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Original article

# Nobiletin inhibits non-small cell lung cancer through TRKC and exhibits a synergistic effect with the HDAC inhibitor

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ABSTRACT

Approximately 85% of all lung cancer cases are classified as non-small cell lung cancer (NSCLC). Given its poor prognosis and resistance to radiotherapy and chemotherapy, there is an urgent need to elucidate its molecular mechanisms to develop novel and more effective therapeutic strategies. In prior research, we identified nobiletin from a compound library and confirmed it as a novel natural BH3 mimetic. Nobiletin synergized with vorinostat to induce autophagy and apoptosis in small-cell lung cancer. In the current study, we further demonstrate that nobiletin, either alone or in combination with vorinostat, exerts inhibitory effects on NSCLC. Specifically, the combination of nobiletin and vorinostat suppressed the proliferation of NSCLC A549 cells. Nobiletin, used alone or with vorinostat, induced apoptosis in A549 cells by mimicking BH3-only proteins, which included down-regulating anti-apoptotic proteins such as B-cell lymphoma-2 (BCL-2) and MCL-1, up-regulating apoptosis-related proteins Cleaved-Caspase-3 and Cleaved-PARP, and increasing BH3-only protein expression. Nobiletin binding to BCL-2 facilitated the dissociation of the Beclin-1/BCL-2 complex, thereby elevating levels of free Beclin-1. Furthermore, the combination of nobiletin and vorinostat enhanced the expression of LC3A/BII and forkhead box O1 (FOXO1), ultimately inducing autophagy in A549 cells. Eukaryotic transcriptome sequencing revealed that the combination treatment primarily inhibits tumor cell proliferation by modulating TRKC protein expression and suppressing phosphorylation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway. Therefore, our results indicate that nobiletin, a natural BH3 mimetic, synergizes with vorinostat to regulate both apoptosis and autophagy in NSCLC.

## 1. Introduction

Lung cancer is currently the most lethal malignancy worldwide, with non-small cell lung cancer (NSCLC) accounting for approximately 85% of all cases. Standard therapeutic approaches for NSCLC include surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy [1]. Despite these interventions, the overall 5-year survival rate remains low, and patients frequently develop drug resistance and disease recurrence [2]. Consequently, novel treatment strategies are urgently needed, and targeting apoptosis and autophagy has emerged as one of the most promising therapeutic avenues in recent years.

B-cell lymphoma-2 (BCL-2) family proteins play a critical role in tumorigenesis and progression. Under cellular stress, Beclin-1 and Bcl-2-associated X protein (BAX) are displaced from BCL-2, thereby initiating autophagy and apoptosis, respectively [3]. BH3-only proteins, induced transcriptionally or post-translationally in response to diverse stressors, either bind to BCL-2 to release BAX/BAK or directly activate these apoptotic effectors to trigger apoptosis. Additionally, Beclin-1 forms a heterodimer with BCL-2 (BCL-2/Beclin-1), which suppresses autophagy; Beclin-1 is essential for autophagy initiation [4]. Aberrant expression of BCL-2 family proteins and epigenetic modifications can promote tumor development and diminish the efficacy of anti-tumor therapies [5–9]. Notably, BCL-2 family inhibitors and epigenetic modulators exhibit synergistic effects across multiple cancer types. Epigenetic therapy can eliminate cancer cells by inducing mitochondrial apoptosis *via* BH3 mimetics and by restoring mitochondrial metabolism upon activation of retrotransposable elements [10, 11].

BH3 mimetic drugs are small molecules that mimic the function of BH3-only proteins by inhibiting anti-apoptotic BCL-2 family members. These agents can induce both autophagy and apoptosis in tumor cells and represent a novel class of anti-cancer therapeutics [12]. However, current clinically used BH3 mimetics

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exhibit a narrow anti-tumor spectrum and are associated with serious adverse effects, including tumor lysis syndrome, neutropenia, anemia, upper respiratory tract infections, and thrombocytopenia [13]. Therefore, discovering BH3 mimetics with a broader therapeutic window, lower toxicity, and unique mechanisms of action is of significant value, and natural products constitute a promising source for such compounds.

Nobiletin, a BH3 mimetic compound identified through pharmacophore-based screening of a library containing over 300 000 small molecules, demonstrated inhibitory activity against both small cell lung cancer (SCLC) and NSCLC cell lines in our activity-based assays. Given the higher incidence and mortality associated with NSCLC, we focused on elucidating nobiletin's effects and underlying mechanisms in this subtype [4, 14]. Nobiletin is a polymethoxy flavonoid extracted from the Rutaceae plant *Citrus reticulata* Blanco and is a key active constituent of traditional Chinese medicines such as Pericarpium Citri Reticulatae and Fructus Aurantii Immaturus. This natural product exhibits favorable safety and diverse pharmacological activities, including antioxidant [15], anti-inflammatory [16, 17], and anti-tumor effects [18]. Histone deacetylase inhibitors (HDACi) induce histone acetylation or hyperacetylation, which relaxes condensed chromatin and enhances the expression of tumor suppressor genes. We hypothesized that HDACi might also up-regulate BH3-only proteins, thereby promoting tumor cell apoptosis and potentially synergizing with BH3 mimetics. Previous studies have explored the combined use of BH3 mimetics and HDACi in cancer treatment [4, 19]. Indeed, HDAC inhibition or combination with HDACi has been shown to enhance BH3 mimetic-induced apoptosis in various tumor cells, including drug-resistant lines, potentially through up-regulation of BH3-only proteins such as NOXA and BIM or by facilitating BAK release from BCL-XL and MCL-1 [20, 21]. These findings suggest that combining BH3 mimetics with HDACi may delay the emergence of drug resistance and expand the utility of BH3 mimetics in solid tumors, offering a viable alternative strategy for NSCLC therapy.

Our prior work demonstrated that nobiletin functions as a BH3 mimetic and, in combination with vorinostat, induces apoptosis and autophagy in SCLC cells. Using molecular docking, molecular dynamics simulations, and surface plasmon resonance (SPR) assays, we confirmed that nobiletin exhibits BH3 mimetic activity and compared its profile with that of ABT-263 (navitoclax) [4]. In the present study, we found that the combination of nobiletin and vorinostat (SAHA) inhibits proliferation and induces apoptosis and autophagy in A549 cells. Mechanistically, this effect appears to involve suppression of *NTRK3* gene expression and inhibition of phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway phosphorylation, thereby promoting apoptosis and autophagy in NSCLC. Furthermore, the nobiletin-SAHA combination synergistically up-regulated BH3-only proteins, potentiating nobiletin's intrinsic BH3 mimetic activity and resulting in a markedly enhanced anti-tumor response.

## 2. Materials and methods

### 2.1. Cell culture and agents

Human A549 lung cancer cells and mouse Lewis lung carcinoma (LLC) cells were obtained from Procell (Wuhan, China). A549 cells were cultured in Dulbecco's modified Eagle medium/ Ham's F-12 nutrient mixture (DMEM/F12) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (GEMINI, USA) and 1% penicillin-streptomycin (Gibco, USA) at 37 °C in a 5% $CO_2$ atmosphere. LLC cells were maintained in DMEM (Gibco, USA) supplemented with 10% FBS and penicillin-streptomycin under identical conditions.

Nobiletin (analytical standard, high-performance liquid chromatography (HPLC) ≥ 98%, CAS: 478-01-3, #B20199), gossypol (analytical standard, HPLC ≥ 97%, CAS: 303-45-7, #B20869), and SAHA (analytical standard, HPLC ≥ 99%, CAS: 149647-78-9, #B23745) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).

### 2.2. Cell viability and combination index (CI) calculation

The effects of nobiletin and SAHA, alone or in combination, on A549 cell viability were evaluated using the cell counting kit-8 (CCK-8) assay (Biosharp, China). A549 cells were seeded in 96-well plates at a density of 10 000 cells per 100 μL per well. After treatment with varying concentrations of nobiletin and SAHA for 24 h, cells were incubated with 10 μL of CCK-8 solution for 2 h, and absorbance was measured at 562 nm.

The CI for the two drugs was calculated using Compusyn software, based on the Chou-Talalay method for drug combination analysis [22].

### 2.3. Flow cytometry

A549 cells were seeded in 6-well plates at a density of $2 \times 10^6$ cells per well and treated with nobiletin, SAHA, or their combination for 24 h. Following treatment, 1-10 × $10^5$ cells were collected and incubated with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) reagent (Elabscience, China) for 30 min in the dark. Apoptosis was then analyzed by flow cytometry (BD Biosciences, USA).

### 2.4. Hoechst 33342 staining

Cells were seeded in 6-well plates at a density of $2 \times 10^6$ per well and allowed to adhere for 24 h. After treatment with nobiletin, SAHA, or their combination, cells were washed twice with phosphate-buffered saline (PBS) and incubated with 1 mL of Hoechst 33342 staining solution (Solarbio, CA1120) per well for 30 min in the dark. Morphological changes in A549 cells were observed under a fluorescent inverted microscope.

