Original Article

Bufalin inhibits epithelial-mesenchymal transition and increases radiosensitivity of non-small cell lung cancer via inhibition of the Src signaling

Zijian Wang[1], Fen Liu[2], Chen Huang[2], Jiaqi Zhang[2], Jibiao Wu[1]

[1]College of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, Jinan, China; [2]Cancer Institute, Jining First People's Hospital, Jining Medical University, Jining, China

Contributions: (I) Conception and design: J Wu; (II) Administrative support: J Wu; (III) Provision of study materials or patients: Z Wang; (IV) Collection and assembly of data: Z Wang, F Liu; (V) Data analysis and interpretation: C Huang, J Zhang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Jibiao Wu. College of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, 53 Jingshi Road, Jinan 250014, China. Email: wujibiao1963@163.com.

Background: Epithelial-mesenchymal transition (EMT) is a biological process involved in tumor migration, invasion, and radiotherapy resistance. Bufalin can affect the proliferation, apoptosis and invasion of tumor cells by regulating multiple signaling pathways. Whether bufalin can increase radiosensitivity through EMT deserves further investigation.

Methods: In this study, we investigated the effect of bufalin on the EMT and radiosensitivity of non-small cell lung cancer (NSCLC) and the underlying molecular mechanism. NSCLC cells were treated with bufalin (at a dose of 0–100 nM) or irradiated with 6 MV X-rays (4 Gy/min). The effects of bufalin on cell survival, cell cycle, radiosensitivity, cell migration, and invasion were detected. Western blot was used to analyze the gene expression changes of Src signaling in NSCLC cell induced by Bufalin.

Results: Bufalin significantly inhibited cell survival, migration, and invasion and induced G2/M arrest and apoptosis. Cells co-treated with bufalin and radiation manifested a higher inhibitory effect compared to those treated with radiation or bufalin alone. Furthermore, the levels of p-Src and p-STAT3 were considerably reduced following bufalin treatment. Interestingly, elevated p-Src and p-STAT3 were observed in cells treated with radiation. Bufalin inhibited radiation-induced p-Src and p-STAT3, whereas the knockdown of Src abrogated the effects of bufalin on cell migration, invasion, EMT, and radiosensitivity.

Conclusions: Bufalin inhibits EMT and enhances radiosensitivity through targeting Src signaling in NSCLC.

Keywords: Epithelial-mesenchymal transition (EMT); non-small cell lung cancer (NSCLC); bufalin; radiosensitivity; Src signaling

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Introduction

Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases, of which about 25% are diagnosed as locally advanced disease (III stage NSCLC) (1). Radiotherapy is currently considered the best treatment option for advanced lung tumors. Although the tumor can be locally controlled, metastasis after radiation therapy remains the main cause of death (2).

Epithelial-mesenchymal transition (EMT) is a biological process that promotes the malignant behavior of tumor cells after radiation (3-5). The main characteristics of EMT include the transformation of cell morphology

from epithelial type to interstitial type, down-regulation of E-cadherin (epithelial marker), and up-regulation of N-cadherin and vimentin (interstitial cell markers). Inhibition of E-cadherin expression has a key role during the process of EMT. The major transcription factors involved in inhibiting E-cadherin expression and inducing EMT include Snail, Slug, and Twist (6). Src signaling have been reported to regulate the resolution and initiation of EMT programs (7). Recent studies have shown that radiation can induce EMT to lead to acquired radiation resistance (3,8,9). However, the underlying mechanisms of these effects remain unknown. Understanding the underlying mechanism of EMT in radiation resistance may help to create more effective anti-NSCLC therapeutic drugs.

