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## Original Research Article

# Unveiling the antimetastatic activity and molecular mechanism of montanine in lung cancer cells via the integration of network pharmacology approaches with in vitro and in vivo investigations

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## ABSTRACT

**Objective:** Non-small cell lung cancer (NSCLC), a leading cause of cancer-related mortality, presents a poor prognosis due to its tendency to metastasize and its resistance to standard therapies. Montanine (MTN), a naturally occurring alkaloid, has recently shown potential as an anticancer agent. However, the specific mechanisms through which it exerts its anticancer effects are still largely unknown. This study investigates the anti-NSCLC potential of MTN by utilizing integrative network pharmacology alongside in vitro and in vivo experimental validations.

**Methods:** Genes targeted by MTN in NSCLC were identified and subjected to functional enrichment analysis to elucidate the most enriched signaling pathways. A protein-protein interaction network was constructed to identify the interactions of potential targets. Adrenoceptor  $\beta_2$  (ADRB2) expression in lung cancer was analyzed using data from The Cancer Genome Atlas and Gene Expression Omnibus datasets. The effects of MTN on NSCLC cell lines, including migration, proliferation, viability and apoptosis, were assessed in vitro. MTN-related mechanisms were investigated using quantitative reverse transcription-polymerase chain reaction and Western blotting. Furthermore, an in vivo lung cancer metastasis experiment was performed in rats using a tail vein assay.

**Results:** MTN at nontoxic doses significantly reduced cell migration and lung colonization in an in vivo metastasis study ( $P < 0.05$ ). Network pharmacology revealed 29 targets of MTN in NSCLC, highlighting ADRB2 as a primary target linked to poor prognosis in lung cancer. In vitro studies confirmed that MTN notably upregulated ADRB2 expression and downregulated epithelial-mesenchymal transition (EMT) signaling pathways, which was further enhanced by terbutaline sulfate, an ADRB2 activator.

**Conclusion:** MTN is a promising therapeutic agent for NSCLC by suppressing the migration via ADRB2 and EMT mechanisms.

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## 1. Introduction

Non-small cell lung cancer (NSCLC), the most common type of lung cancer, presents a formidable treatment challenge and has a poor prognosis [1]. Despite advancements in treatment modalities, approximately 80%–85% of diagnosed patients worldwide exhibit low survival rates [2]. While some subtypes of NSCLC exhibit slower growth, the overall aggressiveness of this cancer type is concerning. NSCLC is notorious for its high metastatic potential and it often spreads beyond the lungs to distant organs such as the brain, liver and bones [3]. Therefore, inhibiting these aggressive behaviors represents a promising therapeutic approach for treating late-stage NSCLC.

Cancer metastasis is mainly driven by the regulation of epithelial–mesenchymal transition (EMT) [4], which enables the transformation of epithelial cells into mesenchymal-like cells, thereby increasing their migratory and invasive abilities [5]. Throughout the EMT, cancer cells alter their cell–cell adhesion and polarity, facilitating the detachment from the primary tumor and invasion of surrounding tissues. This morphological shift, coupled with the degradation of basement membranes and enhanced survival signals, enables cancer cells to traverse the bloodstream and potentially initiate secondary tumors in distant organs [4–6]. EMT-driven metastasis significantly contributes to the aggressive behavior observed in NSCLC, highlighting the critical need for research aimed at disrupting EMT to prevent cancer spread.

Clinical studies have indicated that adrenoceptor  $\beta_2$  (ADRB2) is crucial in regulating various cancer processes, including its growth, migration and invasion, and the immune system's response [7–10]. While activating ADRB2 generally promotes tumor development, some findings suggest that its activation might inhibit tumor aggressiveness [10–12]. Recent research has indicated that activating ADRB2 signaling can inhibit lung cancer cell proliferation [7]. Furthermore, research has demonstrated that lung cancer patients with high ADRB2 expression levels have higher overall survival rates compared to those with low expression levels [8,13]. Therefore, the precise impact of ADRB2 signaling on lung cancer progression remains unclear.

Montanine (MTN) or 2-methoxypancracine, a natural alkaloid primarily found in plants of the Amaryllidaceae family, has attracted considerable attention due to its possible medicinal benefits [14]. Research suggests that MTN exhibits various pharmacological activities, including anti-inflammation, antioxidant and cytotoxic effects in cancer cells [15]. MTN has shown promise in inhibiting cancer cell growth across different cell lines, but its precise anticancer mechanisms are not fully understood [14,16].

Network pharmacology is a comprehensive approach that utilizes extensive databases and computational strategies to identify potential molecular targets, complex signaling pathways and protein–protein interactions (PPIs) of natural compounds for treating various diseases [17,18]. This approach has effectively elucidated the molecular mechanisms of different compounds by identifying multiple potential targets and pathways [19]. This study identified the mechanism of action and molecular pathways influenced by MTN in NSCLC using network pharmacology and in vitro and in vivo experiments. The findings of this study could offer valuable insights into its potential applications as an anticancer agent.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum

(FBS), and a 100 U/mL penicillin/streptomycin solution were purchased from Gibco (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Invitrogen (Waltham, MA, USA). 4',6-Diamidino-2-phenylindole (DAPI), an ADRB2 agonist terbutaline sulfate (TER), and other reagents were acquired from Sigma Aldrich (St. Louis, MO, USA).

### 2.2. MTN preparation

MTN was isolated from *Hippeastrum reticulatum* (L'Hér.). Its chemical structure was characterized using nuclear magnetic resonance (NMR) spectroscopy on a Bruker Avance 500 spectrometer (Bruker, MA, USA). The detailed NMR data and structural analysis can be found in our previous report [20]. The purity of MTN was determined to be 99.96% through high-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis using an Agilent 1260 Infinity II preparative LC system (Agilent Technologies, Santa Clara, CA, USA). The analysis followed the peak area method described in our previous work [20]. This purity assessment was conducted prior to the bioactivity studies, ensuring a comprehensive comparison of MTN with other components in the sample.

MTN was initially dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution. MTN solutions of various concentrations were then prepared and diluted in cell culture media for each experimental group. The final DMSO concentration in the MTN solutions was less than 0.5%, ensuring that there were no harmful effects from the solvent on the cells being evaluated. The chemical structure of MTN was retrieved from the PubChem database (<https://www.ncbi.nlm.nih.gov/pccompound>) and entered into the pkCSM web server (<https://biosig.lab.uq.edu.au/pkcsml/>) in simplified molecular-input line-entry system (SMILES) notation to identify its associated pharmacokinetic parameters [21].

### 2.3. Cell culture

Normal bronchial epithelial BEAS-2B cells and human lung cancer cell lines A549, H23 and H460 were procured from the American Type Culture Collection (Manassas, VA, USA). A549-luciferase cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). H23 and H460 cells were cultured in RPMI-1640 medium, while A549, A549-luciferase and BEAS-2B cells were grown in DMEM with 2 mmol/L L-glutamine, 100 U/mL penicillin/streptomycin, and 10% FBS. The cells were cultured at 37 °C in a humidified environment including 5% CO<sub>2</sub>.

### 2.4. Cytotoxicity assay

A549, H460, H23 and BEAS-2B cells were seeded into a 96-well plate at a density of  $8.0 \times 10^3$  cells per well and allowed to adhere and grow overnight in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. The MTT assay was performed with slight modification [22,23]. After overnight incubation for cell attachment, the cells were treated with MTN (0–100  $\mu$ mol/L) for 24 h, which is a duration equal to or shorter than the doubling time of these cell lines. The cells were then incubated with 5 mg/mL MTT and the plates were incubated for 4 h at 37 °C to allow for MTT reduction. The medium containing MTT was then carefully removed, and 100  $\mu$ L of DMSO was added to each well to dissolve the formazan crystals, followed by gentle shaking to ensure complete dissolution. Absorbance was measured at 570 nm using a CLARIOStar microplate reader (BMG Labtech, Hesse, Germany). The percentage of cell viability relative to untreated control wells was calculated and plotted against the MTN concentrations to determine the cytotoxic effects.