### 2.5. Transmission electron microscope

Cells were treated with nobiletin, SAHA, or their combination for 24 h, then harvested using a cell scraper, fixed in 2.5% glutaraldehyde, and washed with 0.1 mol·$L^{-1}$ phosphate buffer. Samples were dehydrated through a graded ethanol series, embedded, and imaged using an H-7650 transmission electron microscope (Hitachi, Japan).

### 2.6. Western blotting analysis

Cells were lysed on ice for 30 min in RIPA Lysis Buffer (Solarbio, R0010) containing 1% PMSF, followed by centrifugation to collect the supernatant. Total protein concentration was determined using a BCA protein assay kit, and equal amounts (30 μg) of protein lysate were separated by 10% SDS-PAGE and transferred onto a 0.22 μm PVDF membrane (Roche, 030100400011). Membranes were washed with Tris-buffered saline (TBS)-0.1% Tween buffer and blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBST) for 90 min at room temperature.

Membranes were then cut based on the molecular weights of the target proteins and incubated overnight at 4 °C with the following primary antibodies, each diluted at 1:1000: BAX (Cat No. 5023T), BIM (Cat No. 2933T), BAD (Cat No. 9239T), BID (Cat No. 2002T), BCL-2 (Cat No. 3498T), MCL-1 (Cat No. 5453T), Cleaved-PARP (Cat No. 5625T), PARP (Cat No. 9532T), Caspase-3 (Cat No. 9662), Caspase-9 (Cat No. 9508T), Cleaved-Caspase-3 (Cat No. 9664T), H3K9ac (Cat No. 9649T), H3K14ac (Cat No. 7627T),

H3K18ac (Cat No. 13998T), H3K27ac (Cat No. 8173T), p62 (Cat No. 5114T), p53 (Cat No. 2527T), Beclin-1 (Cat No. 3495T), forkhead box O1 (FOXO1, Cat No. 2880T), LC3A/B (Cat No. 4108T), phosphorylated (p)-AKT (Cat No. 4060T), AKT (Cat No. 9272T), p-PI3K (Cat No. 17366T), PI3K (Cat No. 4292T), p-mTOR (Cat No. 5536T), mTOR (Cat No. 2983T), and *β*-Actin (Cat No. 4967S). After washing, membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (goat anti-rabbit IgG, Cat No. ZB-2301; goat anti-mouse IgG, Cat No. ZB-2305) diluted 1:10,000 in TBST. Protein bands were visualized using ECL reagents, and densitometric analysis was performed using Image*J* (v1.8.0) software.

### 2.7. Tumor-bearing mouse model

Male C57BL/6J mice (6-7 weeks old, 20 ± 2 g) were obtained from Henan Sikebesi Biotechnology Co., Ltd. (SCXK 2020-0005). After one week of acclimatization under standard housing conditions, mice received a subcutaneous injection of $1 \times 10^6$ LLC cells in 100 μL PBS into the right axilla.

When tumors became palpable (about 7 days post-inoculation), mice were randomized into five treatment groups ($n$ = 5 per group): vehicle control (0.5% CMC-Na), nobiletin 75 mg·kg$^{-1}$·d$^{-1}$, nobiletin 150 mg·kg$^{-1}$·d$^{-1}$, SAHA 60 mg·kg$^{-1}$·d$^{-1}$, or nobiletin + SAHA (75 mg·kg$^{-1}$·d$^{-1}$ + 60 mg·kg$^{-1}$·d$^{-1}$). All compounds were administered daily by oral gavage for 18 consecutive days. Mice were euthanized when any tumor reached 1500 mm$^3$; orbital blood was collected, and subcutaneous tumors, along with major organs, were harvested. Tumor weights were recorded to calculate the tumor weight index.

### 2.8. Wound healing assay

A549 cells were seeded evenly in 6-well plates and allowed to reach 85%-100% confluence. A straight wound was created by scratching the monolayer with a pipette tip perpendicular to the well. After gently washing three times with PBS to remove detached cells, fresh medium containing nobiletin and/or SAHA was added. Images were captured immediately at five representative fields per well. After 24 h, cells were washed three times with PBS, and images were acquired at the same locations.

### 2.9. Statistical analyses

Data are presented as mean ± SD and were analyzed using SPSS 19.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism v8.01 (CA, USA). One-way ANOVA was used for multiple comparisons. Statistical significance was defined as $^{*}P < 0.05$ *vs* Control, $^{**}P < 0.01$ *vs* Control; $^{\#}P < 0.05$ *vs* nobiletin and SAHA alone, $^{\#\#}P < 0.01$ *vs* nobiletin and SAHA alone.

## 3. Result

### 3.1. Inhibitory effect of nobiletin and SAHA on the proliferation of A549 cells

Preliminary studies indicated that the natural product nobiletin exerts potent inhibitory effects on small-cell lung cancer cell viability [4]. In this study, we evaluated nobiletin's ability to suppress A549 cell proliferation alone or in combination with SAHA. Both nobiletin and SAHA significantly inhibited A549 cell proliferation at concentrations exceeding 1 μmol·L$^{-1}$ (Figs. 1A–1B). When SAHA was fixed at 1 μmol·L$^{-1}$ and combined with nobiletin (0.1-80 μmol·L$^{-1}$) for 24 h, the combination exhibited greater inhibitory activity than nobiletin alone (Fig. 1C).

CompuSyn software was used to calculate the dose-effect curve of nobiletin combined with SAHA. The results showed that the inhibitory rate of the nobiletin-SAHA combination on A549 cells was higher than that of either agent alone (Fig. 1D). The CI

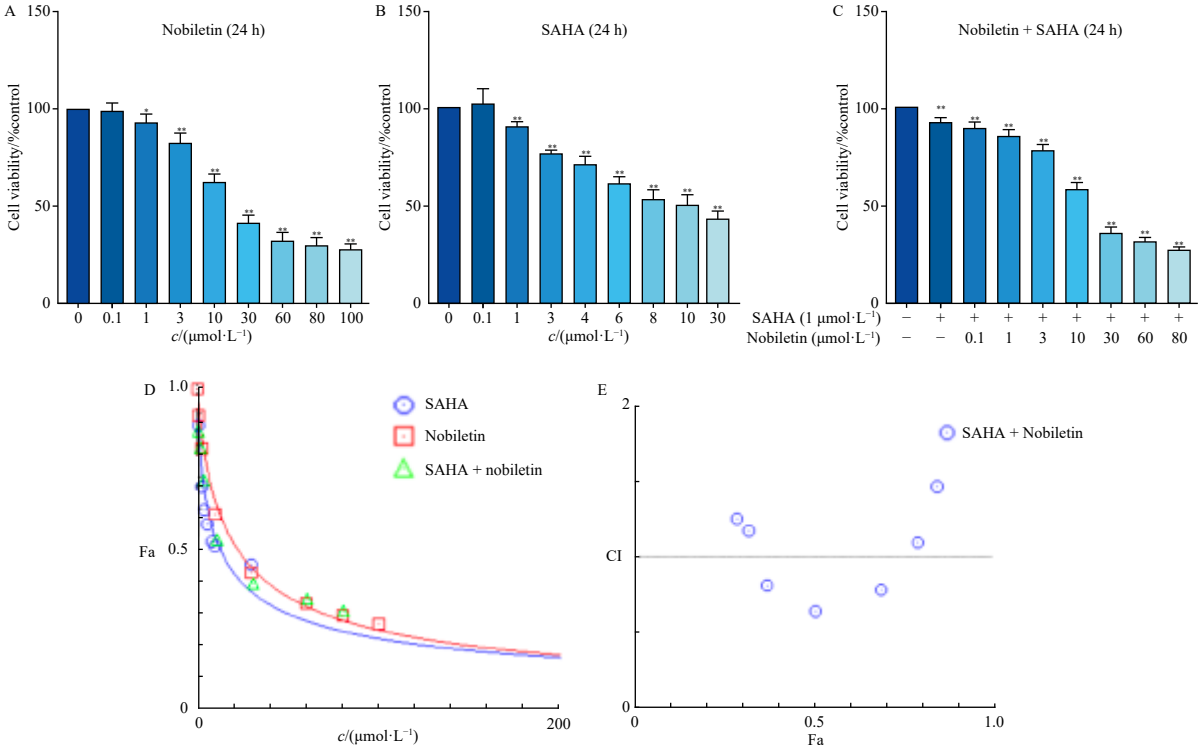



**Fig. 1** Synergistic inhibitory effects of nobiletin combined with SAHA on the proliferation of A549 cells. (A) Inhibitory effect of nobiletin (0.1–80 μmol·L$^{-1}$) on A549 cell viability after 24 h of treatment. (B) Inhibitory effect of SAHA (0.1–30 μmol·L$^{-1}$) on A549 cell viability after 24 h of treatment. (C) Effect of increasing concentrations of nobiletin (0.1–80 μmol·L$^{-1}$) combined with SAHA (1 μmol·L$^{-1}$) on A549 cell viability after 24 h of treatment. (D) Dose-effect curves for nobiletin and SAHA in A549 cells. (E) Combination index (CI) values fornobiletin and SAHA in A549 cells. CI < 1 indicates synergism. Data are presented as mean ± SD ($n$ = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* the control group.

value was calculated using the Chou-Talalay method implemented in CompuSyn software. The drug concentrations and combination indices are listed in Table 1. The lowest CI value (0.63) was observed with 1 μmol·L$^{-1}$ SAHA combined with 10 μmol·L$^{-1}$ nobileti, indicating the strongest synergistic effect (Fig. 1E).