Bufalin is an effective anti-cancer agent that has various biological activities. Its structure is similar to digitalis toxin (10-12). Some studies have demonstrated that bufalin exerts significant anti-tumor activity by inhibiting cell proliferation and angiogenesis and inducing programmed cell death (13-15). More recently, studies have discovered that bufalin can inhibit the invasion and metastasis of hepatocellular carcinoma by regulating the PI3K/Akt/Mtor/HIF-1α axis (16,17). The aim of this study was to explore the effect of bufalin on the EMT and radiosensitivity in NSCLC and the underlying molecular mechanism. We found that bufalin inhibits EMT and increases radiosensitivity in NSCLC cells. We also showed that Src signaling is a new major target of bufalin, which reduced p-Src and largely abrogated Src activation triggered by radiation. We present the following article in accordance with the MDAR reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1859/rc).

Highlight box

Key findings

- Bufalin was found to inhibit EMT-related effects via Src-related signaling pathways to enhance radiosensitivity.

What is known and what is new?

- EMT can enhance cell invasion and metastasis and induce tumor cells' resistance to radiation; Src has been shown to have a critical role in the regulation of the resolution and initiation of EMT programs; bufalin inhibition of STAT3 activity, Src is an upstream activator of STAT3.
- Bufalin enhances the radiosensitivity of NSCLC cells; bufalin inhibits EMT and abrogates radiation-induced EMT; Src mediates bufalin action in cell mobility and EMT.

What is the implication, and what should change now?

- The interaction of Src and STAT3 with bufalin should be addressed by future research; assessment efficacy of bufalin in preclinical and clinical context.

Methods

Reagents and plasmids

Bufalin [high-performance liquid chromatography (HPLC) ≥98%] was obtained from the National Standard Network (Beijing, China) and dissolved in dimethyl sulfoxide (DMSO) to a 10 mM concentration and then stored at −20 ℃. Final concentrations of bufalin were diluted using culture medium (DMSO <3%, 0–200 nM).

Lentiviral short hairpin RNA (shRNA) was generated by inserting shRNA targeting sequence (GACAGACCTGTC CTTCAAGAA) into the pLKO.1 vector.

Antibodies

The antibodies used in this study were purchased from Cell Signaling Technology (CST; Danvers, MA, USA), including anti-Src (#2123), anti-p-Y416-Src (#2102), anti-STAT3 (#12640), anti-p-STAT3 (#9145), anti-E-cadherin (#3195), anti-snail (#3879), anti-snail2 (#9585), anti-vimentin (#5741), anti-N-cadherin (#13116), anti-Vinculin (#4650), anti-GAPDH (#5174), and anti-β-actin (#3700).

Cell culture

We acquired 3 human NSCLC cell lines, H1299, A549, and Hcc827, from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) or Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotics in a humidified atmosphere containing 5% CO_2/95% air at 37 ℃.

Irradiation

Cells were incubated with or without bufalin (at a dose of 0–100 nM) for 24 hours. Then, 6 MV X-rays at an exposure rate of 4 Gy/min were used to irradiate the cells at room temperature; the radiation field was 15 cm × 15 cm with the aid of a local irradiation apparatus.

Cell migration and invasion assays

A wound-healing assay was used to measure cell migration. Briefly, NSCLC cells were grown to 90% confluency in the medium containing bufalin (0, 25, 50, or 100 μM). A sterile 1,000 μL pipette tip was then used to scratch 3 separate wounds through the cells. Images of the scratches were obtained using an inverted microscope at ×10 magnification at 0 and 24 hours. Cell migration was quantified and expressed as an average percentage of closure of the scratch area.

The cell invasion assay was evaluated using transwell Matrigel invasion chambers (#3422; Corning Costar Corporation, Cambridge, MA, USA) coated with 100 μL of 1:6 diluted Matrigel. The number of invading cells on the filters was counted in 5 random fields per filter at a 100× magnification in triplicate wells for each group.

Flow cytometry

Flow cytometry was used to analyze the effect of treatment of cell cycle and cell apoptosis. Briefly, cells (4×10^5/well) in culture medium were seeded in a 6-well plate, then incubated at 37 ℃ for 24 hours. Cells were then washed with cold phosphate-buffered saline (PBS) and resuspended in a binding buffer and then incubated with a fresh medium containing various concentrations of bufalin (0 to 100 nM). Apoptotic cells were detected with ANXA5-FITC/PI Detection Kit (Becton, Dickinson, and Co. BD Biosciences, San Jose, CA, USA) and flow cytometry. Distribution of cell cycle was accessed by flow cytometry according to manufacturer's instruction.