## 2.5. Apoptosis assay

A549, H460 and H23 cells were seeded into 6-well plates at a density of  $2.4 \times 10^5$  cells per well and incubated overnight at 37 °C with 5% CO<sub>2</sub> to allow attachment. After 24 h, the cells were treated with the MTN at the experimental concentrations for 24 h. Following treatment, cells were harvested, washed with phosphate-buffered saline (PBS), and resuspended in 500 µL of annexin V-binding buffer. To each sample, 5 µL annexin V-fluorescein isothiocyanate (FITC) and 5 µL propidium iodide (PI) were added, followed by gentle vortexing and incubation for 15 min in the dark at room temperature. The samples were then analyzed using an EPICS-XL flow cytometer (Beckman Coulter, Indianapolis, IN, USA). Data were analyzed to distinguish between viable cells, early apoptotic cells, late apoptotic and necrotic cells.

## 2.6. Cell proliferation assay

A549, H23 and H460 cells were seeded into 96-well microplates at a low density of  $2.0 \times 10^3$  cells per well to ensure adequate space for proliferation during the treatment period. The following day, the cells were treated with MTN at non-cytotoxic concentrations ranging from 0 to 12.5 µmol/L for 24, 48 or 72 h. After that, 5 mg/mL MTT solution was added to each well, and the cells were incubated for an additional 4 h at 37 °C. Formazan crystals formed during this period were dissolved with 100 µL DMSO, and absorbance was measured at 570 nm using a CLARIOstar microplate reader (BMG Labtech, Hesse, Germany). The values of optical density were used to calculate as a relative cell proliferation compared to the control group.

## 2.7. Transwell migration assay

The Transwell migration assay was performed to assess the impact of MTN on the migration of A549, H23 and H460 cells. Briefly, cells at a density of  $1.5 \times 10^5$  cells per well were seeded into the upper chambers of Transwell inserts placed in 24-well plates containing MTN (0–12.5 µmol/L) and allowed to adhere, while the lower chamber contained 10% FBS medium to serve as a chemoattractant. The assemblies were incubated for 24 h. Following incubation, the cells that had migrated to the lower surface of the membrane were fixed with methanol, and subsequently stained with DAPI to enhance visualization. At least five random fields of migrated cells were then imaged and quantified using an ECLIPSE Ts2 fluorescence microscope (Nikon Instruments, New York, NY, USA). The quantity of migrating cells is expressed as relative values compared to the control group.

## 2.8. Tail vein metastasis

The in vivo study was approved by and conducted following the Institutional Animal Care and Use Committee of Chulalongkorn University (protocol No. 2373049). Five-week-old female BALB/c nude mice were obtained from Nomura Siam International (Bangkok, Thailand). These mice were maintained in a specialized pathogen-free environment and were randomly divided into groups of five. During the experiment, a total of  $5.0 \times 10^6$  A549-luciferase cells, pretreated with or without 6.25 µmol/L MTN for 24 h, were resuspended in 200 µL PBS and intravenously injected into the tail vein. After 3 days, the mice were euthanized, and the IVIS® Spectrum In Vivo Imaging System (PerkinElmer, Waltham, MA, USA) was used to visualize the presence of lung metastasis. The tumor nodules were quantified based on luminescence intensity per lung. The lung tissues were then subjected to histological analysis using hematoxylin and eosin staining. Lung tissue sections were imaged using an ECLIPSE Ti-U inverted microscope (Nikon

Instruments). The tumor areas were quantified by ImageJ software (version 1.54 g, National Institutes of Health, Bethesda, MD, USA).

## 2.9. Target identification of MTN and NSCLC-related genes

The structural formula of MTN was retrieved from the PubChem database, and the SMILES notation for MTN was then submitted to the SwissTargetPrediction database (<https://swisstargetprediction.ch/>) to identify associated target genes [24]. The target genes that are related to NSCLC were obtained from multiple databases, including the DisGeNET (<https://disgenet.com/>) [25], GeneCards (<https://www.genecards.org/>) [26], Online Mendelian Inheritance in Man (<https://www.omim.org/>) [27], and Therapeutic Target Database (<https://ttdd.idrblab.cn/>) [28], with a focus on *Homo sapiens*. The Venny 2.1 program (<https://bioinfo.gp.cnb.csic.es/tools/venny/>) was used to identify common targets of MTN and NSCLC. These overlapping targets were identified as the predicted targets for MTN in NSCLC therapy.

## 2.10. Construction of interaction network between potential targets

To investigate the interaction between potential targets, the common targets of MTN and NSCLC were imported into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (<https://string-db.org/>) with a confidence score threshold of  $\geq 0.4$ , specifying *Homo sapiens* as the selected species [29]. The Cytoscape software (v3.10.2, Cytoscape Consortium, San Diego, CA, USA) was used to visualize the protein interaction network [30]. Hub targets were identified using the degree-based algorithm in the cytoHubba plugin [31].

## 2.11. Gene expression and survival analysis

*ADRB2* mRNA expression and clinical data from individuals diagnosed with lung cancer were retrieved from The Cancer Genome Atlas (TCGA, <https://www.cancer.gov/tcga>) and Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) [32,33]. In the TCGA database, RNA-seq data were categorized into tumor lung samples ( $n = 994$ ) and normal lung samples ( $n = 110$ ). The GEO analysis included three datasets: GSE18842 (45 normal and 46 tumor samples), GSE19804 (60 normal and 60 tumor samples), and GSE19188 (65 normal and 91 tumor samples). To investigate the association of survival with *ADRB2* expression in lung adenocarcinoma patients, the Kaplan–Meier plotter tool (<https://km-plot.com/analysis/>) was used. Hazard ratios with 95% confidence intervals and log-rank *P* values were computed, and an auto-selection process was performed to classify patients.

## 2.12. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses

To investigate the core mechanism and pathways of MTN as an anti-NSCLC agent, Gene Ontology (GO, <https://www.geneontology.org/>) functions including biological processes, cellular components, and molecular functions, and Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.genome.jp/kegg/>) pathway enrichment analyses were conducted. Both GO and KEGG analyses were performed using the STRING database, with the species limited to *Homo sapiens*. A bubble plot was generated using a ggplot2 script in RStudio v1.4.1717 (Posit Software, Boston, MA, USA) to display all enrichment data.

## 2.13. Quantitative reverse transcription-polymerase chain reaction

A549 and H23 cells were treated with MTN (0–12.5 µmol/L) for 24 h, and RNA was extracted using GENEzol reagent (Geneaid

Biotech, Taipei, Taiwan, China) according to the manufacturer's protocol. The extracted RNA was subsequently reversely transcribed into cDNA utilizing ProtoScript® II reverse transcriptase (New England BioLabs, Ipswich, MA, USA). The synthesized cDNA was subjected to quantitative reverse transcription-polymerase chain reaction amplification using the SensiFAST™ SYBR® No-ROX kit (Bioline Reagents, Taunton, MA, USA) in combination with specific primers (Table 1). Amplification was performed on a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA) under the following cycling conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing at primer-specific temperatures indicated in Table 1 for 10 s. The expression levels of target mRNAs were quantified using the  $2^{-\Delta\Delta C_t}$  method.

#### 2.14. Immunoblotting analysis

A549 and H23 cells at a density of  $3.0 \times 10^5$  cells per well were seeded into a 6-well plate. The cells were treated with MTN (0–12.5 µmol/L) for 24 h on the next day. After that, lysis buffer was added to each well, and cell lysates were collected as previously described [34]. The protein concentration was then evaluated using the bicinchoninic acid assay kit (Visual Protein, Taipei, Taiwan, China). Samples containing 60 µg of protein were denatured at a temperature of 95 °C for 5 min, separated using 10% sodium dodecyl sulfate–polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. Non-specific bindings were blocked with 5% skim milk in Tris-buffered saline with Tween™ 20 (TBST), and the membranes were incubated with primary antibodies overnight at 4 °C. On the following day, membranes were washed three times with TBST and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. The antibodies used in this study are listed in Table 2. Protein bands were detected using an Immobilon® Western chemiluminescent HRP substrate (Sigma–Aldrich, Darmstadt, Germany), and the visualized protein expression levels were quantified using ImageJ software (version 1.54 g).

