

ORIGINAL ARTICLE

Baicalein inhibits non-small-cell lung cancer invasion and metastasis by reducing ezrin tension in inflammation microenvironment

Xiaolong Zhang^{1,2} | Qinli Ruan¹  | Yiqian Zhai¹ | Dandan Lu¹ | Chen Li¹ | Yahan Fu¹ | Zihui Zheng¹ | Ying Song³ | Jun Guo¹

¹School of Medicine and Holistic Integrative Medicine, Nanjing University of Chinese Medicine, Nanjing, China

²Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China

³Department of Respiratory Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

Correspondence

Ying Song, Department of Respiratory Medicine, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Gulou District, Nanjing, China.
Email: songzhang_ying@163.com

Jun Guo, School of Medicine and Holistic Integrative Medicine, Nanjing University of Chinese Medicine, 138 Xianlin Road, Qixia District, Nanjing, China.
Email: guoj@njucm.edu.cn

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Abstract

Baicalein, a flavonoid phytochemical, has been shown to be effective as an anti-metastatic agent for various cancers, especially for non-small-cell lung cancer (NSCLC). However, the underlying mechanism of how baicalein targets cellular processes during NSCLC cell invasion and metastasis remains elusive. In this study, we found that non-cytotoxic concentrations of baicalein still retained anti-dissemination activity both in vitro and in vivo. Using a genetic encoding tension probe based on Förster resonance energy transfer (FRET) theory, baicalein was shown to significantly decrease ezrin tension by downregulating cellular ezrin S-nitrosylation (SNO) levels in NSCLC cells in the inflammatory microenvironment. Decreased ezrin tension inhibited the formation of an aggressive phenotype of NSCLC cell and leader cell in collective migration, and subsequently suppressed NSCLC dissemination. Baicalein restrained SNO-mediated ezrin tension by decreasing iNOS expression levels. Overall this study demonstrates the novel mechanism used by baicalein to suppress NSCLC invasion and metastasis from a mechanopharmacology perspective and illustrates a new direction for drug development.

KEYWORDS

baicalein, ezrin, mechanopharmacology, NSCLC, tension

1 | INTRODUCTION

NSCLC constitutes approximately 85% of lung cancers and remains a large threat to health worldwide.¹ Increasing numbers of NSCLC therapeutic strategies have been developed in recent years,

however tumor metastasis is still the major effect inducing a limited prognosis and poor overall survival rate. At present, most studies have focused on changes in metastatic biochemical signals,^{2,3} but less attention has been paid to intracellular mechanical activities in NSCLC motility.

Abbreviations: CM, cytokine mixture; CXCL12, C-X-C motif chemokine 12; FRET, Förster resonance energy transfer; iNOS, inducible nitric oxide synthase; NSCLC, non-small-cell lung cancer; SNO, S-nitrosylation.

Xiaolong Zhang and Qinli Ruan contributed equally to this work.

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Ezrin, a member of the ezrin-radixin-moesin (ERM) family, is usually considered a membrane-cytoskeleton linker protein. Several studies have found high expression levels in NSCLC and demonstrated the key regulatory function of ezrin in promoting NSCLC invasion and metastasis.^{4,5} Ezrin is usually located in membrane protrusive structures such as filopodia, lamellipodia, and invadopodia⁶ and is responsible for their aggressive behavior, harboring higher levels of ezrin tension during NSCLC cell invasion and metastasis.⁷ Leader cells are the frontline cells of migrating clusters during the collective migration of cancer cells, and this type of cell is usually identified by its distinct morphology such as ruffling lamellipodia, large focal adhesions and aligned cytoskeletal structure.⁸ Leader cells also differ from other following cells in their ability to finger the leading edge and express unique genes.^{8,9} Ezrin tension is increased in leader cells compared with other following cells and promotes dissemination of NSCLC cells.⁷ These studies suggested that mechanical modulation in ezrin tension could be a potential drug target for NSCLC. Therefore it would be feasible to control NSCLC metastasis by adjusting ezrin tension.

Intracellular mechanical activities can be regulated by chemical signaling. For example, protein S-nitrosylation (SNO) plays an important role in modulating cellular functions such as invasion and metastasis.^{10,11} Inducible nitric oxide (NO) synthase (iNOS), the major endogenous NO donor contributing to cellular SNO in the NSCLC microenvironment,¹² was found to enhance ezrin SNO levels that could promote NSCLC cell invasion and metastasis by increasing cellular ezrin tension.⁷ Therefore, it is necessary to study the effects of drugs on this mechanical signal pathway, to further improve therapeutic measurements of NSCLC metastasis from a mechanical force perspective.

Baicalein, the main pharmacological component derived from the root of *Scutellaria baicalensis*, has been reported to have multiple anti-cancer activities,¹³ especially in preventing cancer invasion and metastasis.^{14,15} In recent years, impressive discoveries have been made when investigating baicalein's potential function in inhibiting NSCLC dissemination.^{16,17} Marked effects of baicalein in suppressing iNOS expression have also been demonstrated in some studies,^{18,19} however the underlying mechanism of whether baicalein can modulate iNOS-mediated ezrin tension and subsequently regulate NSCLC invasion and metastasis remains to be elucidated.

In this study, we investigated the underlying anti-dissemination mechanism of baicalein through ezrin SNO-related mechanical modification. Using an intramolecular ezrin tension probe based on Förster resonance energy transfer (FRET) theory, we detected mechanical change in ezrin tension and observed the metastatic potential of NSCLC with or without baicalein treatment.

2 | MATERIALS AND METHODS

2.1 | Reagents

Commercially available antibodies used included ezrin (Cell Signaling Technology, Danvers, USA), α -tubulin (Boster, Wuhan, China),

NOS2 (Immunoway, Wuhan, China), and secondary anti-rabbit and anti-mouse antibodies (Cell Signaling Technology, Danvers, USA). Chemical reagents used were baicalein (Aladdin, Shanghai, China), human IL-6 (Bioss, Beijing, China), human IFN- γ (NOVUS, Colorado, USA), 1400W (Beyotime, Shanghai, China), C-X-C motif chemokine 12 (CXCL12) (Peprotech, Rocky Hill, USA), EZN Endo-free Plasmid DNA Mini Kit II (OMEGA, Doraville, USA), S-nitrosylated Protein Detection Kit (Cayman, Michigan, USA), X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland), geneticin (Sigma, Darmstadt, Germany), and basement membrane matrix (Corning, New York, USA).