Clonogenic survival assays

The clonogenic survival assays were performed as previously described (9). For clonogenic survival assay, cells were cultured in a 6-well plate at 1,000 cells per well for 2 weeks. Cells were then stained with crystal violet, and the colony numbers were counted.

Western blot

Cells were lysed in a lysis buffer. The protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts (20–25 μg) of protein lysate were then resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA), and immunoblotted as formerly described (15).

Immunofluorescence

Cells were seeded onto coverslips in 6-well plates. Following treatment with radiation or radiation plus bufalin, the cells were fixed with ice-cold methanol for 20 minutes and incubated with 0.1% Triton X-100 in PBS for 10 minutes. After pre-blocking with 2% bovine serum albumin (BSA)/PBS for 45 minutes, the cells were incubated with primary antibodies overnight at 4 ℃ and then with secondary antibodies conjugated with Alexa-Fluor-488 or Alexa-Fluor-594 (Thermo Fisher, Waltham, MA, USA). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Statistical analysis

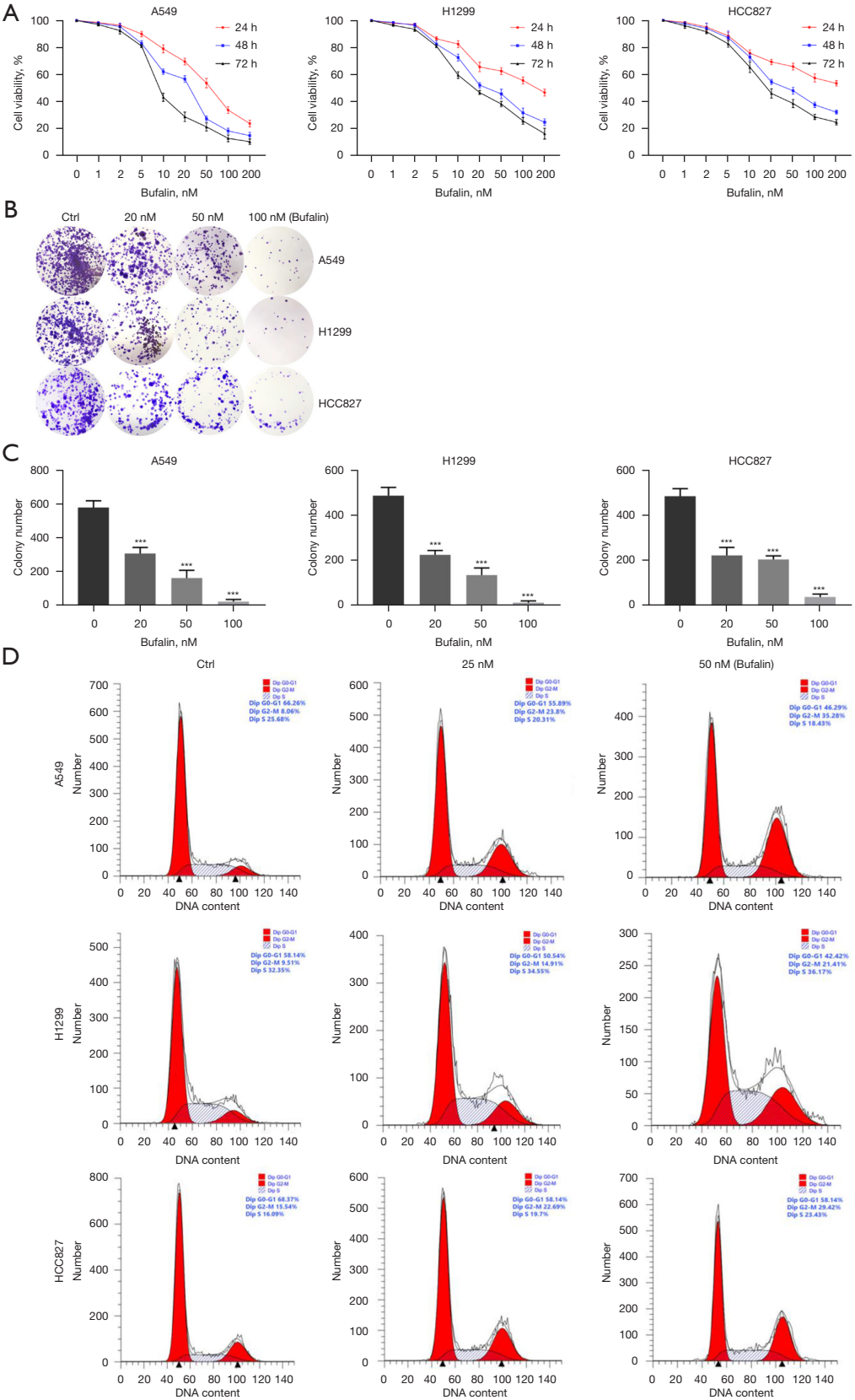
All quantitative data were presented as mean ± standard deviation (SD). Student's *t*-test was used to compare the group differences. A P value ≤0.05 was considered statistically significant.