#### 2.15. Statistical analysis

Statistical analyses were conducted using Prism 8 (GraphPad Software, San Diego, CA, USA). The results are shown as the mean ± standard error of the mean. The statistical differences between the two groups were assessed using Student's *t*-test, while those of multiple groups were evaluated using a one-way analysis of variance followed by Tukey's multiple comparison test with  $P < 0.05$ .

**Table 1**

List of primer sequences for quantitative reverse transcription-polymerase chain reaction analysis.

| Gene       | Primer sequence (5'–3')          | $T_m$ (°C) | Size (bp) |
|------------|----------------------------------|------------|-----------|
| E-cadherin | F: 5'-TTAAACTCCTGGCTCAAGCAATC-3' | 60.3       | 139       |
|            | R: 5'-TCCTATCTTGGGCAAGCAACTG-3'  |            |           |
| N-cadherin | F: 5'-GACCGAGAATCACCAATGTG-3'    | 57.1       | 94        |
|            | R: 5'-GCGTTCCTGTCCACTCATA-3'     |            |           |
| VIM        | F: 5'-ACCTGCAATCTTTCAGACA-3'     | 51.2       | 74        |
|            | R: 5'-GATTCCACTTTCGCTTCAAGG-3'   |            |           |
| Slug       | F: 5'-AGCATTCAACGCCTCCA-3'       | 56.7       | 117       |
|            | R: 5'-GGATCTCTGGTTGGTATGAC-3'    |            |           |
| Gapdh      | F: 5'-ACATCGCTCAGACACCAT-3'      | 61         | 143       |
|            | R: 5'-TGATGTTGAGGTCAATGAAGGG-3'  |            |           |

bp: base pairs; E-cadherin: epithelial cadherin; F: forward primer; Gapdh: glyceraldehyde-3-phosphate dehydrogenase; N-cadherin: neural cadherin; R: reverse primer; Slug: Snail family transcriptional repressor 2;  $T_m$ : melting temperature; VIM: vimentin.

**Table 2**

Antibodies used in the immunoblotting analysis.

| Antibody                                   | Source                                       | Identifier | Dilution |
|--------------------------------------------|----------------------------------------------|------------|----------|
| Anti-ADRB2 antibody                        | Abcam (Cambridge, UK)                        | ab182136   | 1:1000   |
| Anti-E-cadherin rabbit monoclonal antibody | Cell Signaling Technology (Danvers, MA, USA) | #3195      | 1:1000   |
| Anti-GAPDH mouse monoclonal antibody       | Cell Signaling Technology                    | #97166     | 1:2000   |
| Anti-N-cadherin mouse monoclonal antibody  | Cell Signaling Technology                    | #14215     | 1:1000   |
| Anti-Slug rabbit monoclonal antibody       | Cell Signaling Technology                    | #9585      | 1:1000   |
| Anti-VIM rabbit monoclonal antibody        | Cell Signaling Technology                    | #5741      | 1:1000   |
| Anti-rabbit IgG HRP-linked antibody        | Cell Signaling Technology                    | #7074      | 1:1000   |
| Anti-mouse IgG HRP-linked antibody         | Cell Signaling Technology                    | #7076      | 1:2000   |

ADRB2: adrenoceptor  $\beta_2$ ; E-cadherin: epithelial cadherin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HRP: horseradish peroxidase; IgG: immunoglobulin G; N-cadherin: neural cadherin; Slug: Snail family transcriptional repressor 2; VIM: vimentin.

### 3. Results

#### 3.1. MTN suppresses lung cancer cell migration

Based on the pharmacokinetic properties predicted using the pkCSM platform, MTN demonstrated the highest possible bioavailability and moderate blood brain barrier penetration. MTN performed well in most tests, suggesting that it has the potential to be considered as a safe candidate in humans (Table S1). NSCLC cell lines A549, H23 and H460 were incubated with varying doses of MTN (Fig. 1A) for 24 h, and the viability of the cells was evaluated. MTN significantly inhibited the survival of NSCLC cells in a dose-dependent manner (Fig. 1B). The half-maximal inhibitory concentration ( $IC_{50}$ ) values of MTN for A549, H23 and H460 cells were shown in Fig. 1B. The cytotoxicity of MTN was also evaluated in BEAS-2B cells, revealing that MTN had low cytotoxic effects on these normal cells, with an  $IC_{50}$  value of  $(72.5 \pm 5.1)$  µmol/L. The selective index was calculated to be between 2.5 and 4, indicating a higher specificity of MTN toward cancer cells compared to normal cells (Fig. S1).

Further, the annexin V-FITC/PI staining assay demonstrated that MTN induced cell death through apoptosis (Fig. 1C). The number of early (annexin-V<sup>+</sup>/PI<sup>-</sup>) and late (annexin-V<sup>+</sup>/PI<sup>+</sup>) apoptotic cells substantially increased in response to MTN treatment in all tested cells. The effect of MTN on cell growth was also investigated, revealing that nontoxic doses of MTN did not exhibit any antiproliferative effects on any of the tested cell lines after 72 h, except at a high dose (12.5 µmol/L), which had negative effects on the proliferation of H460 and H23 cells (Fig. 1D).

Additionally, the impact of MTN on cell migration was examined using a Transwell migration assay. MTN substantially decreased the number of migrating cells in a dose-dependent manner (Fig. 1E). Moreover, a wound healing assay demonstrated that MTN consistently inhibited the migration of NSCLC cells (Fig. S2). These findings suggest that MTN is a potent inhibitor of the migratory behavior of lung cancer cells.

#### 3.2. MTN attenuates lung cancer cell metastasis in vivo

To substantiate the significant impact of MTN on lung cancer metastasis, luciferase-overexpressing A549 cells were pretreated with MTN (6.25 µmol/L) for 24 h, and subjected to an in vivo tail vein metastasis. The amount of lung colonization was assessed using bioluminescence imaging (Fig. 2A). There was a significant

