify the above findings using western blotting after drug treatment for 48 h (Fig. 4C–E). Exposed to 15 μM curcumin together with GBA dramatically increased the expression levels of LC3-II and Beclin-1 compared to the GBA group (##P < 0.01). Taken together, these results indicated

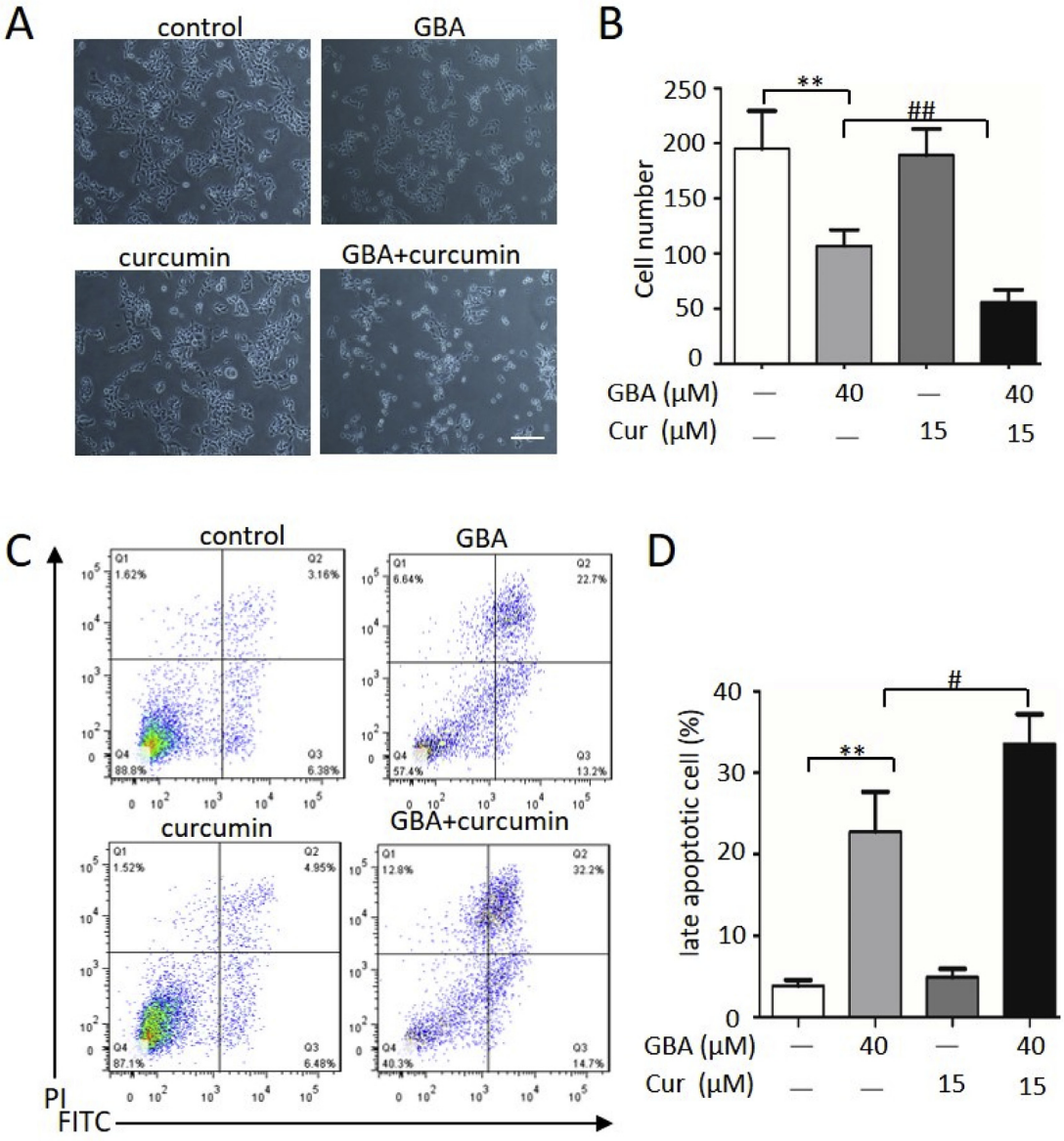

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Fig. 2. Curcumin enhanced galbanic acid-induced apoptosis in A549 cells. (A) A549 cells were treated with 40 μM GBA and/or 15 μM curcumin for 48 h. The morphological changes were observed under a phase contrast microscope. Bar representing all images equals 200 μM. (B) The number of intestinal cells, not cell debris, were counted. **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group. (C) A549 cells were treated with 40 μM GBA and/or 15 μM curcumin for 48 h, and apoptotic cells were measured using flow cytometry. (D) Apoptotic cell values are expressed as mean ± SD of three experiments. **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group. (E) The expressions of c-cas3 (cleaved-caspase 3), c-cas9 (cleaved-caspase 9), Bax, Bcl-2 were analyzed by Western Blotting in A549 cells. (F) The relative expression of c-cas3, c-cas9, Bax, Bcl-2 was quantified by normalizing to GAPDH. All data are represented as mean ± SD, and obtained from three independent experiments. **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group.