**Table 1** Combined dosage index of nobiletin and SAHA

| Cell line | Dose SAHA (μmol·L$^{-1}$) | Dose nobiletin (μmol·L$^{-1}$) | Effect | CI |
|---|---|---|---|---|
| A549 | 1.0 | 0.1 | 0.85 | 1.4776 |
| | 1.0 | 1.0 | 0.79 | 1.095 91 |
| | 1.0 | 3.0 | 0.69 | 0.7803 |
| | 1.0 | 10.0 | 0.51 | 0.633 28 |
| | 1.0 | 30.0 | 0.37 | 0.808 24 |
| | 1.0 | 60.0 | 0.32 | 1.179 03 |
| | 1.0 | 80.0 | 0.28 | 1.253 98 |

Note: CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively.

### *3.2. Inhibitory effects of nobiletin combined with SAHA on apoptosis of A549 cells*

To investigate the mechanism by which nobiletin combined with SAHA inhibits A549 cell proliferation, we performed Hoechst 33342 staining. This analysis revealed that, compared with nobiletin (10 μmol·L$^{-1}$) or SAHA (1 μmol·L$^{-1}$) administered individually, the combination induced apoptosis in A549 cells (Fig. 2A). Features characteristic of apoptosis, including chromatin condensation, nuclear pyknosis, fragmentation, and formation of apoptotic bodies, were observed in the combination group. The fluorescence intensity in the nobiletin-SAHA group was markedly higher than in the control group, indicating increased apoptosis and a greater proportion of apoptotic-positive cells.

Annexin V-FITC/PI flow cytometric analysis showed that 24 h of treatment with either nobiletin or SAHA alone did not significantly alter the apoptotic fraction compared with the control. In contrast, 24 h of combined treatment with nobiletin and SAHA resulted in a significantly higher apoptotic rate relative to the control group (Fig. 2B), indicating that the combination enhances both early and total apoptosis in A549 cells.

Additionally, we assessed the expression of anti-apoptotic proteins BCL-2 and MCL-1 by Western blotting (Fig. 2C). Nobiletin significantly down-regulated MCL-1 expression ($P < 0.05$) but had no significant effect on BCL-2. SAHA alone suppressed both MCL-1 and BCL-2 ($P < 0.05$). The combination of nobiletin and SAHA synergistically down-regulated both MCL-1 and BCL-2 ($P < 0.01$), suggesting that the two agents cooperatively inhibit anti-apoptotic proteins to promote A549 cell apoptosis.

Likewise, we examined the expression of apoptosis-related proteins by Western blotting. The results showed that nobiletin combined with SAHA promoted the activation of Caspase-3 and PARP (Fig. 2D). Compared with single-agent treatments, the combination synergistically reduced full-length Caspase-3 and PARP while increasing Cleaved-Caspase-3 and Cleaved-PARP, thereby inducing apoptosis in A549 cells.

### *3.3. Effects of nobiletin and SAHA on autophagy of A549 cells*

Previous studies have shown that caspase activation during apoptosis can inhibit autophagy. For example, caspases may suppress autophagosome formation by cleaving autophagy-related proteins [23]. To further investigate autophagy induced by nobiletin combined with SAHA, we examined the ultrastructure of A549 cells after 24 h of treatment with nobiletin, SAHA alone, or their combination (Fig. 3A). Autophagic structures were absent in the control group, which exhibited only lysosomes and occasional autophagic vesicles. A few autophagosomes were observed with either SAHA (1 μmol·L$^{-1}$) or nobiletin (10 μmol·L$^{-1}$) alone. In contrast, pronounced accumulation of autophagosomes occurred following combined treatment, indicating that nobiletin and SAHA cooperatively trigger autophagy in A549 cells. Consequently, we evaluated the expression of key autophagy-associated proteins LC3A/B-II, p62, and FOXO1 and included the BH3 mimetic gossypol (4 μmol·L$^{-1}$) as a positive control (Figs. 3B–3C). The combination markedly increased LC3A/B-II, suppressed p62, and elevated FOXO1 protein levels, collectively indicating induction of tumor cell autophagy.

To determine whether nobiletin functions as a BH3 mimetic to induce autophagy, we performed co-immunoprecipitation assays (Fig. 3D). The results showed that both nobiletin alone and in combination with SAHA promoted dissociation of the Beclin-1/BCL-2 complex and increased free Beclin-1 protein levels ($P < 0.01$), thereby inducing autophagy.

### *3.4. Regulation of nobiletin combined with SAHA on histone acetylation and expression of BH3-only protein*

To assess whether the nobiletin-SAHA regimen alters histone acetylation, we quantified H3K9ac, H3K14ac, H3K18ac, and H3K27ac levels by immunoblotting. Nobiletin (10 μmol·L$^{-1}$) alone did not affect acetylation at these sites, whereas SAHA (1 μmol·L$^{-1}$) markedly increased acetylation of H3K9, H3K14, H3K18, and H3K27. Co-administration further enhanced SAHA-induced H3K27ac and up-regulated the tumor suppressor p53. However, nobiletin (10 μmol·L$^{-1}$) did not augment SAHA (1 μmol·L$^{-1}$)-mediated acetylation of H3K9, H3K14, or H3K18 (Fig. 4A).

Furthermore, we examined the effects of nobiletin alone or combined with SAHA on BH3-only proteins (BIM, BID, BAD) and the pro-apoptotic protein BAX in A549 cells. Nobiletin (10 μmol·L$^{-1}$) alone enhanced BIM and BAX expression, while SAHA (1 μmol·L$^{-1}$) modestly increased BIM, BID, and BAD. Combined treatment further amplified the induction of these BH3-only proteins (Fig. 4B).

In summary, the nobiletin-SAHA combination increased acetylation of H3K9, H3K14, H3K18, and H3K27, promoted chromatin decondensation, and up-regulated BH3-only proteins BAD, BIM, and BID. Moreover, nobiletin mimicked BH3-only proteins, thereby synergistically inducing both apoptosis and autophagy in tumor cells.

### *3.5. Inhibitory effect of nobiletin combined with SAHA on LLC-bearing mice and its influence on the expression of BH3-only protein*

To evaluate the *in vivo* anti-tumor efficacy of nobiletin and SAHA, we established subcutaneous LLC xenografts in C57BL/6J mice and monitored tumor volume, body weight, tumor weight, and tumor weight index (Fig. 5A). Tumor growth curves revealed significant suppression of tumor progression in mice treated with nobiletin (75 mg·kg$^{-1}$·d$^{-1}$, i.g., 18 days) combined with SAHA (60 mg·kg$^{-1}$·d$^{-1}$, i.g., 18 days) compared with the control group at day 18 (Figs. 5B–5E). Both tumor weight and tumor weight index differed significantly between the combination and control groups, confirming that the two drugs jointly inhibit tumor growth in LLC-bearing mice. Western blotting analysis showed that the combination synergistically elevated BIM expression in transplanted tumors, exceeding the effect of either agent alone, consistent with our *in vitro* findings that SAHA enhances BH3-only protein expression. Additionally, the combination increased BAX and down-regulated TGF-β1, thereby suppressing tumor growth (Fig. 5F).

### *3.6. Effect of nobiletin combined with SAHA on PI3K/AKT/mTOR signaling pathway in NSCLC*

To elucidate the synergistic mechanism of the nobiletin-SAHA regimen, we performed eukaryotic transcriptome sequencing to identify genes driving cooperative effects, yielding 313