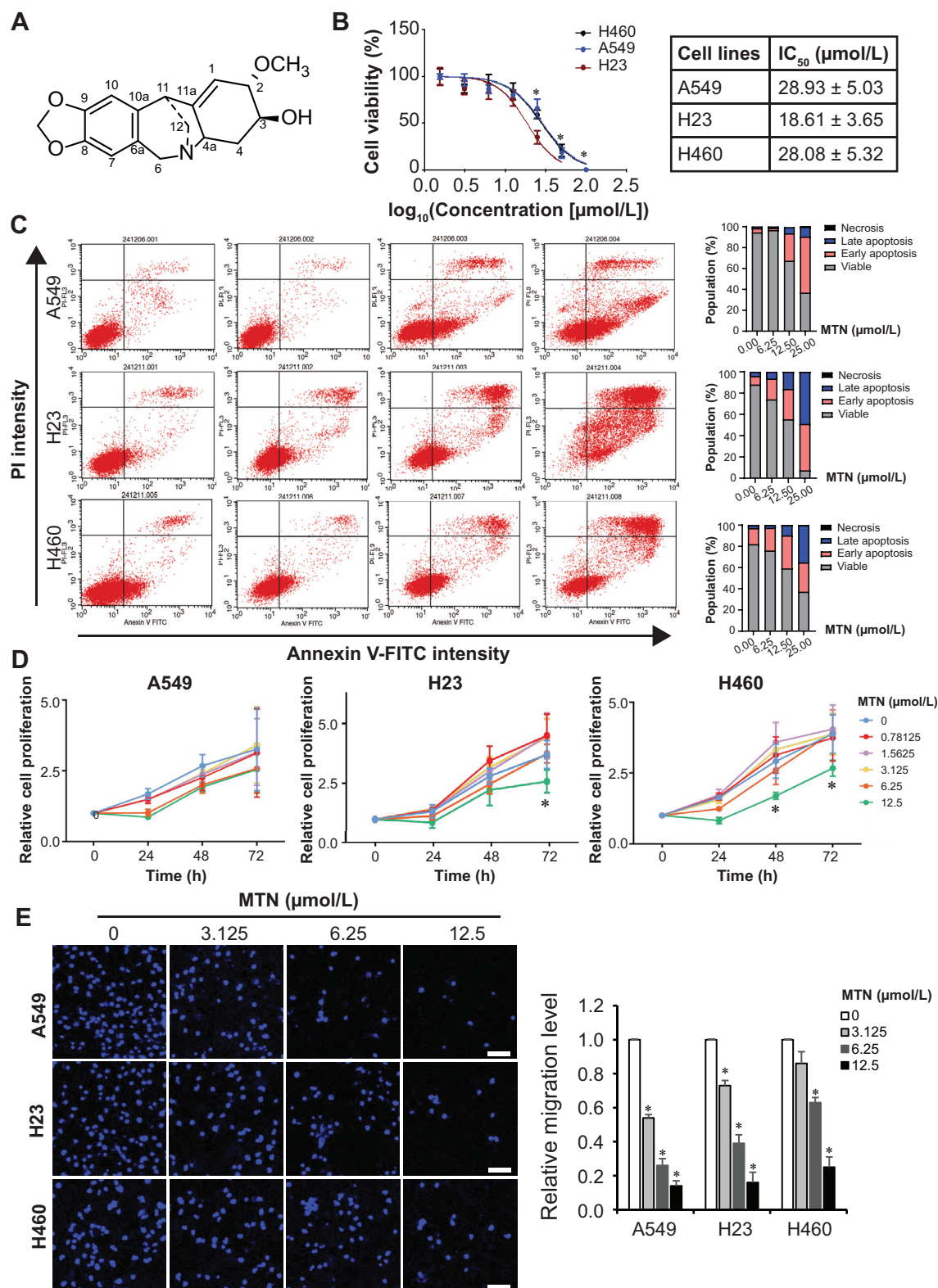


**Fig. 1.** Cytotoxicity and antimigration effects of MTN in non-small cell lung cancer. **A:** Chemical structure of MTN. **B:** Cytotoxicity was assessed using the MTT assay and is expressed as a percentage. A549, H23 and H460 cells were treated with MTN (0–100 μmol/L) for 24 h. **C:** Cell apoptosis was evaluated by annexin-V/PI staining. The populations of early apoptotic cells (annexin-V<sup>+</sup>/PI<sup>-</sup>, orange), late apoptotic cells (annexin-V<sup>+</sup>/PI<sup>+</sup>, blue), necrotic cells (annexin-V<sup>-</sup>/PI<sup>+</sup>, black) and viable cells (annexin-V<sup>-</sup>/PI<sup>-</sup>, grey) are presented as percentages of the total cell number. **D:** Cell proliferation was measured using the MTT assay, and the growth rate was calculated relative to the initial time point (0 h). A549, H23, and H460 cells were treated with MTN (0–12.5 μmol/L) for 24–72 h. **E:** Migrated cells were stained with DAPI and visualized using fluorescence microscopy (scale bar = 10 μm, magnification 40×). A549, H23, and H460 cells were treated with MTN (0–12.5 μmol/L) for 24 h. Data are presented as mean ± standard error of mean ( $n = 3$ ). \* $P < 0.05$ , vs control group. DAPI: 4',6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate; IC<sub>50</sub>: half-maximal inhibitory concentration; MTN: montanine; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI: propidium iodide.

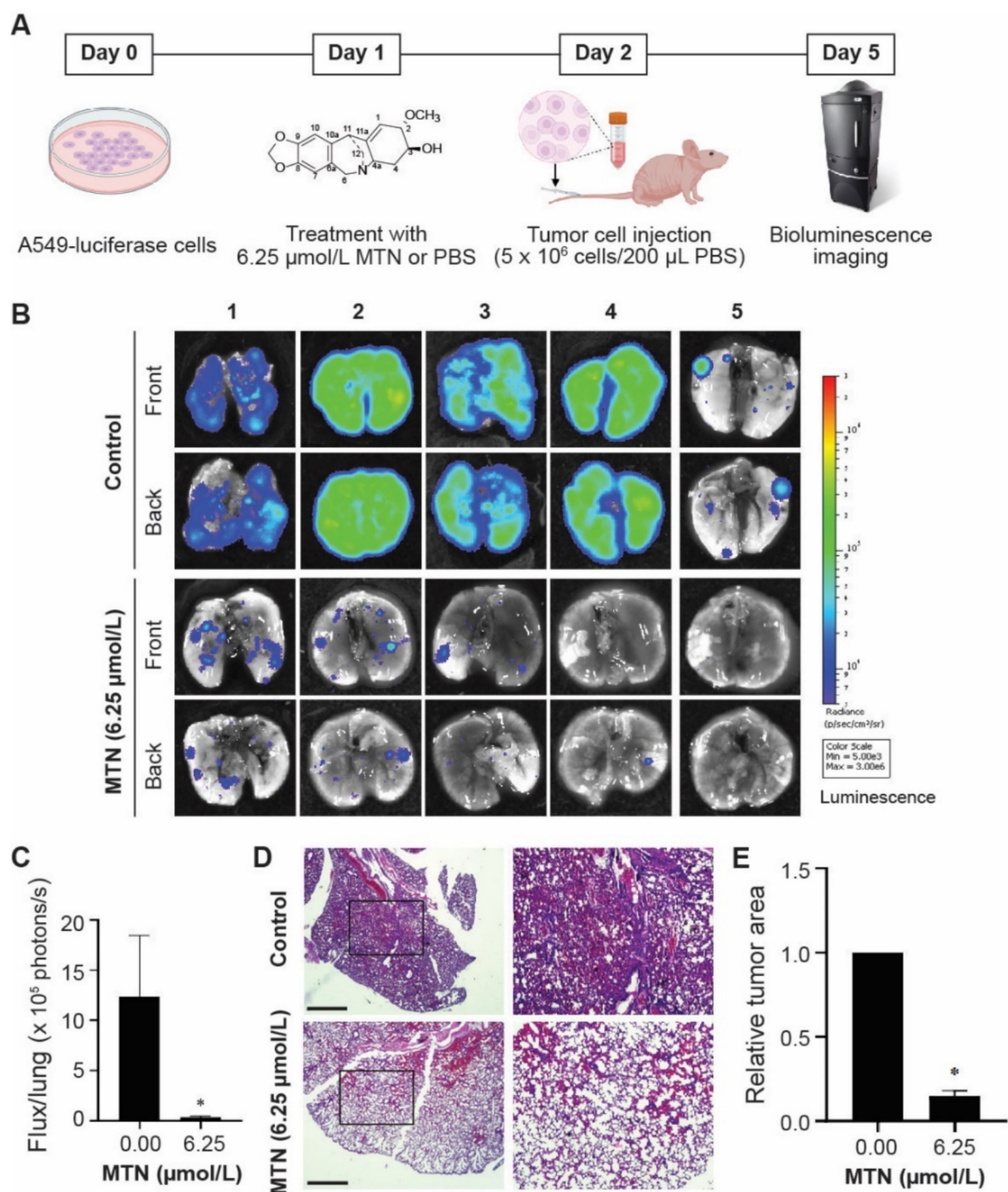


**Fig. 2.** MTN significantly reduces lung cancer metastasis in vivo. **A:** Experimental workflow for assessing lung cancer metastasis in vivo. A549 cells expressing luciferase were pretreated with 6.25  $\mu\text{mol/L}$  MTN for 24 h before being injected into the tail veins of mice. **B:** Metastatic cancer cells within the lung tissues were visualized using the in vivo imaging system. **C:** Quantitative analysis of bioluminescence intensity in both MTN-treated and control groups. **D:** Representative lung sections from each group were stained with hematoxylin and eosin (scale bar = 200  $\mu\text{m}$ , magnification  $10\times$ ). **E:** The tumor area was quantified and expressed as a relative value compared to the non-treated group. Data are shown as mean  $\pm$  standard error of mean ( $n = 5$ ). \* $P < 0.05$ , vs control group. MTN: montanine; PBS: phosphate-buffered saline.

decrease in the number of lung metastatic foci after MTN administration (Fig. 2B). Additional quantitative analysis verified a notable reduction in the lung cancer metastasis in the group treated with MTN, as compared to those of the control group (Fig. 2C), supporting the potent antimetastatic effects of MTN in lung cancer cells. Histological analysis revealed the disruption of the normal alveolar architecture and increased cellular density in the lung tissue of the control group, and these characteristics were significantly reduced

in the MTN-treated group (Fig. 2D and E). Collectively, our results suggest that MTN exhibits a potent antimetastatic activity on NSCLC.