Results

Bufalin decreased cell growth and viability in NSCLC

Previous studies have shown that bufalin can inhibit cell survival and growth of NSCLC (A549 and/or H460 cell lines) (18-20). Another report also demonstrated that bufalin could overcome epidermal growth factor receptor (EGFR)-inhibitor resistance in NSCLC cell lines (21). To further determine whether bufalin inhibits cell proliferation and survival in more NSCLC cell lines, we treated 3 NSCLC cell lines (A549, H1299, and HCC827) with bufalin for 24, 48, and 72 hours, respectively. Cell proliferation was determined with the Cell Counting Kit-8 (CCK-8) and colony formation assays. As shown in *Figure 1A*, bufalin significantly inhibited the proliferation of all 3 cell lines in a dose- and time-dependent manner. The IC_{50} value was nearly 30 nM after 24 hours of bufalin treatment. Colony formation assay further revealed that the number of the colony was considerably reduced by bufalin treatment in a dose-dependent fashion (*Figure 1B*,*1C*).

A previous study suggested that bufalin induces cell cycle arrest in malignant cells, but the effect was different among different cell types (22). We next examined the effect of bufalin on the cell cycle in NSCLC cells. Flow



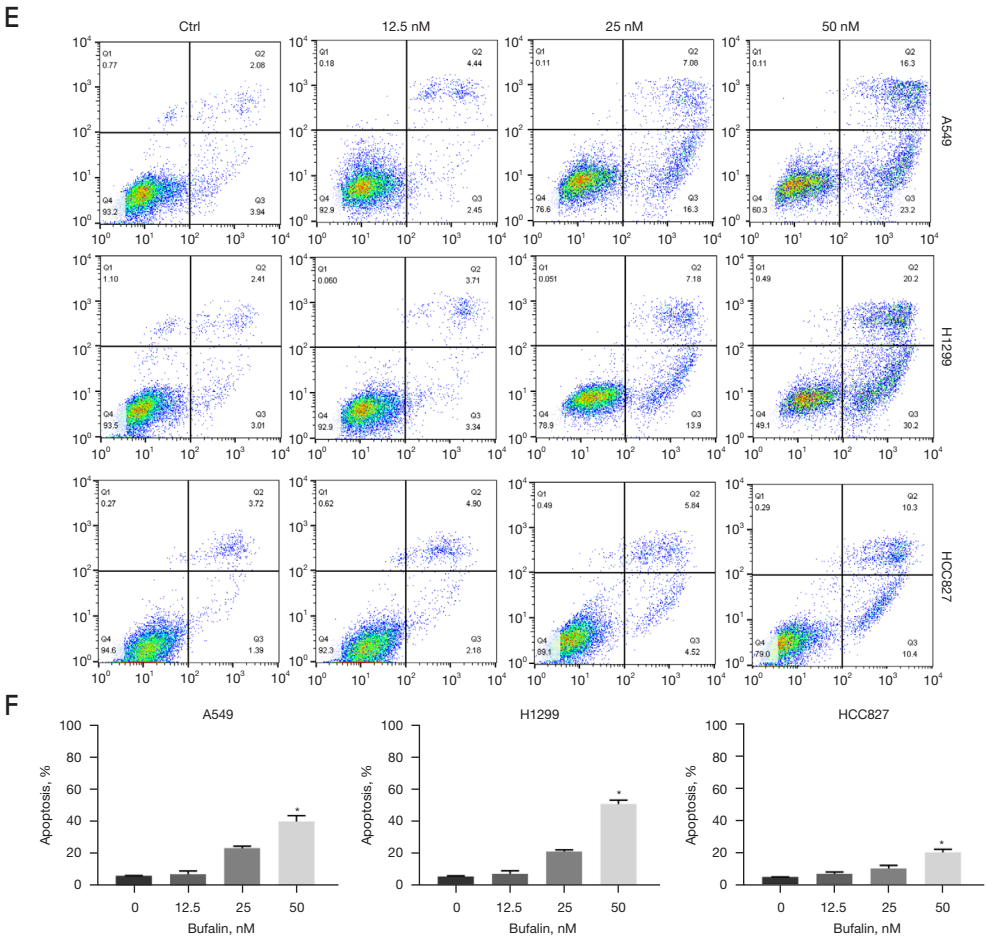
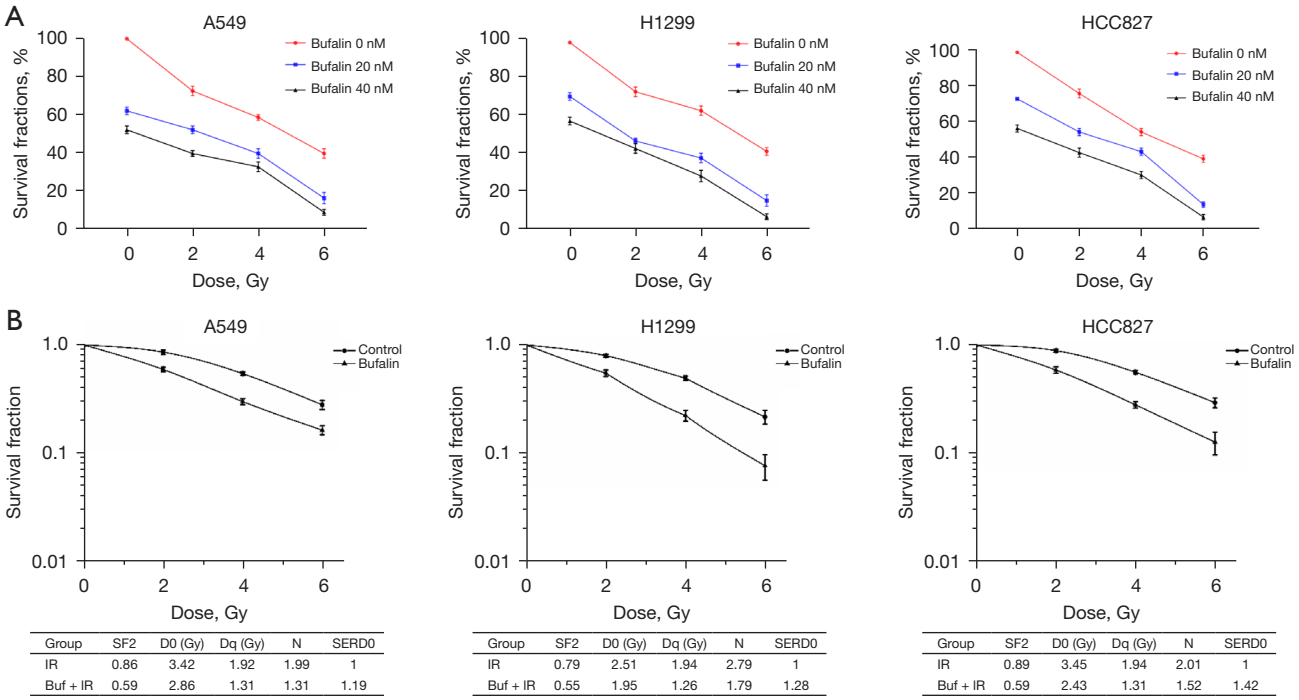