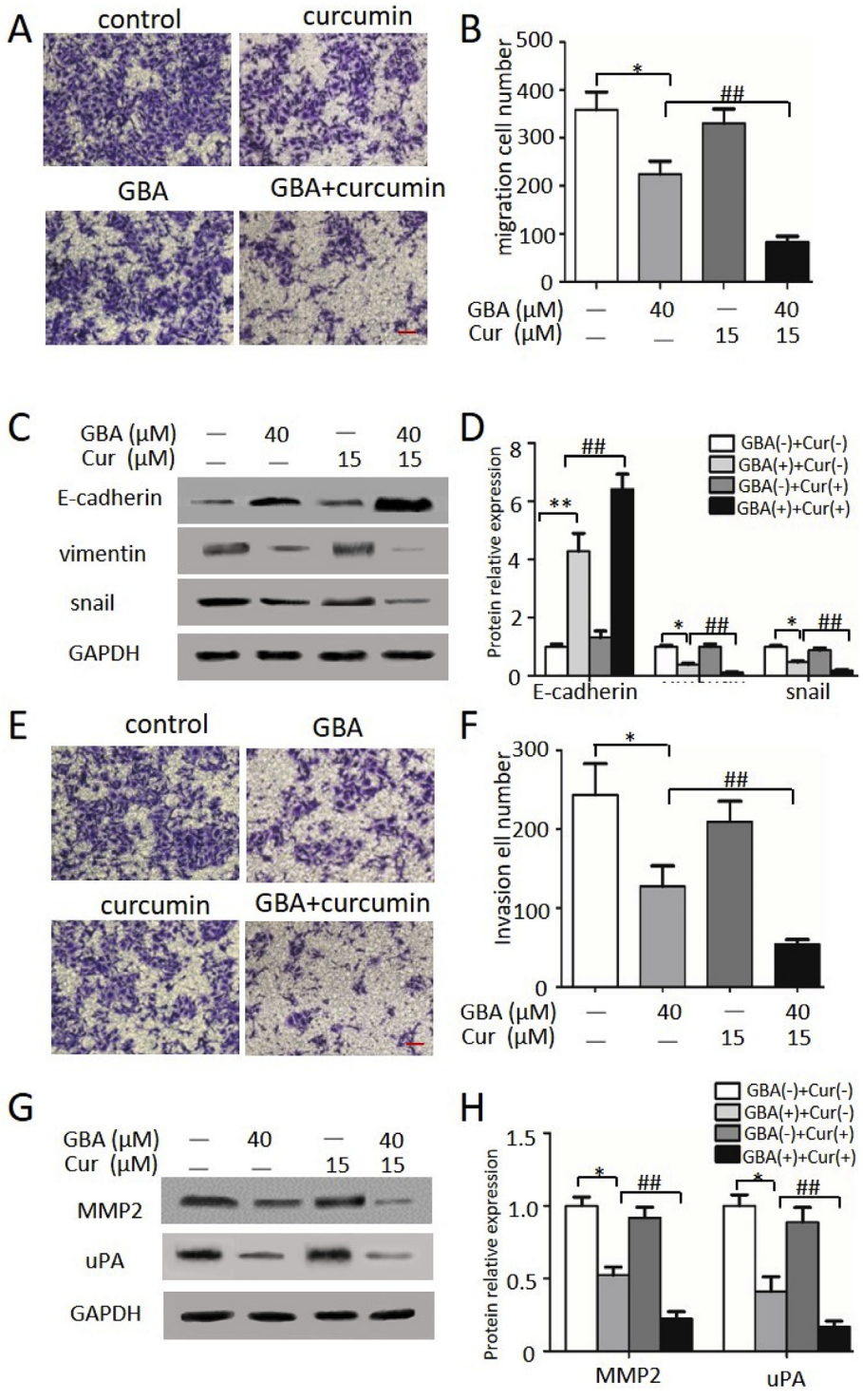

Fig. 3. Curcumin combined with galbanic acid could inhibit cell migration and invasion in A549 cells. (A) Transwell assay was performed to observe the migration capacity of A549 cells after GBA and/or curcumin treated. The migrated cells stained with crystal violet after post-culture for 24 h. The bars represent 200 μm. (B) The number of migrated cells in five different fields were counted from three independent experiments. **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group. (C) The expression of E-cadherin, vimentin, and snail were checked by Western blot analysis in A549 cells. (D) The relative expression of E-cadherin, vimentin, and snail was quantified by normalizing to GAPDH. **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group. (E) Transwell invasion assay was performed after GBA and/or curcumin treated in A549 cells. (F)The number of invasion cells in five different fields was counted from three independent experiments. The bars represent 200 μm. (G) The expression of MMP2 and uPA were examined by western blot analysis. (H) The relative expression of MMP2 and uPA was quantified by normalizing to GAPDH. **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group. All the data are represented as mean ± SD, and obtained from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