### 3.3. Identification of 29 common targets of MTN and NSCLC

A network pharmacology approach was used to explore the molecular mechanism underlying the action of MTN. Fig. 3 outlines

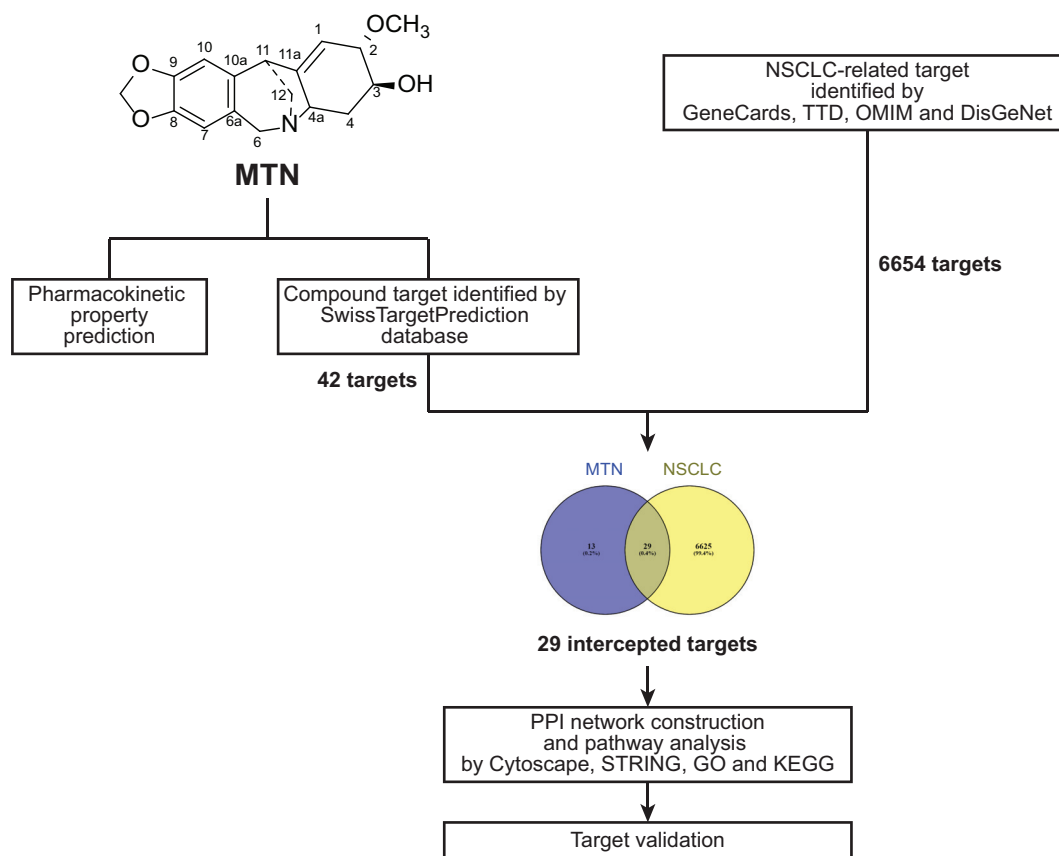


**Fig. 3.** A graphical framework illustrating the network pharmacology approaches used to predict targets of MTN against NSCLC. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MTN: montanine; NSCLC: non-small cell lung cancer; OMIM: Online Mendelian Inheritance in Man; PPI: protein-protein interaction; STRING: Search Tool for the Retrieval of Interacting Genes/Proteins; TTD: Therapeutic Target Database.

the methodology used to predict and analyze MTN-related targets associated with NSCLC. Initially, 42 potential targets linked to MTN were identified (Table S2). Moreover, a comprehensive investigation across multiple databases yielded 6654 targets relevant to NSCLC. A Venn diagram analysis revealed 29 common targets that could serve as targets for MTN treatment in NSCLC (Fig. 4A). Subsequently, by utilizing the STRING database, data pertaining to these 29 common targets were incorporated to construct a PPI network to examine the interactions among these targets. Further refinement of the PPI network was achieved using Cytoscape v3.10.2, with a focus on the top 10 targets based on degree scores (Fig. 4B and Table S3). A detailed overview of the PPI network is provided in Table S3, where the targets are visually represented in a color gradient ranging from red to yellow, corresponding to their degree scores.

#### 3.4. Enrichment analyses of GO and KEGG

In order to gain a deeper understanding of the biological functions of potential targets, we performed GO and KEGG enrichment studies. The top ten terms that had the highest level of enrichment in the categories of biological process, molecular function, and cellular component are displayed (Figs. S3A–C). The enrichment analysis revealed that the identified targets are mostly linked to a range of biological processes, encompassing response to oxygen-containing compounds, cholinergic receptor signaling pathways, G-protein coupled acetylcholine receptor signaling pathways involving adenylate cyclase inhibition, and trans synaptic signaling pathways. Regarding molecular functions, the targets exhibited functions primarily related to receptor activity, particularly trans-

membrane signaling, neurotransmitter activity, G-protein coupling, and adrenoceptor  $\beta$  activity. In terms of cellular components, the targets were predominantly associated with the plasma membrane, integral components of the plasma membrane, and cell junctions.

To explore the underlying mechanisms, KEGG pathway analysis was conducted (Fig. S3D and Table S4). We found that MTN impacts multiple regulatory pathways to inhibit NSCLC progression. Key targets are notably linked to neuroactive ligand receptor interactions, phosphatidylinositol-4,5-bisphosphate 3 kinase (PI3K)/AKT serine/threonine kinase 1 (AKT1) signaling pathway, cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) signaling pathway, adenosine 3',5'-monophosphate (cAMP) signaling pathway, and regulation of the actin cytoskeleton.

#### 3.5. Downregulation of ADRB2 was associated with poor prognosis in NSCLC

Based on its degree of connectivity, ADRB2 has emerged as a primary target of MTN in NSCLC. Analysis of data from the TCGA and GEO databases indicated that there was a notable decrease in the expression of ADRB2 in lung cancer tissues compared to normal ones (Fig. 4C). Subsequently, ADRB2 mRNA expression in NSCLC cells (i.e., A549, H23 and H460) and normal human bronchial epithelial cells (i.e., BEAS-2B) was investigated. Consistent with the bioinformatics analysis, ADRB2 mRNA level was markedly lower in NSCLC cells than in normal ones (Fig. 4D). ADRB2 protein expression was lower in both the membrane and cytoplasmic fractions of NSCLC cells compared to normal ones (Fig. S4). Notably, ADRB2 was not detected in the nuclear fraction of either normal or cancer cells. Survival