Figure 1 Bufalin decreased cell growth and viability in NSCLC. (A) Three NSCLC (A549, H1299, and HCC827) cell lines were incubated with bufalin at various concentrations (0 to 200 nM) for 24, 48, and 72 h, and then, the cell viability was assayed by CCK-8 assay. (B,C) Representative images of colonies stained with crystal violet after treatment with DMSO, 20, 50, and 100 nM bufalin. The colony numbers are shown on the right (magnification, ×1). (D-F) After treating cells with different concentrations of bufalin for 48 hours, the cell cycle and apoptosis were examined by flow cytometry. The apoptosis rates are shown on the right. *, P<0.05; ***, P<0.01 *vs.* untreated controls. Ctrl, control; NSCLC, non-small cell lung cancer; CCK-8, Cell Counting Kit-8; DMSO, dimethyl sulfoxide.

cytometry analysis showed that the percentage of cells in the G2-M phase increased in A549, H1299, and Hcc827 cells after treatment with bufalin (50 nM at 48 h) (*Figure 1D*). Moreover, compared with the control group, the proportion of apoptosis in the 50 nM bufalin treatment group was significantly increased (*Figures 1E,1F*). These findings suggest that bufalin inhibits NSCLC tumor growth by inducing G2-M cell cycle arrest and promoting programmed cell death.

Bufalin enhances the radiosensitivity of NSCLC cells

Radiotherapy is a critical component in the treatment strategy for NSCLC patients. We next investigated the effect of bufalin on radiosensitivity in NSCLC cells. The A549, H1299, and HCC827 cells were treated with bufalin in combination with and without radiation and radiation alone. After incubation of 24 hours, cell viability was assayed and showed that radiation (2/4/6 Gy) reduced cell survival in a dose-dependent manner. However, the inhibitor effect of a bufalin and radiation was significantly stronger than radiation or bufalin alone (*Figure 2A*). We also found that the combinational treatment decreased colony formation was more pronounced when compared to radiation alone (*Figure 2B*). These data revealed that bufalin and radiotherapy have a synergistic effect in NSCLC.



Group	SF2	D0 (Gy)	Dq (Gy)	N	SERD0
IR	0.86	3.42	1.92	1.99	1
Buf + IR	0.59	2.86	1.31	1.31	1.19

Group	SF2	D0 (Gy)	Dq (Gy)	N	SERD0
IR	0.79	2.51	1.94	2.79	1
Buf + IR	0.55	1.95	1.26	1.79	1.28

Group	SF2	D0 (Gy)	Dq (Gy)	N	SERD0
IR	0.89	3.45	1.94	2.01	1
Buf + IR	0.59	2.43	1.31	1.52	1.42

Figure 2 Bufalin enhances the radiosensitivity of NSCLC cells. (A) A549, H1299, and HCC827 cells were treated with bufalin in combination with and without radiation (2/4/6 Gy) and radiation alone for 48 hours; cell viability was assayed by CCK-8 assay ($P<0.01$ *vs.* untreated controls). (B) Radiation survival curves of untreated cells and bufalin treated cells after irradiation with 2, 4, and 6 Gy for 48 hours. The radiobiology parameters (D0, Dq, N, and SF2) and radiosensitivity ratios SERD0 of the 3 NSCLC cells were calculated using the formula $SF = 1 - (1 - e^{-D/D0})N$. Data are the mean. Buf, bufalin; IR, infrared radiation; Buf, bufalin; SF2, survival fraction of 2 cGy irradiation dose; D0, mean lethal dose value; Dq, quasi-threshold dose; N, extrapolation number; SERD0, sensitivity enhancement ratio of D0; NSCLC, non-small cell lung cancer; CCK-8, Cell Counting Kit-8.