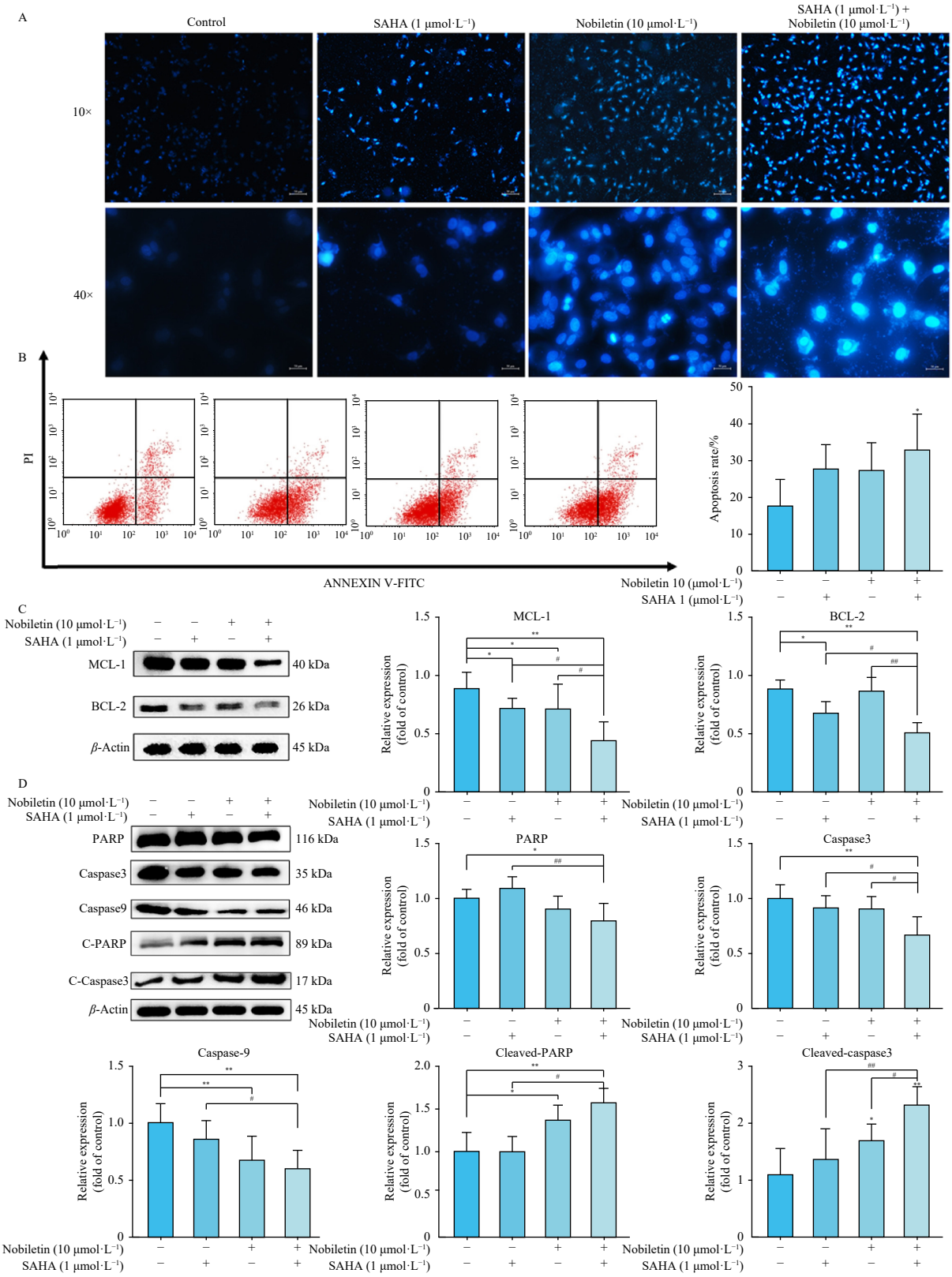



**Fig. 2** Effects of nobiletin combined with SAHA on apoptosis in A549 cells. (A) Fluorescence microscopy after Hoechst 33342 staining showed apoptotic morphological changes in A549 cells treated with nobiletin (10 μmol·L$^{-1}$) and SAHA (1 μmol·L$^{-1}$), either alone or in combination. Normal nuclei are shown in blue, and apoptotic nuclei in bright blue. Scale bar = 50 μm. (B) Apoptosis in A549 cells after treatment with nobiletin (10 μmol·L$^{-1}$) and SAHA (1 μmol·L$^{-1}$), alone or in combination, was quantified by flow cytometry using Annexin V/PI staining. Each bar represents the mean ± SD of three independent experiments. (C) Protein expression levels of the anti-apoptotic proteins BCL-2 and MCL-1 in A549 cells after the indicated treatments. (D) Protein expression levels of apoptosis-related proteins Caspase-3, PARP, Caspase-9, cleaved PARP, and cleaved Caspase-3 in A549 cells after the indicated treatments. Data are presented as mean ± SD ($n$ = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* nobiletin- and SAHA-alone treatment for the alone group.

differentially expressed genes (Fig. 6A). Protein-protein interaction (PPI) network analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment indicated that these genes predominantly converge on the PI3K/AKT axis, suggesting that the combination suppresses tumor growth *via* modulation of PI3K/AKT signaling (Fig. 6B). Western blotting confirmed that,

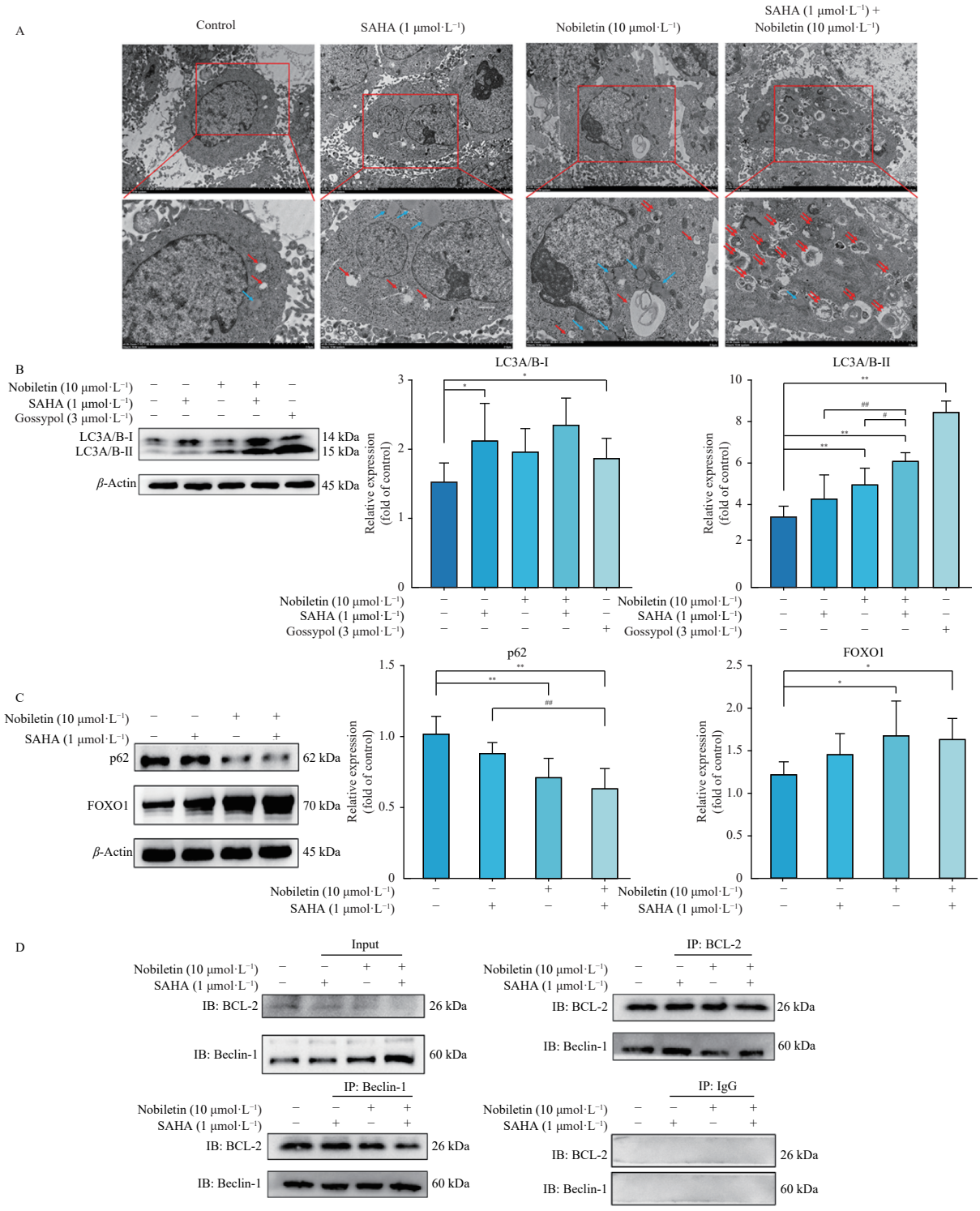



**Fig. 3** Effects of nobiletin and SAHA on autophagy of A549 cells. (A) Nobiletin (10 μmol·L$^{-1}$) and SAHA (1 μmol·L$^{-1}$), either alone or in combination, induced autophagy-related morphological changes in A549 cells, as shown by TEM. The red single arrow indicates an autophagic vacuole, the blue single arrow indicates a lysosome, and the red double arrow indicates an autolysosome. (B) Protein expression levels of LC3A/B-I and LC3A/B-II in A549 cells after treatment with nobiletin (10 μmol·L$^{-1}$), SAHA (1 μmol·L$^{-1}$), or their combination. (C) Co-IP analysis of BCL-2 and Beclin-1. The immunoprecipitates were analyzed by Western blotting with anti-BCL-2 and anti-Beclin-1 antibodies. Data are presented as mean ± SD ($n$ = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* nobiletin- or SAHA-alone reatment group.

relative to monotherapies and vehicle controls, nobiletin-SAHA co-treatment synergistically reduced phosphorylation of PI3K, AKT, and mTOR, effectively inhibiting the oncogenic PI3K/AKT/mTOR pathway and arresting tumor cell proliferation (Fig. 6C).