2.2 | Cell line culture

Human NSCLC cell lines, A549 and H1299, were purchased from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and authenticated by the Genetic Testing Biotechnology Corporation (Suzhou, China) using short tandem repeat profiling. A549 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, New York, USA) with added 10% fetal bovine serum (FBS) (Gibco, New York, USA) and a mixture of 100 units/mL penicillin and 100 μ g/mL streptomycin (Gibco, New York, USA). H1299 cells were cultured in RPMI-1640 medium (Enogene, Nanjing, China), supplemented with 10% FBS and a mixture of 100 units/mL penicillin and 100 μ g/mL streptomycin. Cell culture was maintained in a humidified atmosphere with 5% CO₂ in air at 37°C.

2.3 | Cell viability assay

Here, 200 μ L cell suspensions (5×10^4 cells/mL) were cultured in 96-well plates and treated for 24 h with different concentrations of baicalein, MTT was added to each well and the cells incubated for 4 h, cell culture medium was then discarded and DMSO added to dissolve crystal violet. Optical density (OD) values were measured at 490 nm. Cell viability = $(1 - OD_{\text{Test}}/OD_{\text{Control}}) \times 100\%$.

2.4 | Cell invasion and metastasis assays

For the invasion assay, NSCLC cells were pretreated using baicalein at concentrations of 2.5, 10, or 40 μ mol/L for 24 h, Cells in each group were cultured with serum-free medium for 24 h and seeded into the upper chamber of a transwell apparatus at a density of 2×10^4 cells/chamber; cell culture medium containing 20% FBS was added to the bottom chamber. At 24 h later, cell that migrated to the lower membrane surface were fixed and stained with 4% paraformaldehyde and 0.4% crystal violet, successively. Cell numbers in the central and 5 neighboring fields were counted under a light microscope. For the migration assay, 50 μ L Matrigel was pre-coated in the upper chamber, and the following procedures were similar to those used in the invasion assay.

2.5 | Plasmid transfection and stable cell line development

The pEGFP-N1 ezrin plasmid was purchased from Addgene (#20680). Construction of the ezrin-cpstFRET tension probe has been described previously.⁷ NSCLC cells were transfected with plasmids according to the manufacturer's procedures and cultured in medium containing 1000 mg/mL geneticin for 7 d to screen transfected cells. Then, the remaining cells were subcultured in 500 mg/mL geneticin for 15 d and cell fluorescence was checked. If 80%–90% of cells showed fluorescence, the cell line was used for the next experiments.

2.6 | Experimental animal model establishment and xenograft studies

Thirty-two female SPF grade BALB/c nude mice, which weighed from 16 to 18 g per mouse, were purchased from the Institute of Comparative Medicine of Yangzhou University (License No. SYXK [SU] 2014-0003). After adjustable feeding for 7 d, the mice were assigned either to a control group or 3 baicalein treatment groups (2.5, 10, and 40 mg/kg). On day 0, 1×10^6 A549 cells expressing GFP-tagged wild-type ezrin were resuspended in 50 μ L medium containing 10 μ L Matrigel. The medium was injected into the pleural cavity to establish the NSCLC mice model. On day 1, mice in the treatment groups received baicalein by intragastric gavage at doses of 2.5, 10 or 40 mg/kg, respectively, for 28 d. Mice in the control group were given equal volumes of normal saline in the same way. Cancer progression was observed and quantified using ChemStudio PLUS software (Jena, Jena, Germany) at different time points. On day 28, mice were anesthetized. Lungs were excised, observed by the naked eye, fixed, sectioned, and stained with H&E. Histological analyses and lung nodules in serial sections were quantified under a Zeiss microscope axio scope A1 (Zeiss, Oberkochen, Germany). During the experiment, all animals were kept in a SPF barrier environment at a room temperature of 21–23°C, with a relative humidity of 60% and 12 h of light.

2.7 | Immunoprecipitation-based detection of protein-SNO

A S-Nitrosylated Protein Detection Kit (Cayman, Michigan, USA) was used for ezrin SNO detection. NSCLC cells were pretreated with lysate buffer and centrifuged, and the supernatants were separated and subjected to acetone precipitation. The precipitated pellets were resuspended in buffer that contained reducing and labeling reagents, and subjected again to acetone precipitation. The final products (nitrosylated proteins) were resuspended in NP-40 lysis buffer, incubated with anti-ezrin antibodies, and immunoprecipitated with protein A/G agarose beads overnight to extract nitrosylated ezrin. Immunoprecipitates were denatured by boiling in loading buffer and

cooled down before western blotting analysis. Total ezrin level was preliminarily normalized. Total ezrin and ezrin SNO levels were detected. The negative control group omitted the reducing and labeling reagents.

2.8 | Immunofluorescence

NSCLC cells were fixed and permeabilized as routine, blocked with 4% bovine serum albumin, and labeled using rabbit-derived ezrin antibody and mouse-derived tubulin antibody. Subsequently, the samples were incubated with fluorescein isothiocyanate (FITC)-conjugated or tetramethylrhodamine (TRITC)-conjugated homologous secondary antibodies. 4,6-Diamidino-2-phenylindole (DAPI) was used for DNA staining. FITC-conjugated phalloidine was used for microfilament labeling. All samples were imaged under an inverted fluorescence microscope DMi8 (Leica, Wetzlar, Germany).

2.9 | Western blotting

After treatment with baicalein for 24 h, NSCLC cells were collected and lysed. Cell lysates were denatured by boiling in loading buffer and separated proteins were transferred to nitrocellulose membranes (Bio-Rad, California, USA). After blocking in 5% non-fat skimmed milk for 1 h, the blot was incubated at 4°C overnight with mouse- or rabbit-derived primary antibodies, and then probed with homologous secondary antibodies for 1 h. Immunoreactive bands were detected using an enhanced chemiluminescence gel imaging system (Bio-Rad, California, USA). Densitometric analysis was performed for normalization. The positive control group only used 1400W, which is a specific inhibitor of iNOS.