Bufalin inhibits EMT and abrogates radiation-induced EMT

A previous study has suggested that EMT can enhance cell invasion and metastasis and induce tumor cells' resistance to radiation, chemotherapy, and immunotherapy (23). Our results showed that bufalin treatment led to a decrease in N-cadherin, vimentin, Snail, and Slug protein levels and an increase in E-cadherin expression in a dose-dependent manner when compared to untreated cells (*Figure 3A* and Figure S1A).

Next, we determined whether bufalin overrides radiation-induced EMT in A549 and H1299 cell lines. As expected, radiation decreased E-cadherin and upregulated N-cadherin, vimentin, Snail, and Slug. However, additional bufalin treatment completely abrogated the changes in these proteins induced by radiation (*Figure 3B* and Figure S1B). Immunofluorescence analysis further showed that bufalin overrode radiation effects on E-cadherin and vimentin (*Figure 3C* and Figure S1C). These findings suggested that bufalin enhancing radiosensitivity could be due to inhibition of EMT induced by radiation in NSCLC.

Bufalin inhibits the Src signaling

Src has been shown to have a critical role in the regulation of the resolution and initiation of EMT programs as well as cell migration and invasion (7,24). In addition, previous studies demonstrated bufalin inhibition of STAT3 activity (25,26). As Src is an upstream activator of STAT3 (27), we next investigated whether bufalin inhibits Src activity in 3 NSCLC cell lines examined. Western blot analysis revealed that bufalin considerably reduced the phosphorylation of Src-Y416, an autophosphorylation tyrosine residue present in the activation loop, which in turn reflected Src activation (*Figure 3D* and Figure S2A,S2B). Accordingly, phosphorylation of STAT3 was inhibited by bufalin. Immunofluorescent staining NSCLC cells with antibodies