### *3.7. Nobiletin combined with SAHA has a synergistic effect by inhibiting TRKC expression*

To identify the key target mediating synergistic NSCLC inhibition by nobiletin and SAHA, we analyzed differentially expressed genes in the combination group. Thirty-one genes were identified as potential contributors to synergy (Fig. 6D). Heatmap analysis revealed that NTRK3 transcript levels were markedly reduced in the combination group compared with monotherapies or vehicle control. Subsequent Western blotting confirmed that nobiletin combined with SAHA inhibited TRKC protein expression. Moreover, quantitative polymerase chain reaction (qPCR) results corroborated the transcriptome data, showing synergistic

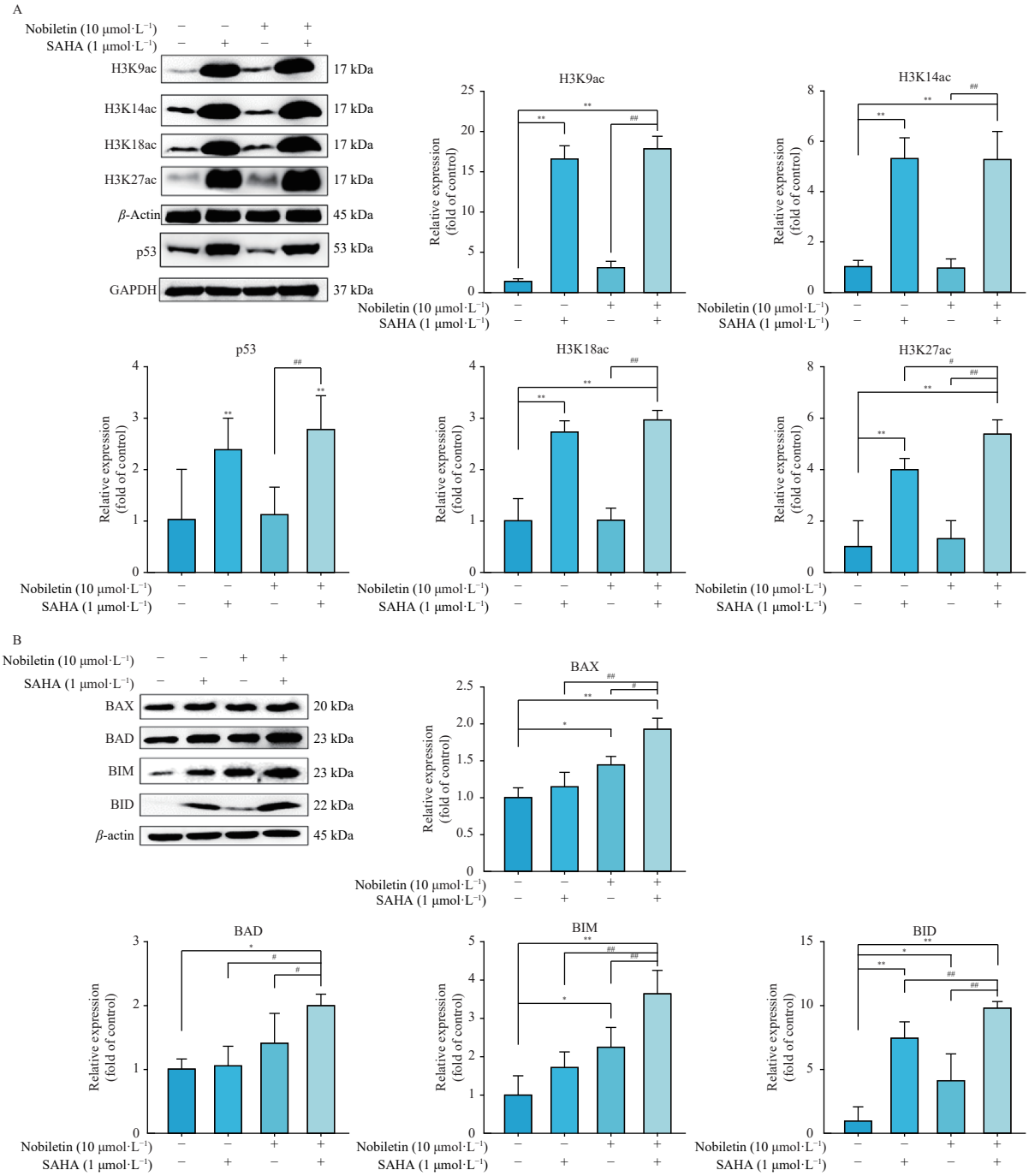



**Fig. 4** Effects of nobiletin combined with SAHA on histone acetylation and BH3-only protein expression. (A) Protein expression levels of H3K9ac, H3K14ac, H3K18ac, H3K27ac, and p53 in A549 cells following treatment with nobiletin (10 μmol·L$^{-1}$), SAHA (1 μmol·L$^{-1}$), or their combination. (B) Protein expression levels of BAX, BAD, BIM, and BID in A549 cells following treatment with nobiletin (10 μmol·L$^{-1}$), SAHA (1 μmol·L$^{-1}$), or their combination. Data are presented as mean ± SD ($n$ = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* nobiletin- or SAHA-alone treatment group).

suppression of *NTRK3* gene expression by the *NTRK3* combination (Fig. 6E).

### *3.8. Nobiletin inhibits the proliferation and migration of A549 cells through down-regulating the expression of TRKC*

To assess TRKC's role in nobiletin-mediated tumor suppression, we transfected A549 cells with NTRK3-targeted knockdown plasmids. *NTRK3* knockdown alone inhibited cell proliferation, and the additional inhibitory effect of nobiletin and SAHA was markedly attenuated, indicating that *NTRK3* expression is essential for A549 cell proliferation (Fig. 7A). We then examined the impact of *NTRK3* knockdown on the AKT/mTOR pathway (Fig. 7B). Unexpectedly, *NTRK3* knockdown significantly suppressed AKT/mTOR activation relative to scrambled control. However, subsequent nobiletin treatment reversed this inhibition (Fig. 7C).

Studies indicate that *NTRK3* fusions are prevalent in numerous tumor cell lines and clinical samples, with expression levels positively correlated with tumor cell migration and invasiveness, potentially *via* the EMT pathway [24, 25]. We therefore evaluated the effects of nobiletin and SAHA on A549 cell migration. SAHA alone minimally inhibited migration, nobiletin exerted a modest effect, but the combination achieved superior inhibition (Fig. 7D). Following *NTRK3* knockdown, baseline cell migration was reduced, and drug treatment no longer significantly affected motility (Fig. 7E). Similarly, nobiletin increased the epithelial marker E-cadherin and decreased mesenchymal markers N-cadherin and Vimentin, whereas SAHA had no notable effect on EMT-related pro-