2.10 | FRET analysis

FRET image acquisition was performed on an inverted fluorescence microscope DMi8 (Leica, Wetzlar, Germany) equipped with a $\times 63$ oil-immersion objective as described previously.⁷ Briefly, cyan and yellow emission wavelengths were detected at the same time (excitation wavelength [EX] = 436 nm and emission wavelength [EM] = 535/30 nm for cyan fluorescent protein (CFP) detection and 470/30 nm for yellow fluorescent protein (YFP) detection). The dipole angle between the donor/CFP and the acceptor/YFP determined the effectiveness of FRET. CFP/FRET ratios (the intensity of the CFP channel divided by the intensity of the FRET channel) were negatively correlated with FRET efficiency, but positively correlated with force.^{20,21}

2.11 | Statistical analysis

Statistical analyses were performed using SPSS statistics program version 20 (IBM Corp., Armonk, NY, USA). All data in the study are

presented as mean \pm standard deviation (SD) unless otherwise indicated. Differences between 2 groups or multiple groups were determined using 2 independent sample *t* tests or one-way analysis of variance (ANOVA); Dunnett *t* test was used in post hoc multiple comparisons. A probability level of .05 was considered to be statistically significant.

3 | RESULTS

3.1 | Baicalein inhibits invasion and metastasis of NSCLC cells at non-cytotoxic concentrations

Baicalein is a flavonoid, and its structure is shown in Figure 1A. To assess the effects of baicalein on cell viability, we first detected the effect of baicalein on cell growth using an MTT assay. NSCLC cells were treated with baicalein at different concentrations (2.5–60 $\mu\text{mol/L}$) for 24 h; baicalein showed no cytotoxicity for cells at concentrations of 2.5–40 $\mu\text{mol/L}$ (Figure 1B). Therefore, 3 non-cytotoxic concentrations were used to assess the anti-disseminative effect of baicalein in the following assays.

To investigate the potential pharmacological effect of baicalein on cellular tension activity, the transwell assay, depending on intracellular tension activity, was performed to test the A549 and H1299 aggressiveness. As shown in Figure 1C,D, baicalein significantly

inhibited A549 cell invasion and metastasis compared with the blank control at either 10 or 40 $\mu\text{mol/L}$ concentrations. Similarly, baicalein could also inhibit H1299 cell invasion and metastasis at 10 or 40 $\mu\text{mol/L}$ concentrations. These data suggested that even low concentrations of baicalein still harbored the potential to prevent NSCLC spread of different cell lines.

3.2 | Baicalein decreases ezrin tension and inhibits the aggressive phenotype of NSCLC cells in inflammatory milieu

Ezrin is responsible for mechanical transduction from plasma membrane to cytoskeleton, therefore it was necessary to investigate the mechanical properties of cells in response to chemoattractant reagents. The chronic inflammatory microenvironment plays an important role in types of cancer progression such as cancer invasion and metastasis.^{11,22} Thus, based on our previous studies, we used a cytokine mixture (CM) consisting of IL-6 and IFN- γ to build an inflammatory environment. We exposed CM-treated or CM and baicalein-treated cells to CXCL12, a widely used chemokine, to build aggressive cell models,^{23,24} and performed time-lapse imaging for 30 min to evaluate change in dynamic ezrin tension. The results indicated that, after CM treatment, both A549 and H1299 cells had faster increasing trends in ezrin tension in response to

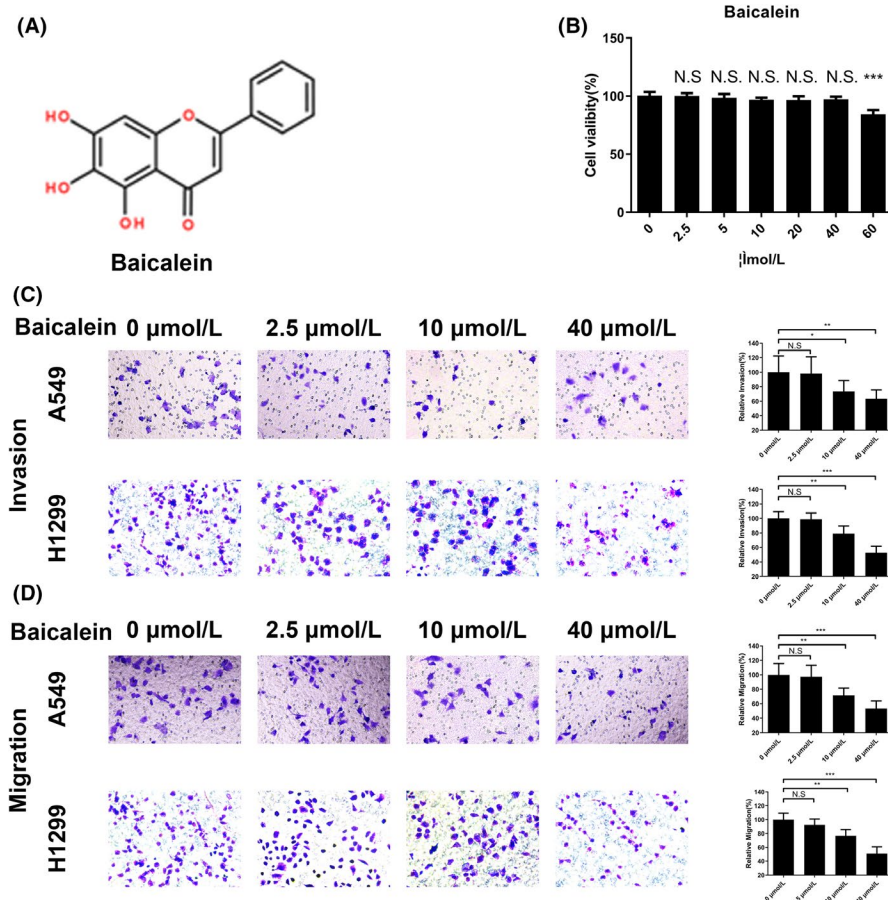


FIGURE 1 Baicalein inhibits NSCLC cell invasion and migration in vitro. A, Chemical structure of baicalein. B, Effects of baicalein on cell viability at different concentrations. C, Effects of baicalein on invasion of A549 and H1299 cells at different concentrations. D, Effects of baicalein on migration of A549 and H1299 cells at different concentrations

CXCL12 inducement than control group cells (Figure 2A,B). This result is consistent with our previous findings,⁷ however when pretreated with CM and baicalein simultaneously the increasing trend in ezrin tension in the 2 cell lines was significantly restrained (Figure 2A,B). These data suggested that baicalein could downregulate NSCLC aggressiveness by inhibiting ezrin-related mechanical transduction.

As a scaffold protein, ezrin is responsible for the interaction between the plasma membrane and the actin cytoskeleton. Ezrin is usually located in protrusive cell structures such as filopodia, lamellipodia, and invadopodia.⁶ These special structures, especially invadopodia, are symbols of cell aggression.²⁵ The 2 types of NSCLC cells were subjected to fluorescent staining after CM or CM and baicalein treatments. After CM stimuli, A549 and H1299 cells displayed a reconstructed microfilament structure and increased pseudopodia (Figure 2C,D). However, when baicalein was added simultaneously, the NSCLC cells regained a relative normal morphology and had less pseudopodia than the CM group; microtubule structures remained stable (Figure 2C,D).