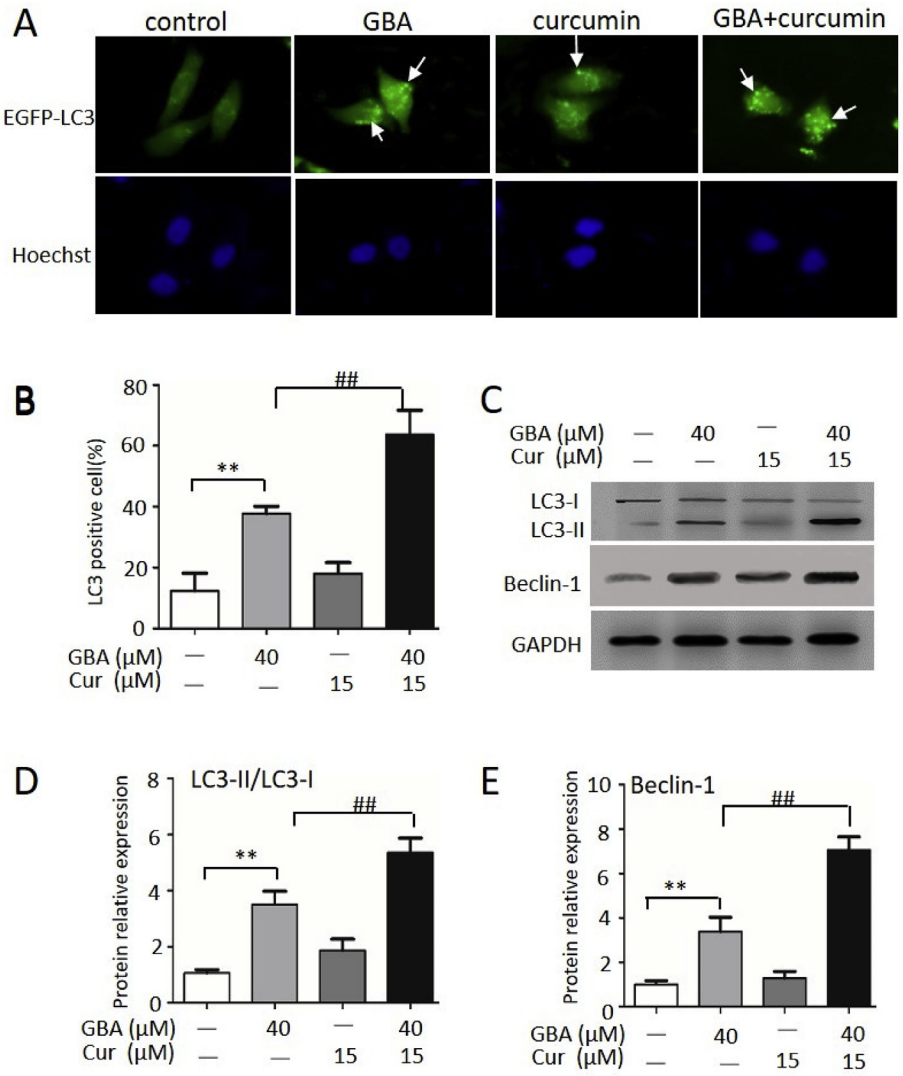

Fig. 4. Curcumin potentiated galbanic acid induced-cell autophagy in A549 cells. (A) Representative photomicrographs showed the formation of autophagosomes performed by GFP-LC3 adenovirus infection following by GBA and/or curcumin treatment under confocal microscopy (Scale bar 40 μm, 400 × magnification). A549 cells containing green and bright puncta as white arrows pointed were regarded as the LC3 positive cells. (B) LC3 positive cells were counted. **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group. (C) The expression levels of the autophagy-related proteins, including LC3B-II and Beclin-1, were investigated by western blots. The quantitative analysis results of LC3B-II/LC3B–I and Beclin-1 were shown in (D) and (E) when normalized to GAPDH. *P < 0.05, **P < 0.01 vs untreated control group; ##P < 0.01 vs 40 μM GBA alone treatment group. All data were obtained from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that curcumin potentiates GBA induced cell autophagy in A549 cells.

3.5. Combination of curcumin and GBA blocked the Akt/mTOR pathway in A549 cells

To further investigate the underlying mechanisms, the AKT/mTOR signaling pathway, which played a pivotal role in regulating cell growth, cell motility, and homeostasis, was being researched. We firstly pretreated A549 cells with 40 μM rapamycin (Ra, a mTOR inhibitor) or 100 nM insulin (an AKT activator) for 2 h. Subsequently the cells were exposed to 15 μM curcumin and 40 μM GBA, separately or in combination, for 24 h. MTT results in Fig. 5A indicated that 40 μM rapamycin significantly inhibited the proliferation of A549 cells (**P < 0.01) and further decreased when combined with GBA and curcumin ($^{\#\#}$P < 0.01). However, when pretreated with 100 nM insulin after exposure with GBA synthesized with curcumin, the cell viability was notably upregulated compared with GBA and curcumin group (*P < 0.01, ##P < 0.01). Thus, based on these results, the Akt/mTOR pathway might be involved in the inhibition effect of GBA and curcumin on A549 cells.