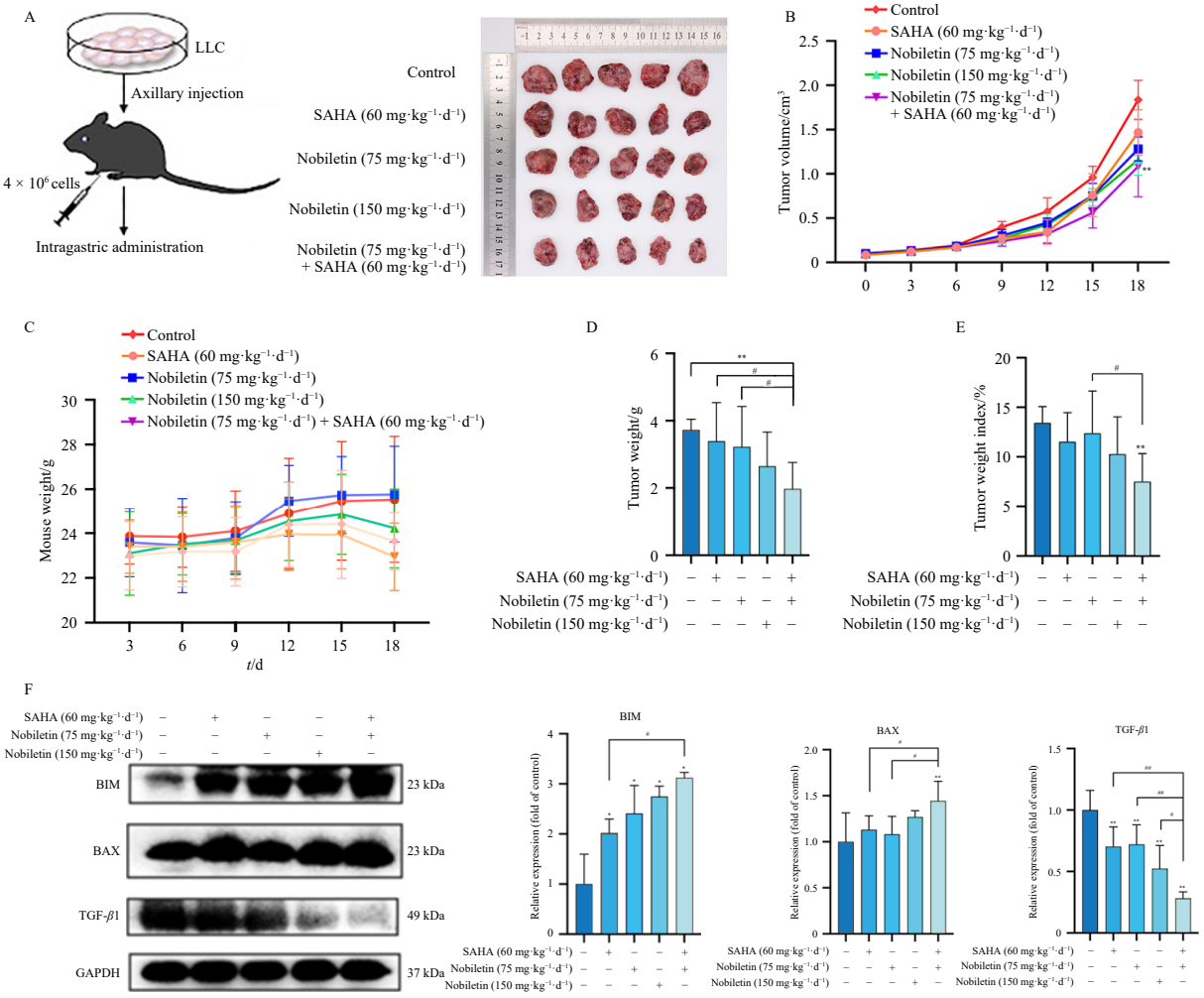



**Fig. 5** Inhibitory effects of nobiletin combined with SAHA in LLC-bearing mice and its effects on BH3-only protein expression. (A) LLC cells were transplanted into C57BL/6J mice ($4 \times 10^5$ cells/mouse). Mice were then administered 75, 150 mg·kg$^{-1}$·d$^{-1}$ nobiletin, 60 mg·kg$^{-1}$·d$^{-1}$ SAHA, and the combination of 75 mg·kg$^{-1}$·d$^{-1}$ nobiletin and 60 mg·kg$^{-1}$·d$^{-1}$ SAHA by gavage for 18 days. (B) Tumor growth curves of mice treated with nobiletin or SAHA alone, or in combination, for 18 days. (C) ody weight changes in mice treated with nobiletin or SAHA alone, or in combination, for 18 days. (D) Tumor volume after 18 days of treatment. (E) Tumor weight index after 18 days of treatment. (F) Effects of nobiletin or SAHA alone, or in combination, on BIM, BAX, and TGF-β1 protein expression in LLC tumor tissues, as determined by Western blotting. Each bar represents the mean ± SD of five independent experiments. GAPDH was used as the loading control, and relative protein expression was normalized to GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* model group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* nobiletin- or SAHA-alone treatment group) .

teins (Fig. 7F). After *NTRK3* knockdown, EMT was dramatically suppressed, and the inhibitory effects of both drugs were diminished (Fig. 7G). Collectively, these findings indicate that nobiletin suppresses A549 cell proliferation and migration by inhibiting the TRKC-mediated AKT/mTOR pathway.

## 4. Discussion

In this study, we investigated the effects of combining the BH3 mimetic nobiletin with the HDAC inhibitor SAHA in NSCLC. Our results demonstrate that this combination effectively suppresses NSCLC growth both *in vitro* and *in vivo*. Specifically, nobiletin, acting as a novel BH3 mimetic, binds BCL-2 to promote the release of Beclin-1 and the pro-apoptotic effector BAX, thereby inducing autophagy and apoptosis. Concurrently, SAHA, as an HDACi, facilitates chromatin relaxation through histone acetylation and enhances expression of BH3-only proteins (BAD, BIM, and BID), complementing nobiletin's actions to produce a synergistic anti-tumor response. Moreover, we found that the combination primarily inhibits NSCLC by suppressing the PI3K/AKT/mTOR signaling pathway, while nobiletin exerts its effects on this pathway and on cell migration mainly through TRKC down-regulation.

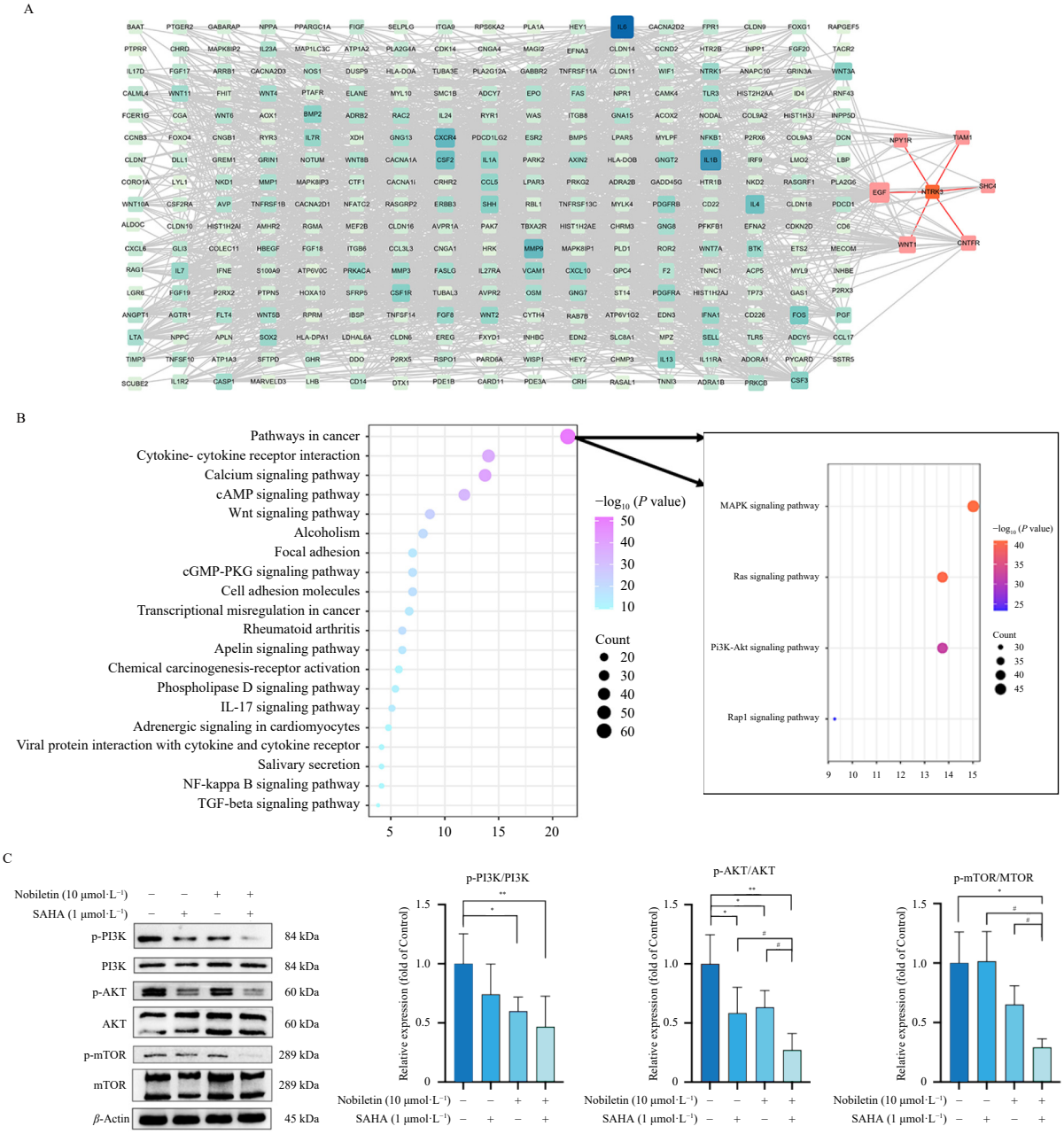
NSCLC accounts for approximately 85% of all lung cancer cases. Its poor prognosis and resistance to radiotherapy, chemotherapy [26], targeted therapy, and immunotherapy [1] contribute to its status as the leading cause of cancer-related mortality worldwide. Thus, elucidating NSCLC molecular mechanisms and developing novel, effective therapeutic strategies are imperative [2]. The concept of "BH3 mimetics" has driven the development of small molecules that induce cancer cell apoptosis by mimicking BH3-only proteins [27, 28]. Promising candidates such as gossypol and its derivatives have undergone clinical evaluation [20]. However, BH3 mimetics often exhibit limited anti-tumor activity as monotherapies [29] and are therefore typically combined with other oncologic treatments to enhance efficacy [30]. Clinically used BH3 mimetics can cause adverse effects, including tumor lysis syndrome [31], neutropenia [32], anemia [33], upper respiratory tract infection, and thrombocytopenia [34], and possess a narrow anti-tumor spectrum. Consequently, identifying BH3 mimetics with broad therapeutic potential, low toxicity, and unique mechanisms, particularly from natural sources, is highly valuable.

Based on our prior work, we employed pharmacophore virtual screening combined with Lipinski's Rule of Five to identify nobiletin, a natural BH3 mimetic, from a natural small-molecule library [4, 14]. Nobiletin has been reported to exert diverse anti-cancer effects and protect normal cells from various toxicants [35, 36]. Our previous studies demonstrated that nobiletin inhibits SCLC growth and induces apoptosis and autophagy both *in vitro* and *in vivo* [4].