3.3 | Baicalein decreases cellular ezrin tension and suppresses leader cell formation

To further investigate the specific mechanism of baicalein in preventing NSCLC invasion and metastasis, we performed a wound healing assay combined with FRET analysis, and tested the ezrin tension of NSCLC cells at different locations during collective cell migration. For both A549 or H1299 cells, CM treatment increased the number of leader cells, but this number was significantly decreased after pretreatment with baicalein (Figure 3A,B). Using FRET, leader cells exhibited relatively high ezrin tension compared with the other following cells under normal conditions (Figure 3C,D). After treatment with CM, the tension gap between the leader cells and the other following cells was more apparent (Figure 3C,D). However, it is worth noting that the difference in ezrin tension between A549 leader and following cells was less significant in response to co-treatment with CM and baicalein, (Figure 3C), and H1299 cells showed a similar decreased trend (Figure 3D). These data suggested that formation of leader cells was correlated positively with ezrin tension, which could be upregulated by CM-induced ezrin nitrosylation, and downregulated by baicalein.

3.4 | Baicalein decreases iNOS-mediated ezrin SNO in NSCLC cells

The basic level of ezrin SNO that was correlated with ezrin tension and NSCLC aggressiveness has been detected in our previous studies.⁷ As baicalein is considered an iNOS inhibitor, it was necessary to demonstrate the effect of baicalein on ezrin SNO in NSCLC. In this study, A549 and H1299 cells were treated with different concentrations of baicalein; the biotin-switch assay suggested that baicalein

could significantly decrease basic levels of ezrin SNO in a dose-dependent manner (Figure 4A,B).

iNOS is considered a major donor of intracellular NO for protein SNO in this inflammatory microenvironment.²⁶ Therefore, we used CM to induce high iNOS conditions for protein SNO. As expected, baicalein suppressed iNOS expression in A549 cells in a dose-dependent manner, and this was consistent with the effects of the iNOS-specific inhibitor 1400W (Figures 4C and S1). The biotin-switch assay indicated that the ezrin SNO level decreased accordingly (Figure 4D,E). That baicalein could inhibit iNOS expression by decreasing the ezrin SNO level was verified again using H1299 cell assays (Figure 4C-E). Combined with the results in Figure 1C,D, baicalein treatment illustrated its inhibitory effects on both NSCLC aggressiveness and the ezrin SNO level. Moreover, to further assess the role of enhanced ezrin SNO levels in cellular ezrin tension during NSCLC invasion and metastasis, we performed the time-lapse FRET assay again with 1400W as a positive control. The results showed that 1400W treatment could prevent the increasing trend of ezrin tension as for baicalein treatment in both NSCLC cell lines (Fig. S1). These data suggested that baicalein may reduce NSCLC dissemination in inflammatory microenvironments by inhibiting iNOS-mediated ezrin SNO and ezrin-related mechanical transduction.

3.5 | Baicalein suppresses NSCLC cells invasion and metastasis in vivo

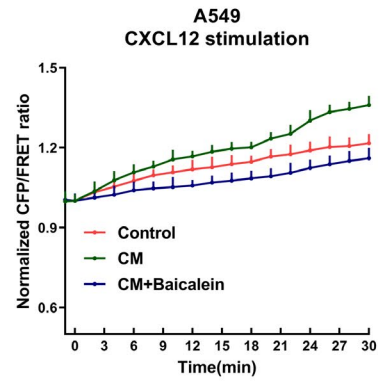
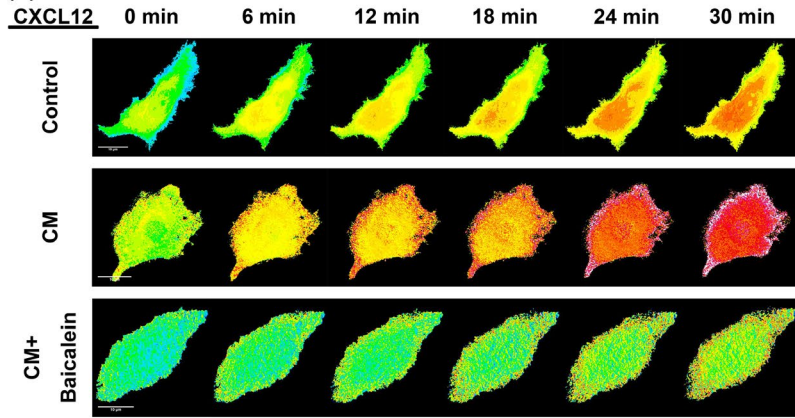
To verify the inhibitory effect of baicalein on metastasis of NSCLC in vivo, an orthotopic implantation assay was performed. Although baicalein had a significant suppressive effect on A549 and H1299 cells, this phytochemical had higher efficacy when reducing ezrin tension in A549 cells (Figure 2), so this cell line was used in the xenograft study.

Three dose gradients were used to test the anti-metastatic efficacy of baicalein. After treatment with baicalein for 28 d, mice administered 10 or 40 mg/kg exhibited less spontaneous metastatic signals than the control group (Figure 5A). Histological observations showed that orthotopic tumors were apparent, with pleomorphic tumor cells in control group, and that the scattered metastatic sites were found in distal lung tissues. With increase in baicalein concentration, the numbers of metastatic sites in distal lung tissues decreased, correspondingly, in a dose-dependent manner. The numbers of intrapulmonary metastasis sites in the 10 and 40 mg/kg baicalein groups were significantly less than in the control group (Figure 5B,C). These results indicated that baicalein could inhibit metastasis of NSCLC in vivo by decreasing ezrin tension.