Furthermore, we examined the underlying proteins including phosphorylation expression of AKT (Ser473), mTOR(Ser2448), and its downstream target P70s6k(Ser371) by western blotting analysis in Fig. 5C. Our statistical results in Fig. 5 C–F revealed that the phosphorylation of AKT, mTOR, and P70s6k were significantly downregulated after curcumin or GBA treatment (**P < 0.01), especially when the treatments were combined ($^{\#\#}$P < 0.01) while the total expression of AKT, mTOR, and P70s6k were not altered (**P > 0.05). What's more, the inhibition effect induced by curcumin or/and GBA treatment could be aggravated when pretreatment with 40 μM rapamycin ($^{\Delta}$P < 0.05), while it also was reversed or at least alleviated by insulin pretreatment ($^{\&}$P < 0.05). These data might support the idea that the anti-cancer effect of curcumin with GBA on A549 cells may be related to the downregulation of Akt/mTOR pathways.

3.6. The rapamycin regulated curcumin and GBA -induced anti-tumor effect via the Akt/mTOR pathway

The above results revealed that the synergetic effect of curcumin and GBA was superior to monotherapy on cell apoptosis, migration, and autophagy, what is more, the Akt/mTOR pathway was involved in the process. In order to further ascertain and identify curcumin and GBA induced function of cell cytotoxicity, migration, and autophagy occurred via the Akt/mTOR signaling pathway. We adopted the mTOR inhibitor rapamycin (40 μM) for 2 h pretreatment followed by the curcumin and GBA combined treatment. The mRNA expression of the cell invasion related gene MMP2 and uPA in Fig. 6A–B indicated that downregulation of the Akt/mTOR pathway by rapamycin could remarkably impact on cell migration and invasion, when compared with