HDACis represent an innovative class of anti-cancer agents. SAHA, one of the most promising HDACi, exhibits significant anti-tumor activity in hematological malignancies and solid tumors, including SCLC [37, 38]. However, HDACi monotherapy shows limited efficacy in solid tumors and is currently approved only for hematologic indications. Some tumor cells resist HDACi by up-regulating anti-apoptotic proteins or activating the PI3K/AKT pathway [39]. Our study aimed to enhance BH3 mimetic efficacy by combining nobiletin with SAHA, thereby modulating histone acetylation and achieving synergistic anti-tumor effects.

To test this hypothesis, we assessed the effects of nobiletin and SAHA alone or in combination on A549 cell viability *in vitro*. Compared with nobiletin alone, the combination of nobiletin (10 μmol·L$^{-1}$) and SAHA (1 μmol·L$^{-1}$) dramatically inhibited A549 proliferation, with the lowest CI indicating optimal synergy. In *in vivo* experiments using an LLC xenograft mouse model, analysis of tumor volume, weight, and tumor weight index revealed that the combination significantly suppressed tumor growth relative to the control group. Notably, nobiletin (75 mg·kg$^{-1}$·d$^{-1}$) combined with SAHA (60 mg·kg$^{-1}$·d$^{-1}$) reduced tumor weight more effectively than either single agent, confirming that the combination inhibits NSCLC growth and proliferation.

BH3 mimetics bind the BH3-binding groove of anti-apoptotic BCL-2 family proteins; BCL-2 is critical in mitochondrial-mediated intrinsic apoptosis, while Beclin-1 regulates autophagy [40]. Hoechst 33342 staining and flow cytometry demonstrated that, relative to monotherapy, the nobiletin-SAHA combination induced pronounced chromatin condensation, mitotic arrest, apoptotic body formation, and an elevated late-apoptosis/total-apoptosis ratio, collectively indicating robust pro-apoptotic synergy. BCL-2 overexpression is a key factor in cancer development and a major barrier to effective therapy [8, 41, 42]. Although BH3 mimetics can induce tumor cell apoptosis by targeting anti-apoptotic proteins [43], they often exhibit weak activity against MCL-1, a protein frequently overexpressed in tumors and essential for tumor cell survival. Our analysis of apoptosis-related proteins revealed that the combination, but not either agent alone, suppressed both MCL-1 and BCL-2. Similarly, the combination synergistically enhanced Caspase-3 and PARP activation, increasing Cleaved-Caspase-3 and Cleaved-PARP levels to drive tumor cell apoptosis. Beyond apoptosis, BH3 mimetics can also promote autophagy [44]. Consistent with prior findings, we observed that the combination



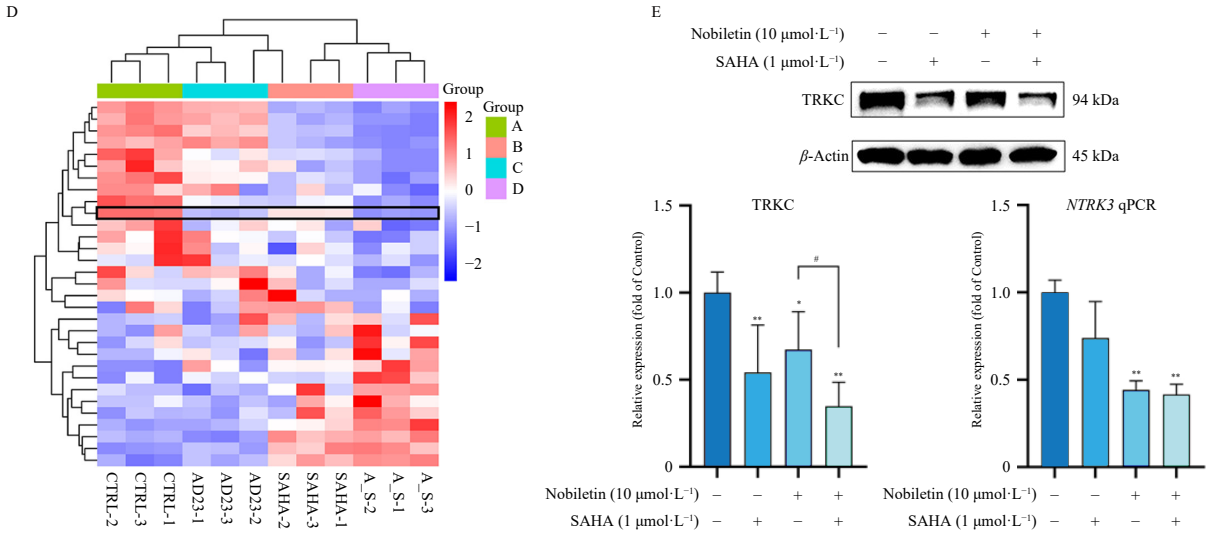
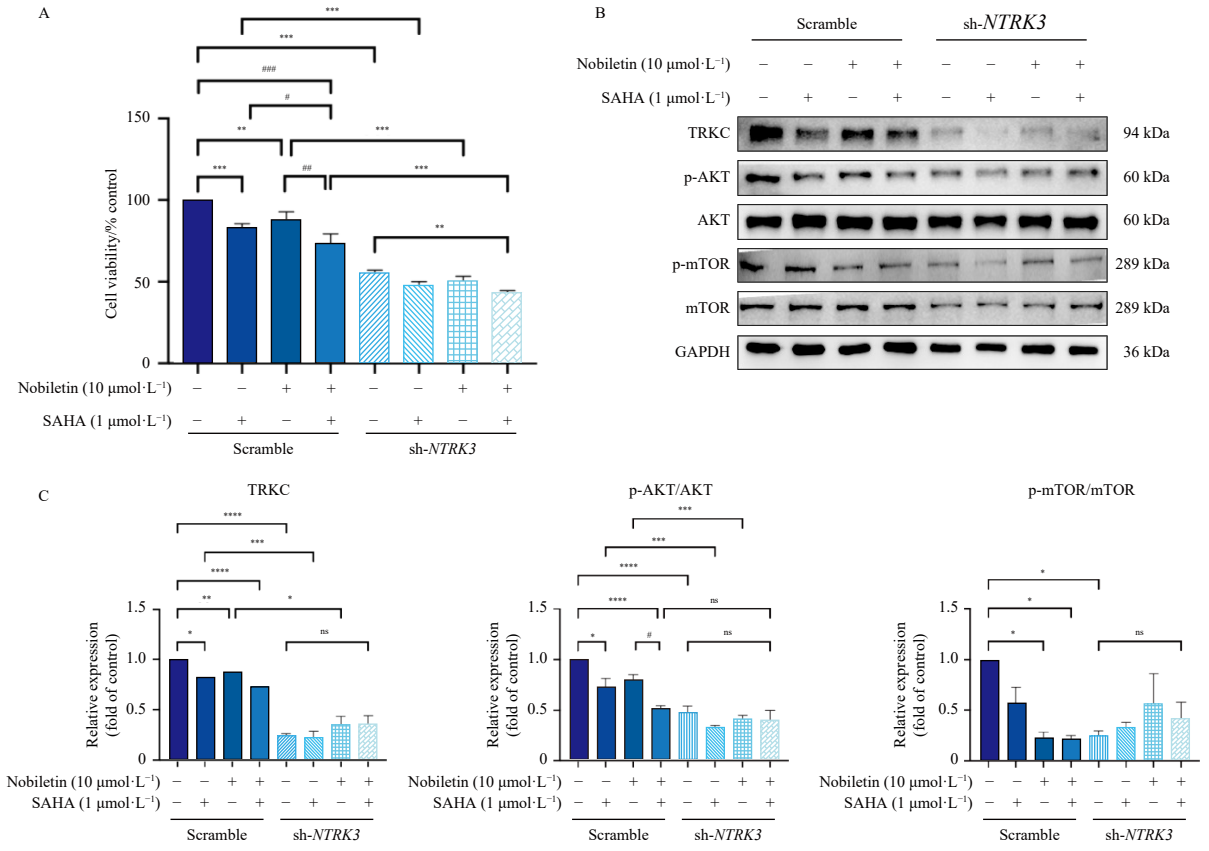



**Fig. 6** Mechanism underlying the synergistic inhibitory effects of nobiletin and SAHA on NSCLC. (A) A549 cells were treated with nobiletin (10 μmol·L$^{-1}$), SAHA (1 μmol·L$^{-1}$), or their combination, after which transcriptome sequencing was performed and protein–protein interaction (PPI) analysis of the differentially expressed genes was conducted. (B) KEGG enrichment analysis of differentially expressed genes identified by transcriptome sequencing. (C) Protein expression levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR in A549 cells after the indicated treatments. (D) Transcriptome analysis identified 31 differentially expressed genes in the combination group; *NTRK3* expression is highlighted by the black rectangle. (E) TRKC protein and *NTRK3* mRNA expression in A549 cells after the indicated treatments, as measured by Western blotting and qPCR, respectively. Data are presented as mean ± SD ($n$ = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* nobiletin- or SAHA-alone treatment group.

reduced p62 and increased LC3-II levels, with autophagosome formation confirmed by transmission electron microscopy. Co-immunoprecipitation further showed that the combination promoted dissociation of the BCL-2/Beclin-1 complex, potentially linked to enhanced BH3-only protein expression, thereby synergistically augmenting tumor cell autophagy.