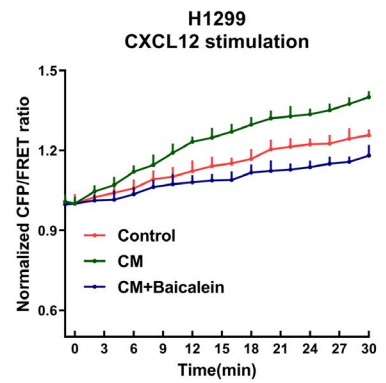
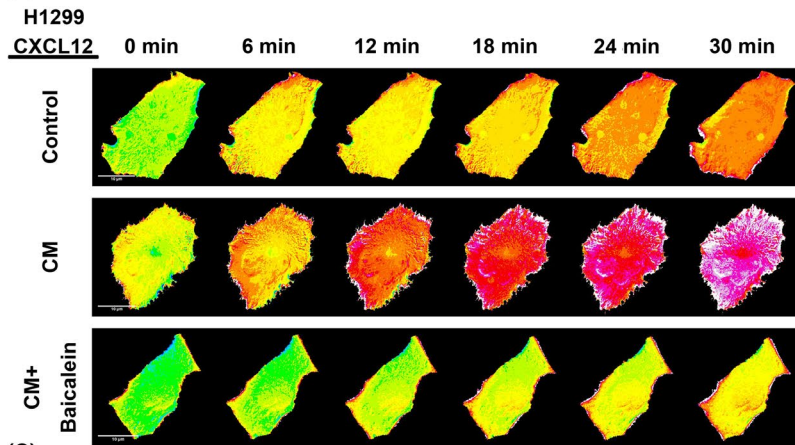
4 | DISCUSSION

In recent years, large numbers of studies have indicated many natural product-derived compounds with promising anti-metastasis activities. Baicalein is the major active flavonoid extracted from

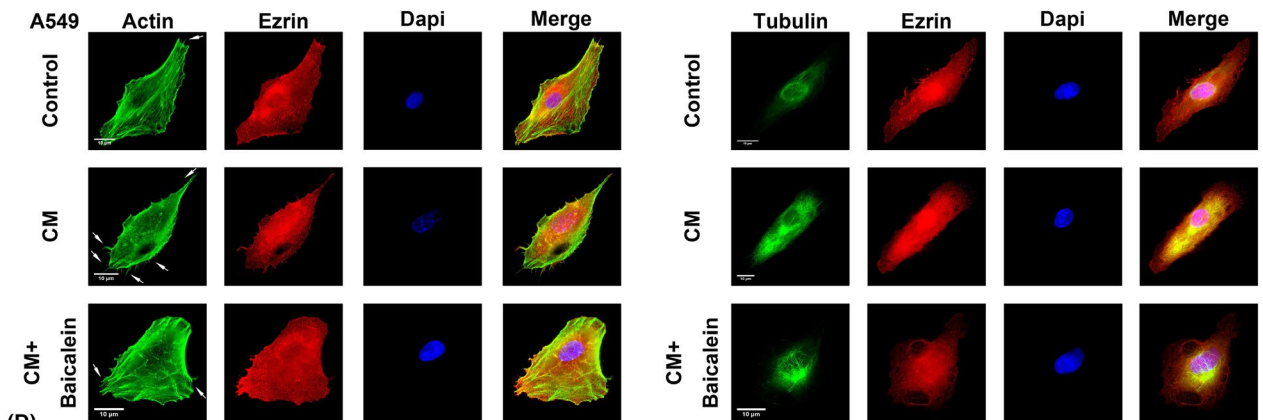
(A) A549



(B)



(C)



(D) H1299

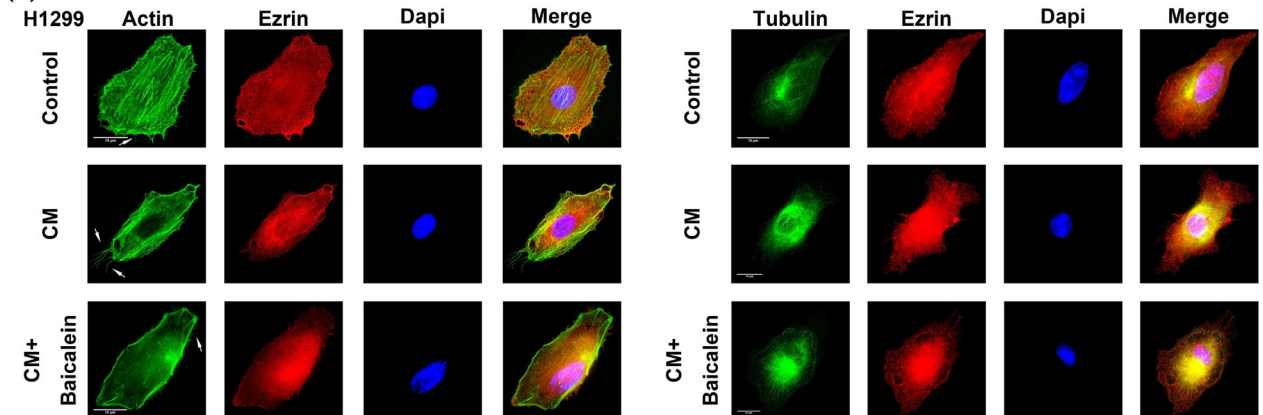


FIGURE 2 Baicalein decreases cellular ezrin tension and inhibits the aggressive phenotype of NSCLC cells. A, 30-min time-lapse images of FRET analyses in A549 cells expressing the ezrin-M-cpstFRET probe treated with CXCL12 after pretreatment with vehicle saline, CM or CM and baicalein, respectively. Calibration bar: 0.8-2.8. Scale bar, 10 μm (left panel). Normalized signals corresponding to A549 ezrin tension vs time under different stimuli (mean \pm SEM, $n \geq 5$) (right panel). B, 30-min time-lapse images of FRET analyses in H1299 cells expressing the ezrin-M-cpstFRET probe treated with CXCL12 after pretreatment with vehicle saline, CM or CM and baicalein, respectively. Calibration bar: 0.8-2.8. Scale bar, 10 μm (left panel). Normalized signals corresponding to H1299 ezrin tension vs time under different stimuli (mean \pm SEM, $n \geq 5$) (right panel). C, Representative images of microfilaments (MF) (left panel)/microtubules (MT) (right panel) and ezrin structures in A549 cells stimulated with CM in the presence or absence of baicalein treatment (FITC-stained MFs/MTs, green; TRITC-stained ezrin, red; nucleus, blue; white arrows: filopodia and lamellipodia structures). Scale bar: 10 μm . D, Representative images of MF (left panel)/MT (Right panel) and ezrin structures in H1299 cells stimulated with CM in the presence or absence of baicalein treatment (FITC-stained MFs/MTs, green; TRITC-stained ezrin, red; nucleus, blue; white arrows: filopodia and lamellipodia structures). Scale bar: 10 μm

the dried root of *S. baicalensis*. Treatment with baicalein blocks the dissemination of various cancers by suppressing epithelial to mesenchymal transition, downregulating ezrin protein expression, and adjusting multiple related signal pathways,^{15,27,28} the related molecular mechanisms of chemical signals is studied.