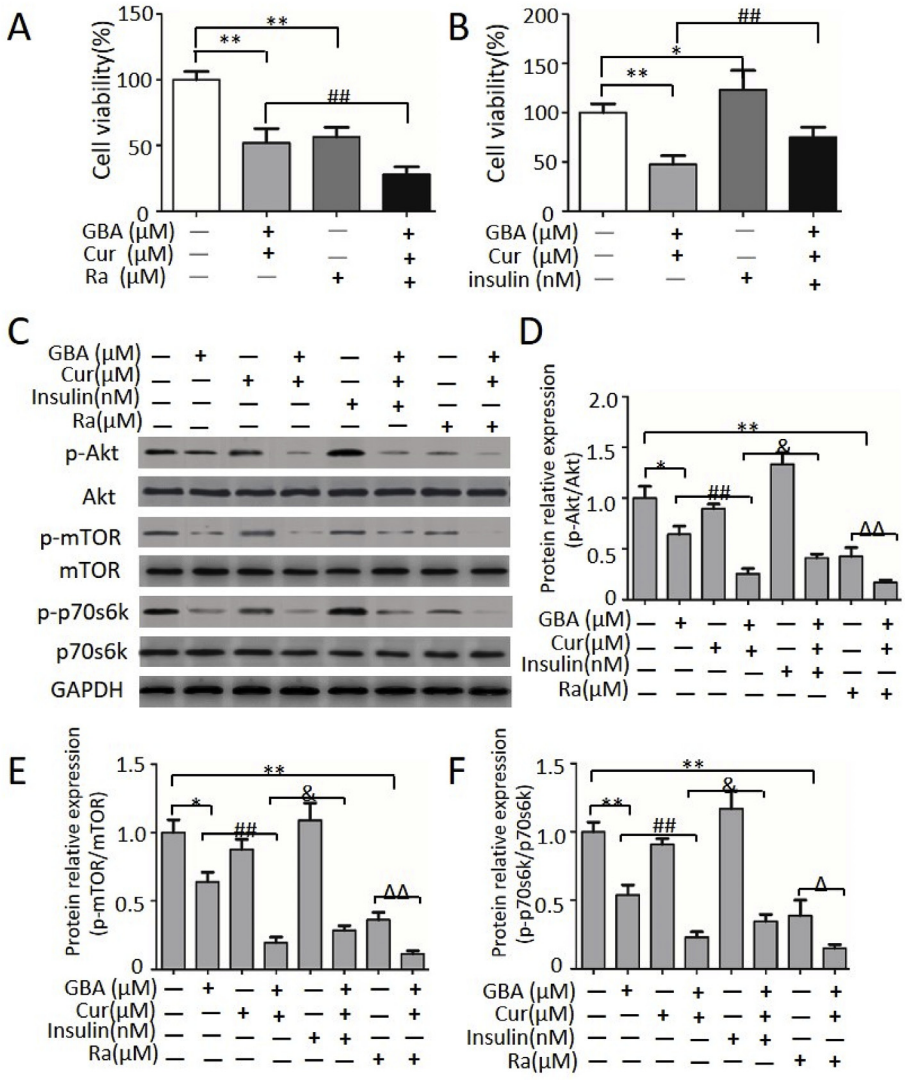

Fig. 5. Combination of curcumin and GBA blocked the Akt/mTOR pathway in A549 cells. A549 cells were preincubated with rapamycin (40 μM) (A) or insulin (100 nM) (B) for 2 h, subsequently exposure to 15 μM curcumin and/or 40 μM GBA, separately, for 24 h. The MTT assay was performed to examine the cell viability. $^{**}P < 0.01$ vs untreated control group; $^{\#\#}P < 0.01$ vs GBA and curcumin synergistically treatment group (C) phosphorylation expression of AKT (Ser473), mTOR (Ser2448) and its downstream target P70s6k(Ser371) by western blotting analysis. (D) The relative expression of p-Akt was quantified by normalizing to Akt. (E) The relative expression of p-mTOR was quantified by normalizing to mTOR. (F) The relative expression of P70s6k was quantified by normalizing to P70s6k. $^{*}P < 0.05$, $^{**}P < 0.01$ vs untreated control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs GBA alone treatment; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs GBA and curcumin synergistically treatment; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs rapamycin alone treatment; All the data are represented as mean ± SD, and obtained from three independent experiments.