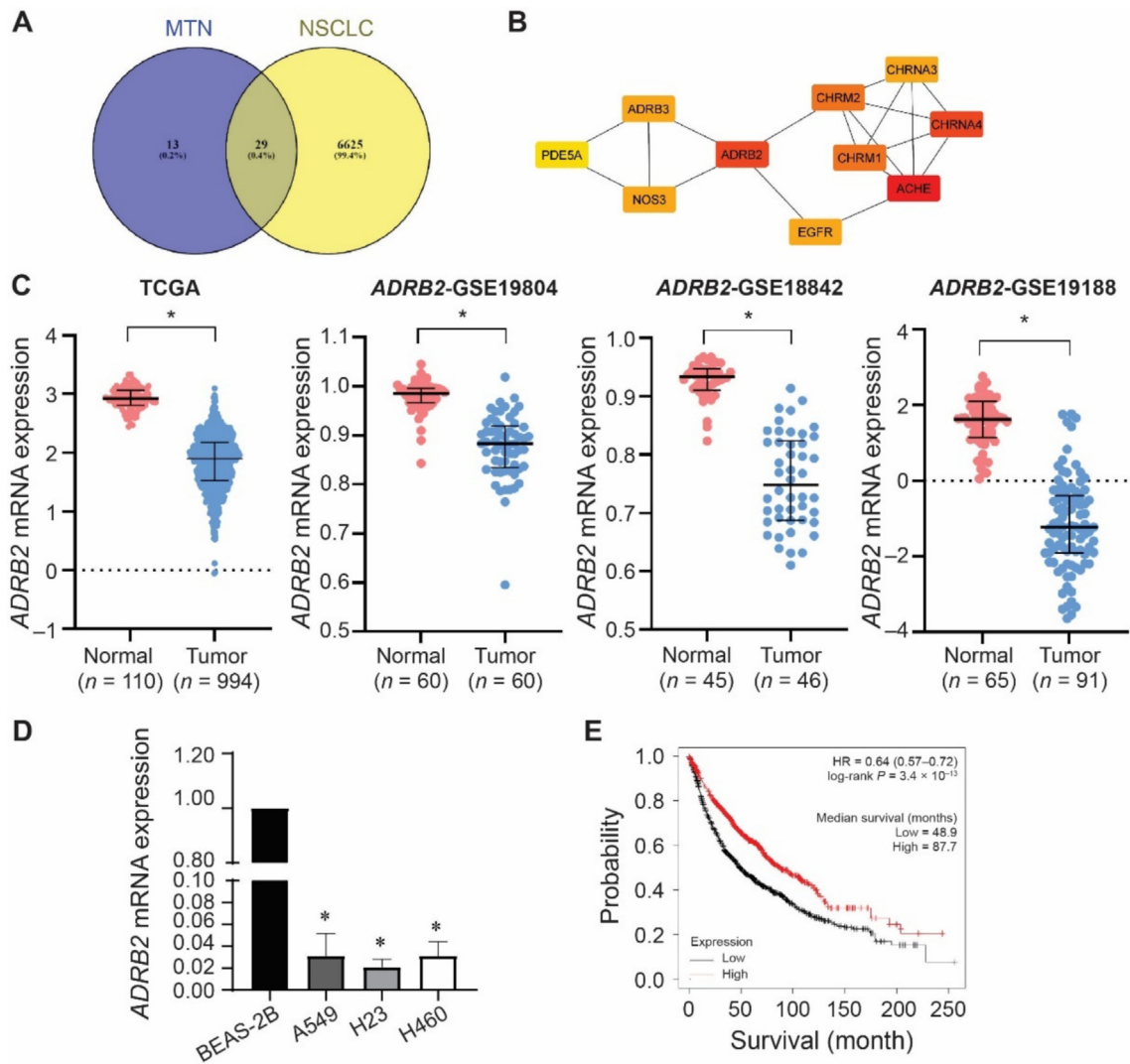


**Fig. 4.** Common targets of MTN and NSCLC and differential *ADRB2* mRNA expression. **A:** Venn diagram illustrates the overlapping targets of MTN and NSCLC. **B:** Top 10 nodes in the PPI network ranked by the degree scores assigned by cytoHubba are displayed. Nodes with higher degree values are shown with redder coloration. **C:** *ADRB2* mRNA expression in both normal lung and lung tumor tissues were sourced from the TCGA and GEO databases. \* $P < 0.05$ , vs control group. **D:** *ADRB2* mRNA expression in NSCLC cells (A549, H23 and H460) and normal bronchial epithelial cells (BEAS-2B) were analyzed by quantitative reverse transcriptase polymerase chain reaction. **E:** Kaplan–Meier analysis comparing lung cancer patients with high and low *ADRB2* expression. Data are shown as mean  $\pm$  standard error of mean ( $n = 3$ ). \* $P < 0.05$ , vs BEAS-2B cells. ACH3: acetylcholinesterase; ADRB2: adrenoceptor  $\beta 2$ ; CHRMA3: cholinergic receptor nicotinic  $\alpha 3$  subunit; CHRMB: cholinergic receptor muscarinic 1; EGFR: epidermal growth factor receptor; GEO: Gene Expression Omnibus; GSE: GEO Series Experiment; HR: hazard ratio; MTN: montanine; NOS3: nitric oxide synthase 3; NSCLC: non-small cell lung cancer; PDE5A: phosphodiesterase 5A; PPI: protein–protein interaction; TCGA: The Cancer Genome Atlas.

analyses further underscored the clinical significance of *ADRB2* expression, indicating that lower level of *ADRB2* was predictive of a poorer prognosis (Fig. 4E). In conclusion, our findings demonstrated the significant downregulation of *ADRB2* in lung cancer and its association with a poor patient prognosis, suggesting that *ADRB2* could be a promising target for lung cancer therapy.

### 3.6. MTN upregulates *ADRB2* expression and decreases EMT marker expression

Given that *ADRB2* is a potential target in lung cancer, the effect of MTN on *ADRB2* levels was examined. Western blotting analysis demonstrated a significant increase in *ADRB2* protein levels in both A549 and H23 cells following MTN treatment (Fig. 5A). Moreover, MTN treatment resulted in a gradual reduction in the mesenchymal markers neural cadherin (N-cadherin), vimentin (VIM), and Snail family transcriptional repressor 2 (Slug), accompanied by an upregulation of the epithelial marker epithelial cadherin (E-

cadherin), in both cell lines. Consistently, MTN treatment also led to decreased mRNA levels of *N-cadherin*, *VIM* and *Slug* and increased levels of *E-cadherin* (Fig. 5B–E). These findings suggest that MTN may exert its antimetastatic effects by modulating *ADRB2* expression and EMT, thereby reducing the migratory capability of NSCLC cells.

To determine if *ADRB2* activation is involved in the inhibitory effects of MTN on cell migration, a selective *ADRB2* agonist, TER, was used in the in vivo studies. The results demonstrated that TER significantly enhanced the inhibitory effect of MTN on cell migration (Fig. 6A). Furthermore, to test the hypothesis that *ADRB2* activation leads to attenuation of the EMT process, the mRNA and protein levels of EMT markers were examined. The findings demonstrated that the increase in *E-cadherin* and decrease in *N-cadherin*, *Slug* and *VIM* in response to MTN were significantly amplified in the presence of TER (Fig. 6B and C). These findings suggest that MTN suppresses cell migration through a mechanism mediated by *ADRB2* and EMT.