The term mechanopharmacology was introduced recently to describe the mechanics between drugs and their target cells, and investigate the role of the cell mechanical state in determining sensitivity to a particular drug.^{29,30} One challenge in mechanopharmacology is the lack of molecular tools that can quantify forces in living cells directly.³¹ FRET, as used here in living cells, is quantitative as it transforms mechanical changes into observable real-time optical signals. The FRET detection platform facilitated exploration of anti-dissemination mechanisms for baicalein from the combined perspectives of mechanopharmacology and chemopharmacology.

Mechanical forces are very important for cancer motility, ie invasion and metastasis.³² Intracellular structural tensions, mainly derived from microfilaments and microtubules, are the major sources of cellular mechanical forces. Ezrin, as a scaffold protein, plays a role in mechanical crosstalk between intracellular and extracellular forces. In the aggressive cell model, high levels of ezrin tension were detected in 2 different NSCLC cells treated with the CM (Figure 2A,B), but baicalein suppressed the increasing trend of ezrin tension (Figure 2A,B).

Protrusive structures are signs of cell aggressiveness, the effect of baicalein on formation of protrusive structures was studied.²⁵ Accompanied by decreased ezrin tension, baicalein was effective in reversing reconstructed microfilaments and decreasing the number of pseudopods in an inflammatory microenvironment (Figure 2C,D). Ezrin acts as a linker between membranes and microfilaments, and is the protein responsible for maintaining structural stability, such as cortical integrity of cells, and for transducing extracellular to intracellular mechanical signals.^{7,33} It was inferred that baicalein could suppress the formation of an aggressive phenotype by decreasing ezrin tension.

Formation of leader cells that act as guides for collective cell migration is an early step in migration,^{34,35} and mechanical forces play key roles in the formation of leader cells.³⁶ Factors that modulate cellular mechanical forces in leader cell formation have not been identified conclusively. Cells have been found to exhibit both cytoskeletal reorganization, especially actin networks, and enhanced cellular forces during change to a leader-like phenotype.^{37,38}

Combined with our previous study that showed that enhanced ezrin tension was positively correlated with NSCLC aggressiveness, we could speculate that ezrin tension also plays a role in formation of leader cells. By applying FRET in a collective cell migration assay, the magnitude of the gap in cellular ezrin tension between leader and other following cells was found to be enlarged significantly after CM treatment (Figure 3C,D). In addition, the number of leader cells was increased in the CM group (Figure 3A,B). Ezrin acts as a membrane-microfilament linker protein and functions to form membrane protrusions, and ezrin SNO facilitates ezrin translocation to cell membranes in an inflammatory microenvironment.^{6,7} Therefore, more effective mechanical transduction after CM stimulation might polarize cytoskeletal tension in cells, and be beneficial for leader cell formation.³⁹ These results suggested that nitrosylation-related up-regulation of ezrin tension promoted leader cell formation.

When NSCLC cells were treated with baicalein, together with decreased ezrin tension, baicalein decreased the number of leader cells (Figure 3A,B) as well as the magnitude of the gap for cellular ezrin tension between leader cells and other following cells (Figure 3C,D). Baicalein suppressed the formation of an aggressive phenotype and of leader cells by decreasing ezrin tension. Combined with the orthotopic implantation assay (Figure 5), it was evident that decreased ezrin tension induced by baicalein hampered NSCLC dissemination.

In this study, baicalein also exhibited an effective inhibition of iNOS expression in an inflammatory microenvironment, (Figure 4C) consistent with findings in former studies.^{18,19} Nitrosylation of ezrin mediated by iNOS was significantly restrained by this phytochemical (Figure 4D,E). As ezrin SNO level is positively correlated with aggressiveness of NSCLC cells,⁷ it was speculated that the anti-dissemination effect of baicalein was related to modulation of ezrin SNO in an inflammatory microenvironment.

In the aggressive cell model, NSCLC cells in the combined CM and baicalein group exhibited less sensitive responses to CXCL12 treatment than found in the CM group (Figure 2A,B). Chemokines are important factors that induce cancer aggression, which is always accompanied by mechanical forces.³² Hence, chemokine-mediated cell motility should not be simply regarded as a chemical signal, but a combination of both chemical and mechanical effects. There is a large amount of evidence indicating the positive relationship between cancer aggressiveness and cell plasticity.⁴⁰⁻⁴³ The more flexible are cell surfaces, the more aggressive cells become. The effect of baicalein on suppressing ezrin tension augmentation suggested