the GBA and curcumin treatment ($^{**}P < 0.01$). Furthermore, the western blot analysis in Fig. 6C showed a significant increase in the level of cleaved caspase 3 in cells exposed to rapamycin plus curcumin and GBA compared with curcumin and GBA treatment($^{**}P < 0.01$). Similarly, the ratio of LC3-II/LC3-I was augmented when cells were pretreated with rapamycin, followed by treatment with curcumin and GBA (Fig. 6C). Thus, these results illustrated that blocking the Akt/mTOR pathway significantly restrained cell viability and motility as well as enhanced cell apoptosis and autophagy. Above all, our findings unequivocally substantiated the fact that blocking the Akt/mTOR signaling pathway was involved in the regulation of curcumin and galbanic acid-induced anti-tumor effect in A549 cells.

4. Discussion

It is well documented that galbanic acid (GBA), a sesquiterpene coumarin, exerts various biological effects, such as anti-inflammatory effect [17], anti-bacterial activity [18] and anti-thrombotic effect [19] as well as the anti-tumor activity in colon carcinoma [6], prostate [7], and lung [8]. In this work, we aimed to investigate a prospective combination therapy increase not only the curative effect but also reduces cytotoxicity. Curcumin, an active component of turmeric, has also displayed potential therapeutic or preventive activities for several human cancers [20–22]. Besides its anti-cancer properties, curcumin has also been considered to be a safe compound due to its low toxicity and good tolerance to patients [23,24], suggesting that curcumin has the promising potential to be a novel anti-cancer agent. Therefore, this investigation was intended to demonstrate that the potential of curcumin as a sensitizer to GBA induced synergistic anti-tumor properties that exerted advantages over a single chemotherapeutic drug.

In the current study, we firstly demonstrated that low-dose curcumin combined with GBA showed significant cytotoxicity in a dose-dependent manner in A549 cells compared to curcumin or GBA alone (Fig. 1). To evaluate the mechanism of cytotoxicity, cell apoptosis, type I form of programmed cell death when treatment was applied. Further experiments on A549 cells were carried out by flow cytometry and cellular morphology observing (Fig. 2). Expectedly, the results showed that curcumin could enhance GBA-induced pro-apoptosis in A549 cells via increasing the number of apoptotic cells and upregulating the level of Bax and pro-apoptotic proteins cleaved-caspase-3 and 9, as well as reducing the expression of anti-apoptotic protein Bcl-2. Besides, cell migration and invasion assay showed that 15 μM curcumin did not affect cell motility in A549 cells while substantially enhanced the inhibitory role of GBA-induced cell migration and invasion and decreased the level of associated protein expression (Fig. 3). According to the anti-tumor activity arrays of curcumin-GBA combined treatment, we concluded that curcumin possesses a synergistic anti-tumor value against NSCLC A549 cells when applied with GBA.

The autophagy, which is a conserved protective process of metabolic decomposition in cells, can induce autophagic cell apoptosis [25].