To elucidate the underlying mechanism, we examined BH3-only protein expression from the perspective of nobiletin as a BH3 mimetic. Both *in vitro* and *in vivo* results demonstrated that the combination synergistically up-regulated BIM, BID, and BAD, enhancing the anti-tumor efficacy of BH3 mimetics. From SAHA's perspective, it markedly increased acetylation of H3K9, H3K14, H3K18, and H3K27. The combination exhibited only SAHA's histone acetylation effects, implying that SAHA inhibits HDAC activity to increase histone acetylation, thereby up-regulating BH3-only proteins and potentiating nobiletin's efficacy for synergistic tumor cell inhibition.

To further dissect the synergistic mechanism, we performed transcriptomic analysis of A549 cells treated with nobiletin alone or in combination with SAHA. Differential gene analysis revealed that nobiletin effectively suppresses *NTRK3* expression. qPCR and Western blotting validation confirmed that the combination in-



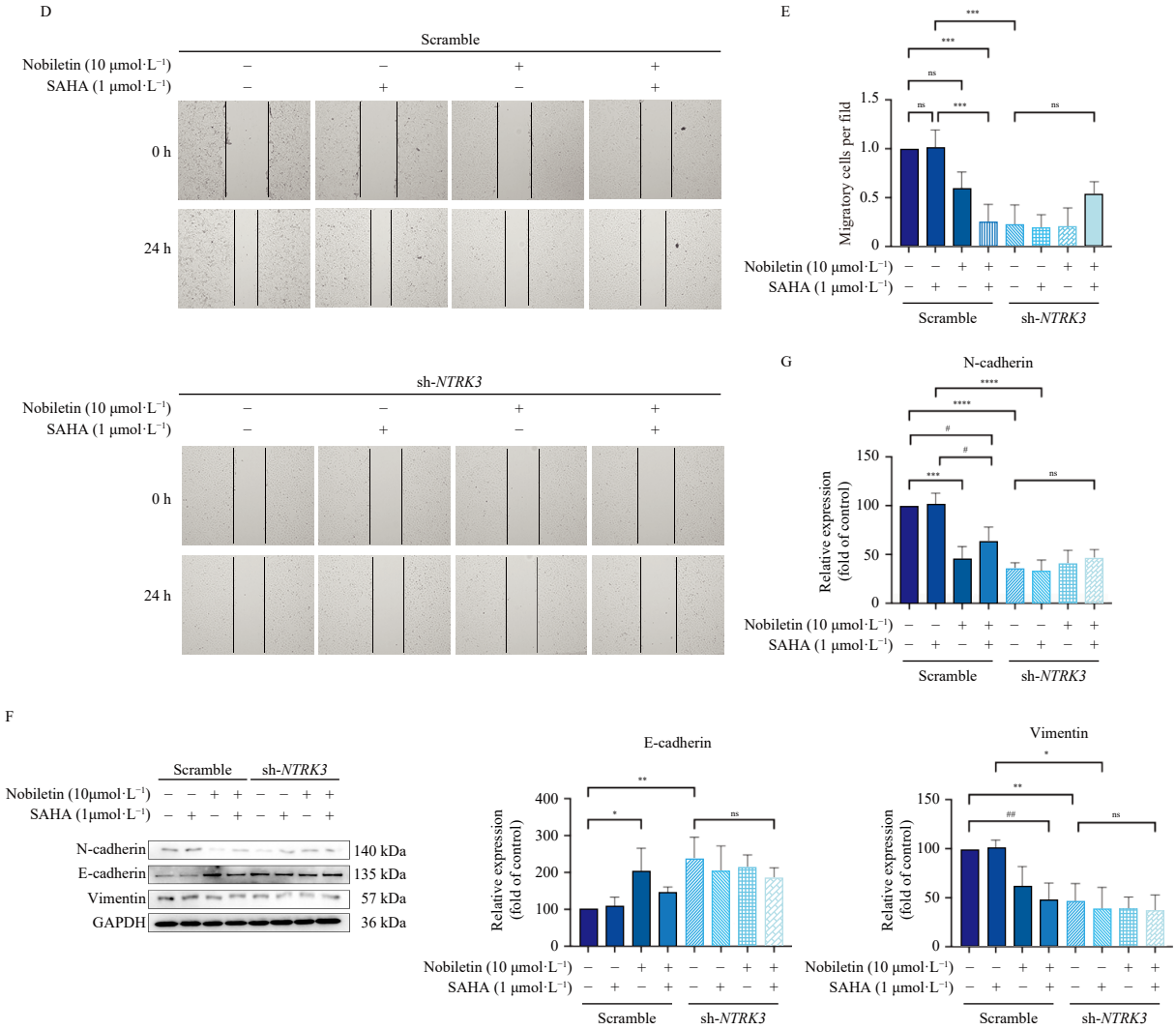



**Fig. 7** Nobiletin inhibits the proliferation and migration of A549 cells by suppressing the TRKC-mediated AKT/mTOR pathway. (A) Effects of nobiletin (10 μmol·L$^{-1}$), SAHA (1 μmol·L$^{-1}$), and their combination on the viability of *NTRK3*-knockdown A549 cells. (B and C) Effects of nobiletin (10 μmol·L$^{-1}$), SAHA (1 μmol·L$^{-1}$), and their combination on TRKC expression and the AKT/mTOR signaling pathway in *NTRK3*-knockdown A549 cells, as determined by Western blotting. (D and E) Effects of nobiletin (10 μmol·L$^{-1}$), SAHA (1 μmol·L$^{-1}$) and their combination on the migration of *NTRK3*-knockdown A549 cells, as assessed by wound-healing assay. (F and G) Effects of nobiletin (10 μmol·L$^{-1}$), SAHA (1 μmol·L$^{-1}$), and their combination on on EMT-related protein expression in *NTRK3*-knockdown A549 cells, as determined by Western blotting. Data are presented as mean ± SD ($n$ = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* nobiletin- or SAHA-alone treatment group.

hibits both *NTRK3* transcription and TRKC protein expression. TRKC, encoded by *NTRK3*, is a member of the tropomyosin receptor kinase (TRK) family and is implicated in the *NTRK3* pathogenesis, metastasis, and progression of multiple cancers [45], including melanoma, NSCLC, and colon cancer. Upon binding its ligand neurotrophin-3 (NT-3), TRKC dimerizes and autophosphorylates, activating its tyrosine kinase function [46]. In tumors, *NTRK3* rearrangements or fusions can cause constitutive TRKC activation, now recognized as a pan-cancer oncogenic driver. TRKC can activate PI3K and AKT [47, 48]. In addition, KEGG analysis of the differentially expressed genes revealed that most were enriched in the PI3K/AKT signaling pathway. We therefore examined the effects of nobiletin, alone or in combination with SAHA, on the PI3K/AKT/mTOR signaling pathway in A549 cells. The results were in agreement with the preceding analyses. Compared with single-agent treatment or vehicle control, combined treatment with nobiletin and SAHA synergistically suppressed activation of the PI3K/AKT/mTOR pathway in A549 cells and induced autophagy. Given the critical role of *NTRK3* in tumor progression, we performed *NTRK3* knockdown by plasmid transfection and observed inhibition of the previously activated PI3K/AKT pathway. Moreover, treatment of *NTRK3*-knockdown cells with nobiletin alone or in combination with SAHA did not result in significant additional changes in this pathway. These findings suggest that nobiletin, either alone or in combination with SAHA, may inhibit NSCLC by targeting the *NTRK3*-mediated PI3K/AKT pathway. Previous studies have shown that aberrant activation of TRKC in various tumors is commonly caused by gene fusions involving *NTRK3*. Such fusions can lead to constitutive activation of TRKC kinase and disruption of its normal regulation [49]. *NTRK3* fusion genes may also alter cell morphology and motility through the regulation of cytoskeleton remodeling-related genes, thereby enhancing the invasive and metastatic potential of tumor cells [25]. In the present study, we found that nobiletin inhibited tumor cell migration by suppressing TRKC expression, and that *NTRK3* knockdown attenuated EMT. However, we did not investigate whether *NTRK3* was involved in gene fusions or identify the possible fusion types. These questions should be addressed in future studies.

## 5. Conclusion

In summary, we confirm that nobiletin is a novel natural BH3 mimetic. The combination of nobiletin and SAHA exhibits significant synergistic inhibition of NSCLC *in vitro*, and *in vivo* studies in mice validate that the combination synergistically suppresses LLC

xenograft growth. Nobiletin binds BCL-2, promoting release of Beclin-1 and the pro-apoptotic effector BAX to induce autophagy and apoptosis. Conversely, SAHA, as an HDACi, enhances chromatin relaxation via histone acetylation, up-regulating BH3-only proteins (BAD, BIM, BID). SAHA thereby synergizes with nobiletin to amplify anti-tumor efficacy. The combination also significantly suppresses tumor cell migration and inhibits the PI3K/AKT/mTOR signaling pathway by down-regulating NTRK3 expression, thereby enhancing its anti-tumor effects.

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## Declaration of competing interest

The authors declare no conflicts of interest.

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