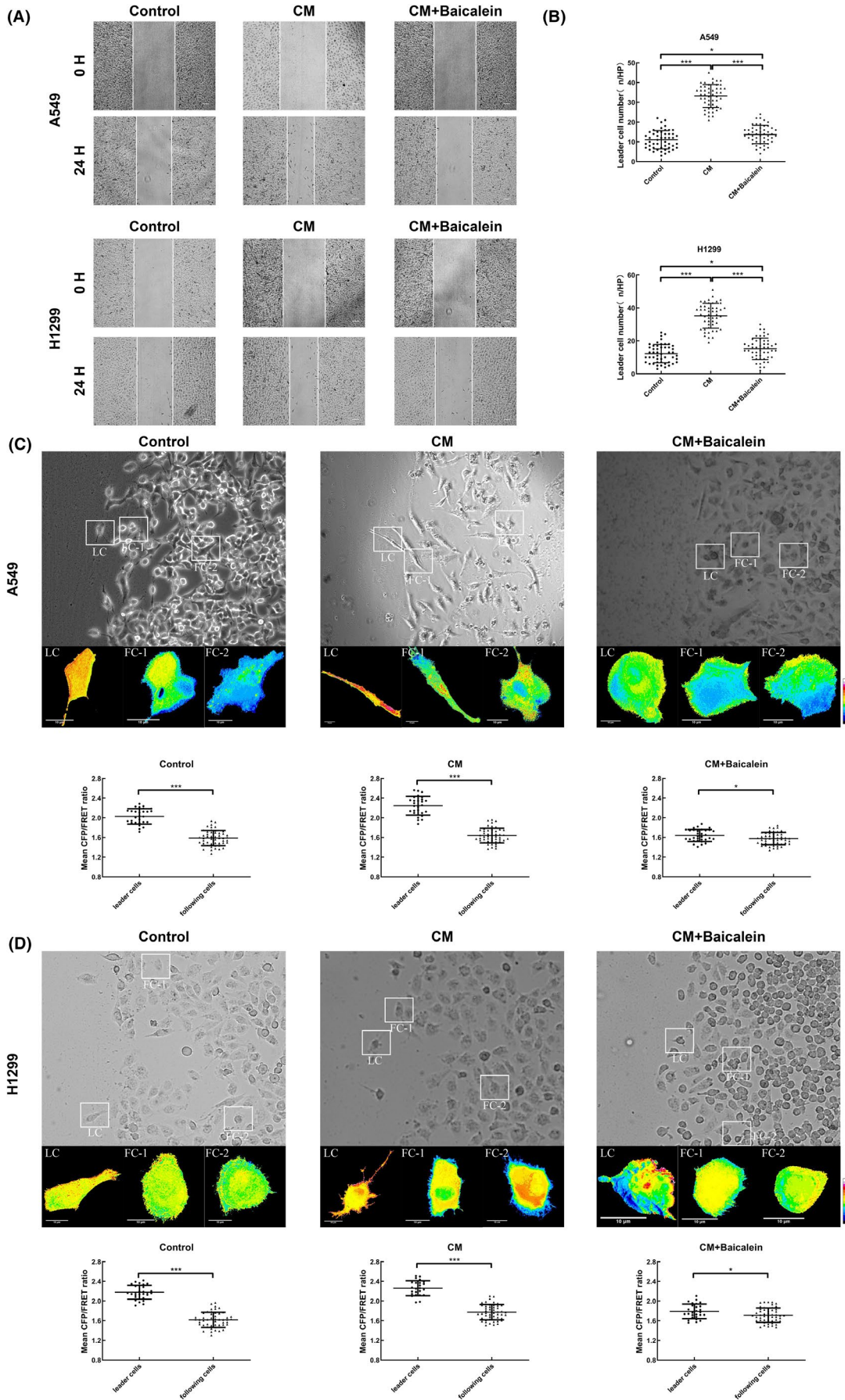


FIGURE 3 Baicalein decreases cellular ezrin tension and the number of leader cells. A, Effects of baicalein on the A549 (upper panel) and H1299 (lower panel) cell collective migration in an inflammatory microenvironment; 0 H and 24 H indicate the beginning and end of the test, respectively. Scale bar: 100 μ m. B, Effects of baicalein on the number of leader cells in each high power field of view in 24 H pictures (A549 upper panel and H1299 lower panel). C, Representative images of wound healing assays for A549 cells treated with vehicle saline, CM or CM and baicalein, respectively. CFP/FRET ratio images of cells in different positions are presented. Calibration bar: 0.8-2.8. Scale bar: 10 μ m (upper panel). Scatter diagrams showing the mean CFP/FRET ratios of both leader cells and following cells (lower panel). D, Representative images of wound healing assays for H1299 cells that were treated with vehicle saline, CM or CM and baicalein respectively. The CFP/FRET ratio images of cells in different positions are presented. Calibration bar: 0.8-2.8. Scale bar: 10 μ m (upper panel). Scatter diagrams showing the mean CFP/FRET ratios of both leader cells and following cells (lower panel). *** P < .001 and * P < .05 for comparison between the leader cell group and the following cell group