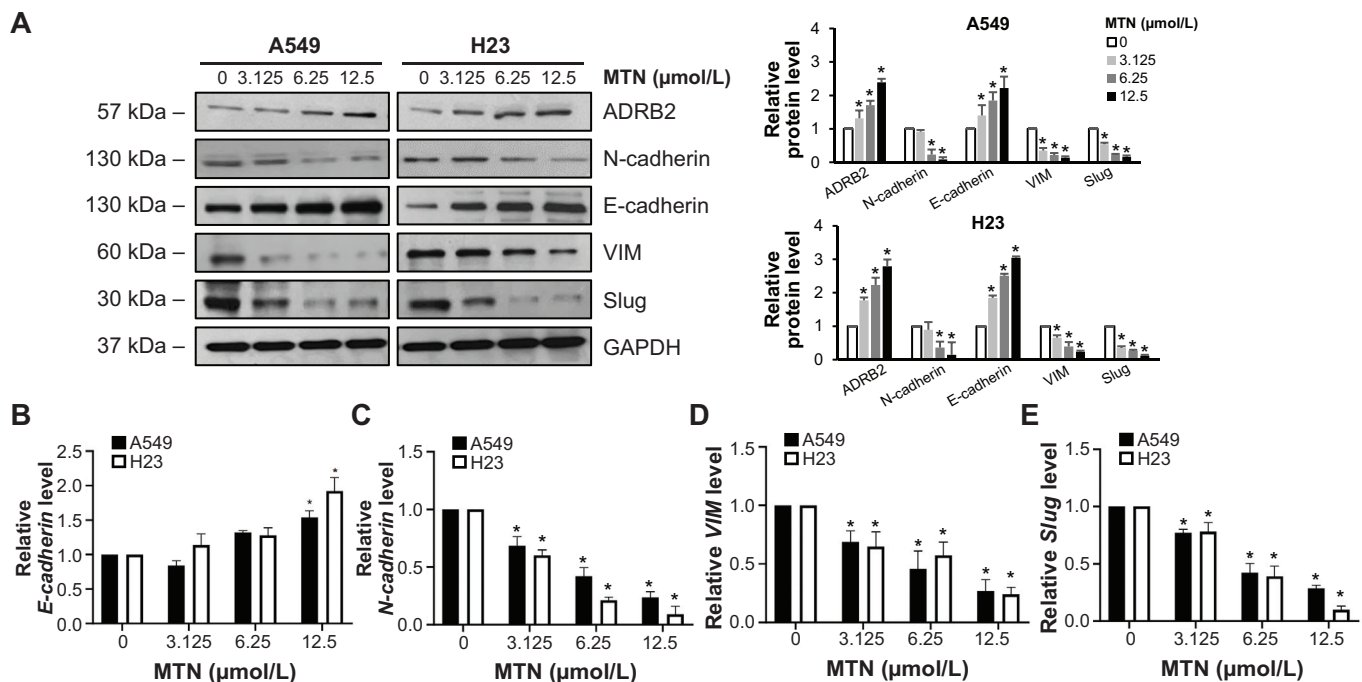


**Fig. 5.** MTN upregulated ADRB2 protein expression and modulated EMT signaling. A549 and H23 cells were treated with MTN (0–12.5 μmol/L) for 24 h. **A:** Protein expression levels of ADRB2, E-cadherin, N-cadherin, VIM, and Slug were analyzed by immunoblotting analysis. **B–E:** mRNA expression levels of EMT markers were measured using quantitative reverse transcriptase polymerase chain reaction. Data are shown as mean ± standard error of mean ( $n = 3$ ). \* $P < 0.05$ , vs control group. ADRB2: adrenoceptor  $\beta 2$ ; E-cadherin: epithelial cadherin; EMT: epithelial–mesenchymal transition; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MTN: montanine; N-cadherin: neural cadherin; Slug: Snail family transcriptional repressor 2; VIM: vimentin.

#### 4. Discussion

As the most frequent type of cancer and a leading cause of cancer-mortality, NSCLC presents significant treatment challenges due to its aggressive nature and high rates of metastasis [35]. Current treatments, including surgery, chemotherapy, radiation therapy, immunotherapy and targeted therapy still have substantial limitations such as drug resistance, severe side effects and poor efficacy, particularly in advanced stages [36,37]. With their diverse chemical structures and unique biological functions, natural compounds are promising sources for drug discovery. MTN, an alkaloid from the Amaryllidaceae family, is one such compound that has significant anticancer effects [16,38]. Our findings are the first to report the potent antimetastasis effect of MTN both in vitro and in vivo. The underlying mechanism involves the upregulation of ADRB2 and subsequent modulation of the EMT process.

By using a network pharmacology approach, we explored the multitarget effects of MTN, focusing on ADRB2 and its ability to inhibit EMT signaling pathways in vitro. This approach is particularly valuable in the context of complex diseases, including cancer, where multiple pathways and molecular targets are involved in disease progression and response to treatment [18]. Network pharmacology enables the mapping of complex interactions within biological systems, revealing how MTN modulates multiple pathways crucial for cancer cell survival, proliferation and metastasis. This multitarget mechanism enhances the therapeutic efficacy of MTN while reducing the likelihood of drug resistance, a significant issue associated with single-target therapies.

EMT is a biological mechanism in which epithelial cells undergo a transformation where they lose their cell polarity and adhesion properties. Instead, they gain mesenchymal traits that increase their ability to migrate and invade surrounding tissues [5]. This shift plays a crucial role in the advancement of cancer, as it allows tumor cells to separate from the main tumor site, infiltrate nearby

tissues, and spread to distant organs, resulting in the formation of metastatic colonies [4]. EMT is characterized by a downregulation of epithelial markers such as E-cadherin and an upregulation of mesenchymal markers such as N-cadherin and VIM. These alterations promote the development of a mobile cancer cell phenotype [39]. In vitro studies showed that MTN modulates key EMT signaling pathways. Additionally, MTN demonstrated significant efficacy in inhibiting lung cancer metastasis in vivo. Animal studies showed that pretreatment with MTN resulted in reduced migratory capabilities of cancer cells, indicating a less aggressive cancer phenotype. Consequently, there was a significant decrease in the quantity of cancerous growths in the lungs, indicating that MTN has a strong ability to suppress cancer metastasis. This is particularly relevant for NSCLC, where metastasis to distant organs is a significant contributor to poor prognosis and mortality [35]. By targeting and inhibiting the EMT process, MTN disrupts the metastatic cascade, thereby reducing the number of migratory cancer cells and improving overall treatment outcomes.

ADRB2 is known as a type of G protein-coupled receptor that plays a role in multiple physiological processes, including the regulation of cardiovascular and respiratory functions [40,41]. Recent studies have highlighted the significance of ADRB2 in cancer biology, particularly its association with EMT and cancer metastasis [9,10]. Interestingly, ADRB2 signaling has been implicated in either promoting or inhibiting EMT and cancer motility [9,10]. For example, ADRB2 activation has been reported to enhance EMT and metastasis potential in head and neck cancer and tongue squamous cell carcinoma [42,43], while it also mediates proto-oncogene tyrosine-protein kinase Src signaling to induce cell motility in hepatocarcinoma and breast cancer cells [44]. Conversely, ADRB2 activation suppresses EMT in oral and breast cancers [10,45]. This dual role may be due to the balance of downstream pathways: cAMP/PKA signaling has an antimigratory role by modulating rhodopsin activity [46,47], whereas extracellu-