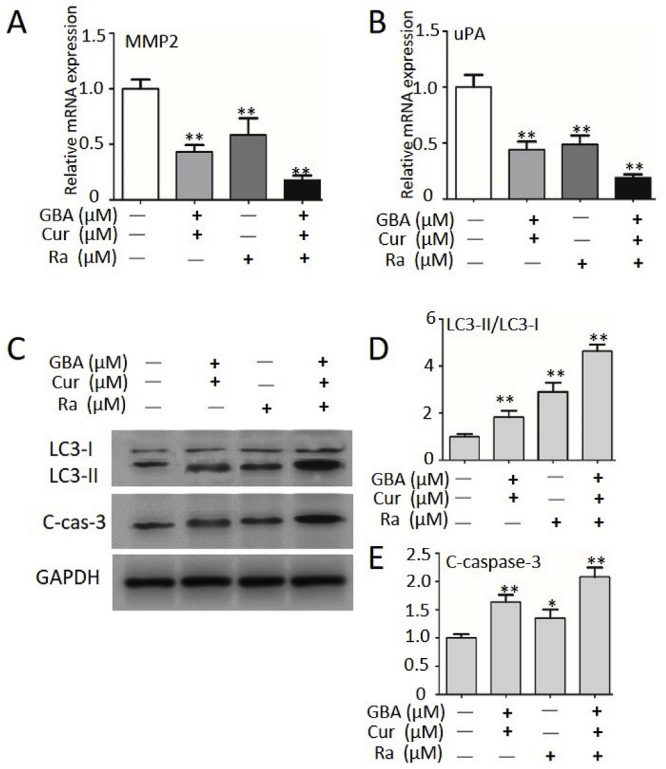

Fig. 6. The rapamycin regulated curcumin and GBA-induced anti-tumor effect via the Akt/mTOR pathway. (A) The mRNA expression level of MMP2 and uPA was examined by qRT-PCR. (B) Quantitative analysis in histograms was presented as a mean ± SD of three experiments. *P < 0.01, **P < 0.01 vs untreated control group. (C)The expressions of LC3-II/LC3-I and c-cas3 (cleaved-caspase 3) were analyzed by western blotting in A549 cells. (D) The relative expression of LC3-II/LC3-I was quantified by normalizing to GAPDH. (E) The relative expression of c-cas3 (cleaved-caspase 3) was quantified by normalizing to GAPDH. **P < 0.01 vs untreated control group. All data are represented as mean ± SD, and obtained from three independent experiments.

The cytotoxicity and chemoresistance of chemotherapeutic agents may be associated with cell autophagy. Our data demonstrated that GFP-LC3 fluorescent particles aggregated autophagosomes were remarkably aggrandized in the curcumin + GBA group compared to the curcumin or GBA single treatment group (Fig. 4A). Then Western Blotting results revealed that the critical autophagy formation proteins LC3-II and Beclin-1 were significantly overexpressed when exposed to the synergetic effect of curcumin and GBA in A549 cells. Based on these results above, we concluded that the combination of curcumin and GBA could notably upregulate the level of autophagy contrast to single dugs treatment in NSCLC A549 cells. However, whether the autophagy induced by curcumin combined with GBA exerted a protective or pro-apoptosis effect on NSCLC cells needs to be further investigated.

Then we explored the underlying mechanisms of the synergistic antitumor effects of curcumin in the presence or absence of GBA in A549 cells. Previous studies have shown that curcumin could inhibit phosphorylation of mTOR as well as its downstream effectors p70S6K. The Akt/mTOR/p70S6K signaling pathway keeps the balance of intracellular homeostasis and plays a vital role in modulating cell growth, migration, and apoptosis in various cancer cells [26,27]. In addition, mTOR (mechanistic target of specific inhibitor rapamycin) [28] functions as the central regulator for cell proliferation, growth, and survival. We firstly investigated the effect of mTOR inhibitor rapamycin and AKT activator insulin on cell viability when treated with curcumin and GBA (Fig. 5A and B). Our work elaborated that rapamycin or insulin could further aggravate or alleviate curcumin and GBA-induced the growth inhibitory in A549 cells, suggesting that there may be a connection between Akt/mTOR/p70S6K and curcumin and GBA in the cytotoxicity of A549 cells. Then the functional expression of Akt/mTOR/p70S6K regulated by curcumin and GBA pretreated with rapamycin or insulin was examined by western blotting (Fig. 5C and D). Expectedly, the rapamycin dramatically downregulated the expression levels of phosphorylated Akt/mTOR/p70S6K, compared with curcumin and GBA treatment group. What's more, the insulin significantly reversed the inhibitory of phosphorylated Akt/mTOR/p70S6K induced by the exposure with curcumin and GBA. Furthermore, we found that rapamycin has a superimposed effect with curcumin and GBA in repressing the mRNA level of cell migration and invasion by qPCR (Fig. 6A and B) and raising the expression of cleaved-caspase 3 and LC3-II by western blotting (Fig. 6C and D). Based on these results, we propose that AKT/mTOR signaling pathways are involved in curcumin and GBA -induced anti-tumor effect in A549 cells.

In conclusion, our work has elucidated that curcumin potentiates galbanic acid-induced anti-tumor effect through inhibition of the Akt/mTOR signaling pathway in non-small cell lung cancer cells. To the best of our knowledge, our current findings present the advantages of combination therapy over a single chemotherapeutic agent, and it would be a novel therapeutic strategy in the eradication of non-small cell lung cancer cells.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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