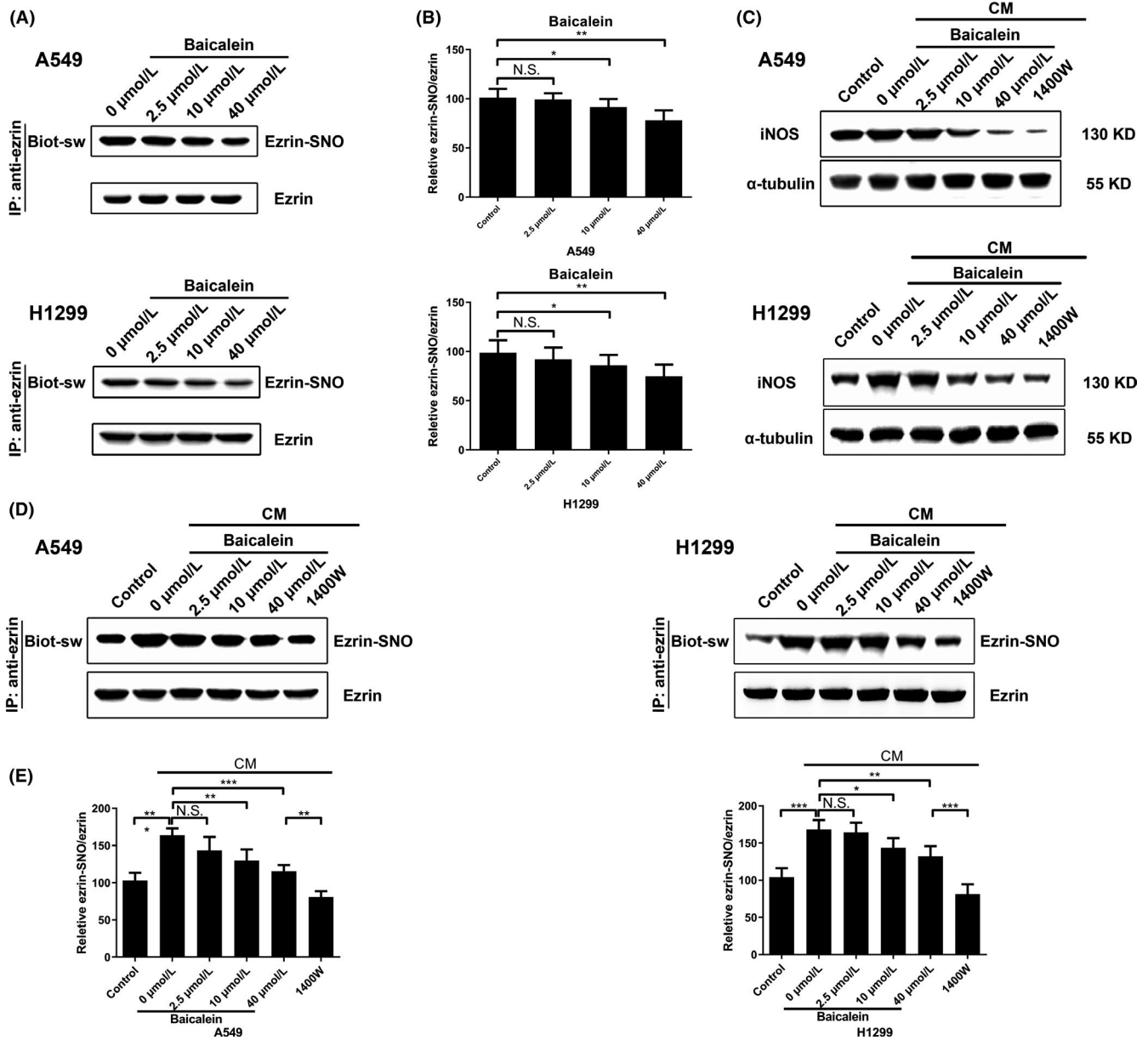


FIGURE 4 Baicalein downregulates iNOS-mediated ezrin SNO in a concentration-dependent manner. A, Levels of ezrin SNO in A549 and H1299 cells treated with baicalein at different concentrations. B, Relative ezrin SNO/ezrin ratio was normalized to the control value (mean \pm SD, n = 3 experiments) with treatment of baicalein. C, Under background CM stimulation, iNOS levels of A549 and H1299 treated with baicalein at different concentrations and α -tubulin levels were used as the loading control. D, Under background CM stimulation, ezrin SNO levels in A549 and H1299 cells treated with baicalein at different concentrations. E, Relative ezrin SNO/ezrin ratio was normalized to the control value (mean \pm SD, n = 3 experiments) with treatment with CM and baicalein. * P < .05, ** P < .01, and *** P < .001 for comparison between each group

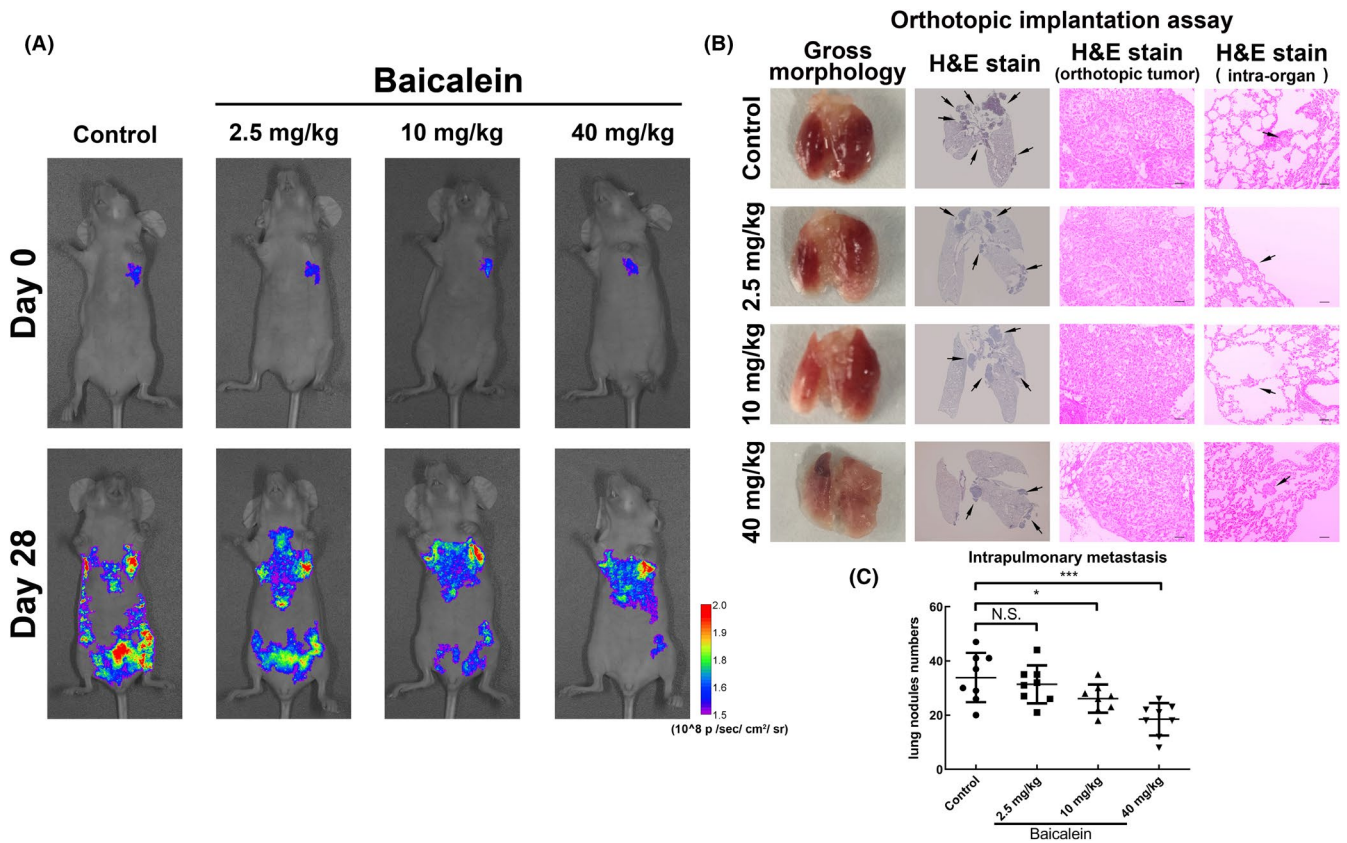


FIGURE 5 Baicalein suppresses NSCLC metastasis in vivo. A, For the orthotopic implantation assay, bioluminescence images of systemic metastases in nude mice on day 0 and day 28 following treatment with baicalein. B, Representative images of H&E-stained histological sections of lungs from nude mice in the orthotopic implantation assay (black arrows: metastatic nodules). Scale bar: 50 μ m. C, Box plot showing the number of lung metastatic nodules from the corresponding mice (mean \pm SD, $n = 8$). Pseudocolor heat-maps indicate the intensity of bioluminescence from low (purple) to high (red) (mean \pm SD, $n = 8$). * $P < .05$, *** $P < .001$ for comparison with the control group

that the plasticity of A549 and H1299 cells in response to chemokine-associated mechanical effects was downregulated, resulting in less aggressive properties.

In summary, baicalein inhibited the formation of protrusive structures and leader cells by the combined effects that decreased both ezrin SNO (chemical signaling) and ezrin tension transduction (mechanical signaling), and hampered NSCLC invasion and metastasis. This study provides a new perspective that therapeutics of cancer dissemination should encompass the combined effects of chemical and mechanical signals. More detailed knowledge on the relationships between chemical and mechanical signals is needed in the future.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

ORCID

Qinli Ruan <https://orcid.org/0000-0002-7893-8096>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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