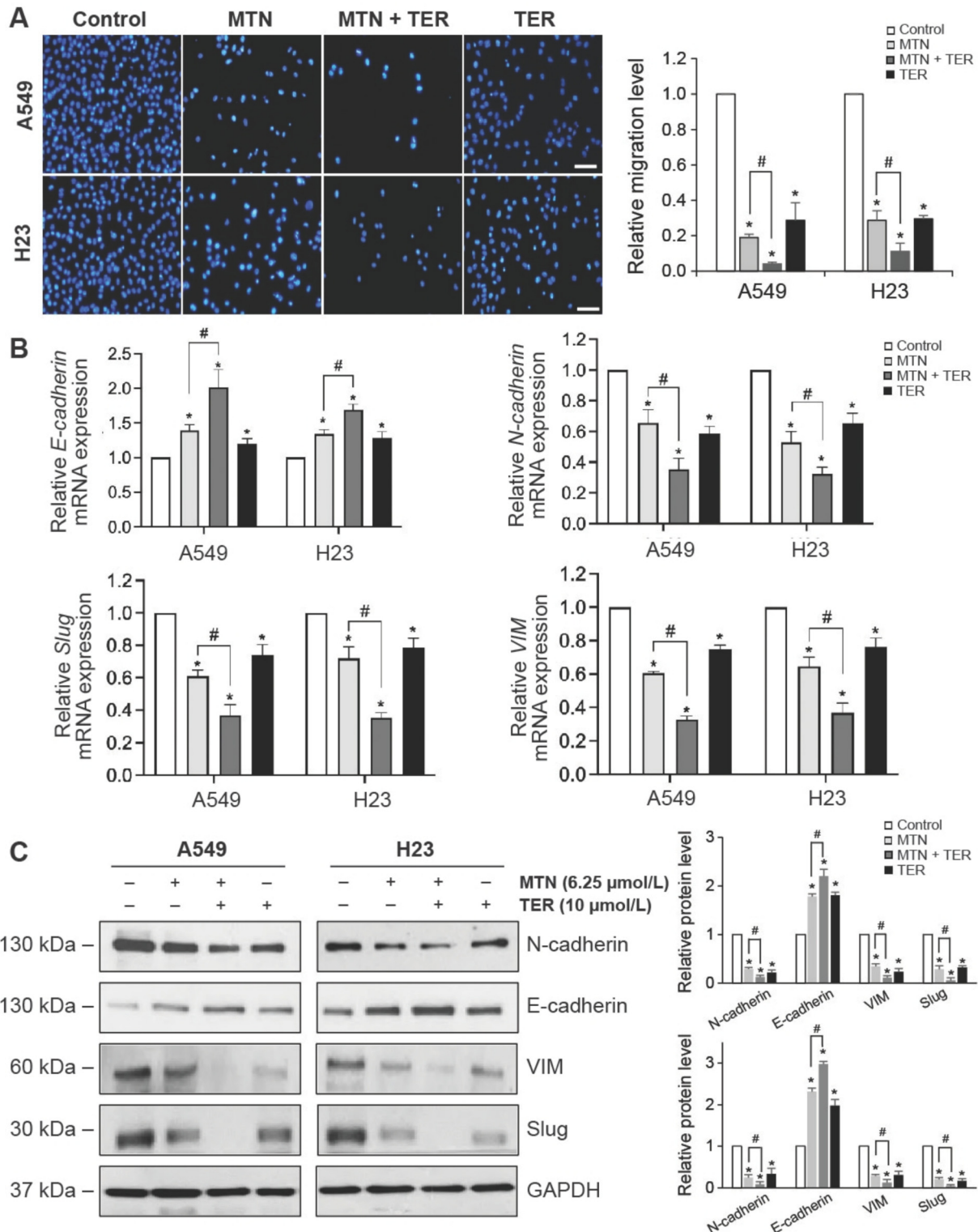


**Fig. 6.** MTN suppresses cell migration through a mechanism related to ADRB2 and EMT in NSCLC cells. **A:** Cell migration was examined using Transwell migration assay (scale bar = 10  $\mu$ m, magnification 40 $\times$ ). A549 and H23 cells were treated with MTN in the presence or absence of an ADRB2 agonist, TER, for 24 h. **B:** mRNA levels of EMT markers were evaluated by quantitative reverse transcriptase polymerase chain reaction. **C:** Protein expression of EMT markers was assessed by immunoblotting analysis. Data are shown as mean  $\pm$  standard error of mean ( $n = 3$ ). \* $P < 0.05$ , vs control group; # $P < 0.05$ , MTN vs MTN-TER group. ADRB2: adrenoceptor  $\beta_2$ ; E-cadherin: epithelial cadherin; EMT: epithelial–mesenchymal transition; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MTN: montanine; N-cadherin: neural cadherin; Slug: Snail family transcriptional repressor 2; TER: terbutaline sulfate; VIM: vimentin.

lar signal-regulated kinase (ERK) signaling is associated with EMT promotion [48]. Recent finding indicated that ADRB2 agonists could inhibit melanoma cell migration through cAMP/PKA signaling [12]. Further, ADRB2 modulates the balance between cAMP/

PKA and ERK signaling, influencing EMT regulation in lung cancer [49]. These finding suggested that the effect of ADRB2 and the molecular mechanism involved might vary depending on cell type and the tumor microenvironment. However, further investigations

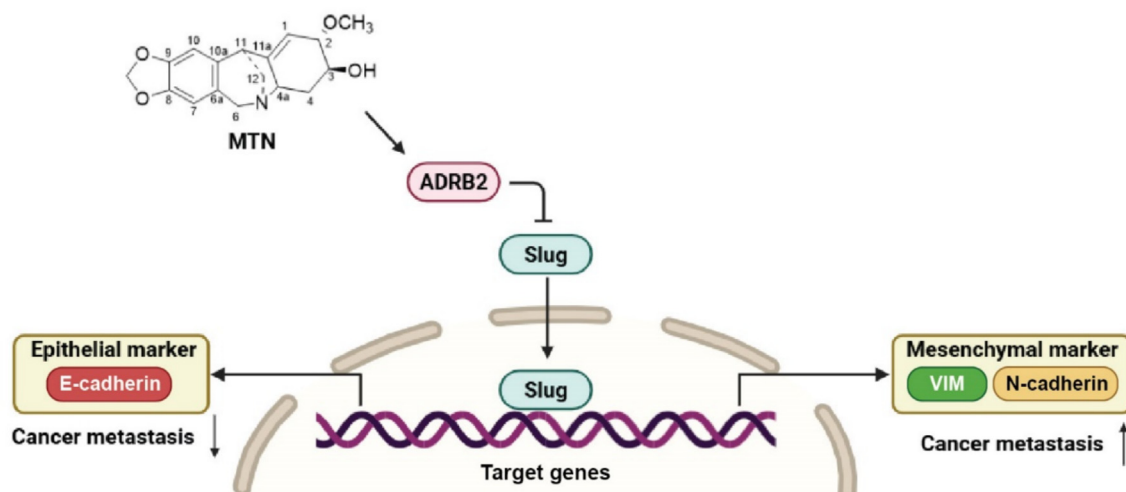


**Fig. 7.** Scheme illustrates the effect of MTN on lung cancer metastasis. MTN upregulates ADRB2, which subsequently attenuates Slug expression. During the EMT process, Slug promotes EMT by downregulating the epithelial marker E-cadherin and upregulating the mesenchymal markers VIM and N-cadherin, thus facilitating cancer metastasis. By suppressing slug expression, MTN effectively inhibits lung cancer metastasis. ADRB2: adrenoceptor  $\beta 2$ ; E-cadherin: epithelial cadherin; MTN: montanine; N-cadherin: neural cadherin; Slug: Snail family transcriptional repressor 2; VIM: vimentin.

into ADRB2 signaling pathways in EMT regulation are necessary to clarify its actual role in lung cancer progression.

An accumulation of evidence shows that the expression of ADRB2 is markedly reduced in breast cancer, lung cancer, and oral squamous cell carcinoma, correlating with poor overall survival and disease-specific survival [8,50,51]. Activation of ADRB2 by pirbuterol, an ADRB2 agonist, has been shown to reduce tumor cell proliferation, induce apoptosis, and promote tumor regression by inactivating the mitogen-activated protein kinase pathway [7]. Additionally, combining the ADRB2 agonist salbutamol with paclitaxel significantly suppresses proliferation and induces apoptosis in MDA-MB-231 breast cancer cells, leading to a substantial reduction in tumor size in animal models [11]. The activation of ADRB2, induced by isoxsuprine, also effectively inhibits the growth and motility of oral squamous cell carcinoma cells in vitro and in vivo by downregulating VIM and N-cadherin, thereby impeding the mesenchymal phenotype [10]. Furthermore, combination therapy with lenalidomide and gefitinib markedly increases ADRB2 expression and reduces gefitinib resistance in gefitinib-resistant lung cancer PC9 cells [52]. Our work also suggested an intriguing suppressive effect of MTN on lung cancer metastasis through the upregulation of ADRB2. These findings suggest that upregulation or activation of ADRB2 could be potential therapeutic interventions in cancer treatment.

## 5. Conclusion

MTN exhibits significant efficacy as an anticancer agent against NSCLC through ADRB2 upregulation and the modulation of EMT signaling pathways (Fig. 7). MTN was able to inhibit lung cancer metastasis both in vitro and in vivo, particularly when used in combination with ADRB2 agonists, underscoring its promise as a novel therapeutic option. Continued research and clinical trials will be crucial for translating these findings into clinically applicable treatments, ultimately improving the prognosis and quality of life of NSCLC patients.

## CRediT authorship contribution statement

**II:** investigation, methodology, visualization, formal analysis, and writing—original draft. **NS:** investigation and writing—review & editing. **HMN, THXH** and **HTN:** investigation and writing—

review & editing. **AW, OW, AC** and **SW:** investigation. **VP:** supervision, project administration, conceptualization, methodology, funding acquisition, visualization, and writing—review & editing.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.joim.2026.01.001>.

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