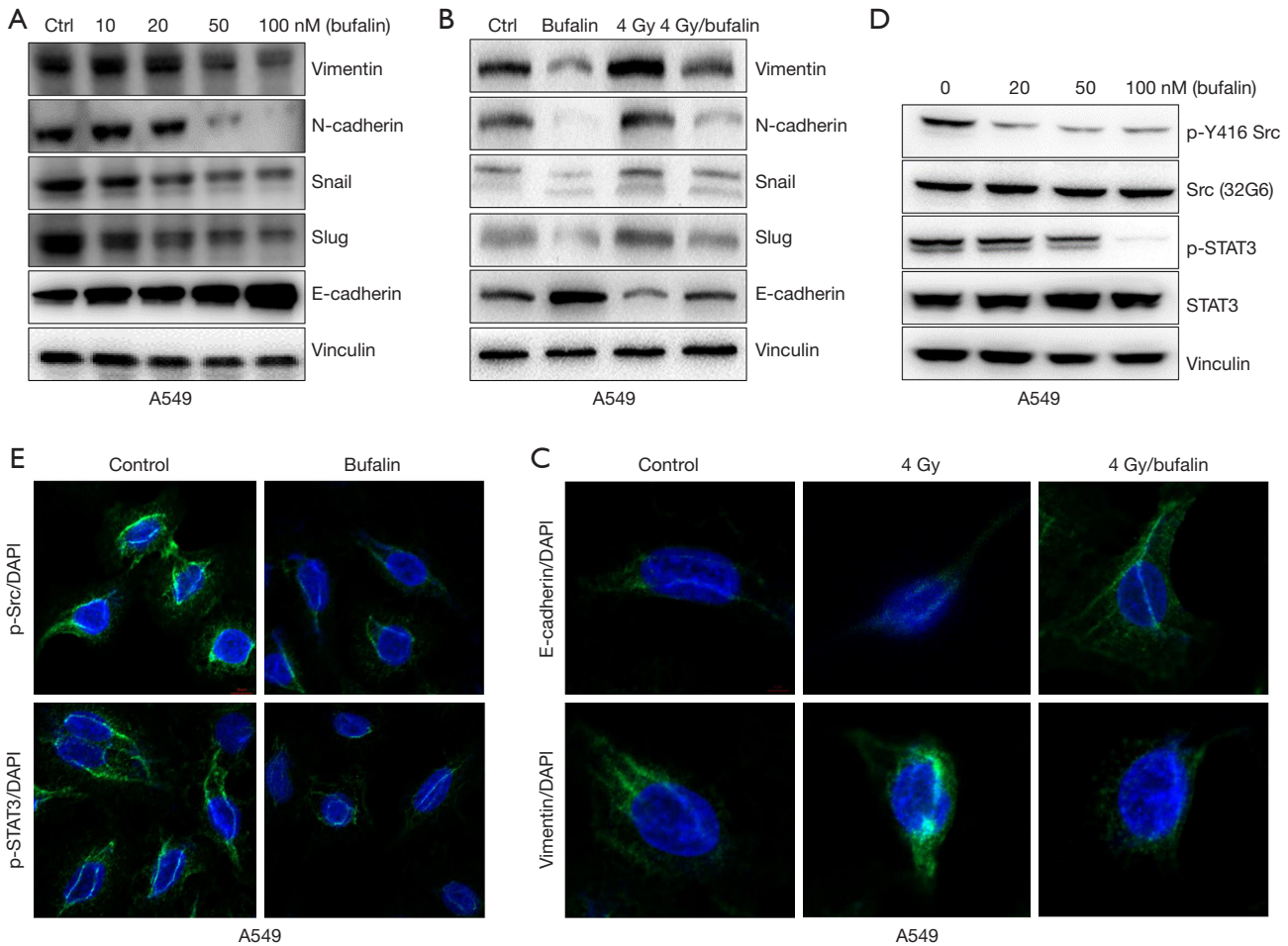


Figure 3 Bufalin inhibits EMT and abrogates radiation-induced EMT. (A) Western blot analysis for protein levels of E-cadherin, N-cadherin, vimentin, Slug, and Snail after treatment of A549 cells with increasing concentrations of bufalin. (B) Western blots to assess protein levels of E-cadherin, N-cadherin, vimentin, Slug, and Snail after treatment of A549 cells with DMSO, bufalin, radiation, and combined treatment (50 nM bufalin + radiation 4 Gy). (C) Cells were treated with radiation (4 Gy) or radiation plus bufalin; the changes of vimentin and E-cadherin in A549 cells were visualized by immunofluorescence microscopy. Scale bar, 20 μm. (D) Western blot analysis for protein levels of p-Y416 Src, Src (32G6), p-STAT3, and STAT3 after treatment of A549 cells with increasing concentrations of bufalin. (E) Representative immunofluorescence micrographs of A549 cells stained with antibodies against p-Y416 Src and p-STAT3. Scale bar, 20 μm. Ctrl, control; EMT, epithelial-mesenchymal transition; DMSO, dimethyl sulfoxide.

against p-Src and p-STAT3 also showed the suppression of p-Src and p-STAT3 by bufalin (*Figure 3E* and Figure S2C,S2D).

Src mediates bufalin action in cell mobility and EMT

Our data revealed that Src was significantly inactivated by bufalin. In order to evaluate whether Src is a major factor in bufalin repression of cell mobility and EMT in NSCLC, Src was knocked down in A549 and H1299 cells; 2 pLKO.1-Src-shRNAs (shSrc-A and shSrc-B) lentiviruses were used. Depletion of Src significantly reduced levels of p-src416 and p-STAT3 in A549 cells (*Figure 4A*). As shown in *Figure 4B*, compared with the control group, depletion of Src significantly affected the effect of bufalin on EMT-related proteins in A549 cells. Notably, compared with the shSrc groups, bufalin treatment failed to repress expression of N-cadherin and vimentin, and weakly increased E-cadherin level and repressed the expression of Slug and Snail. This indicates that bufalin almost completely inhibits vimentin

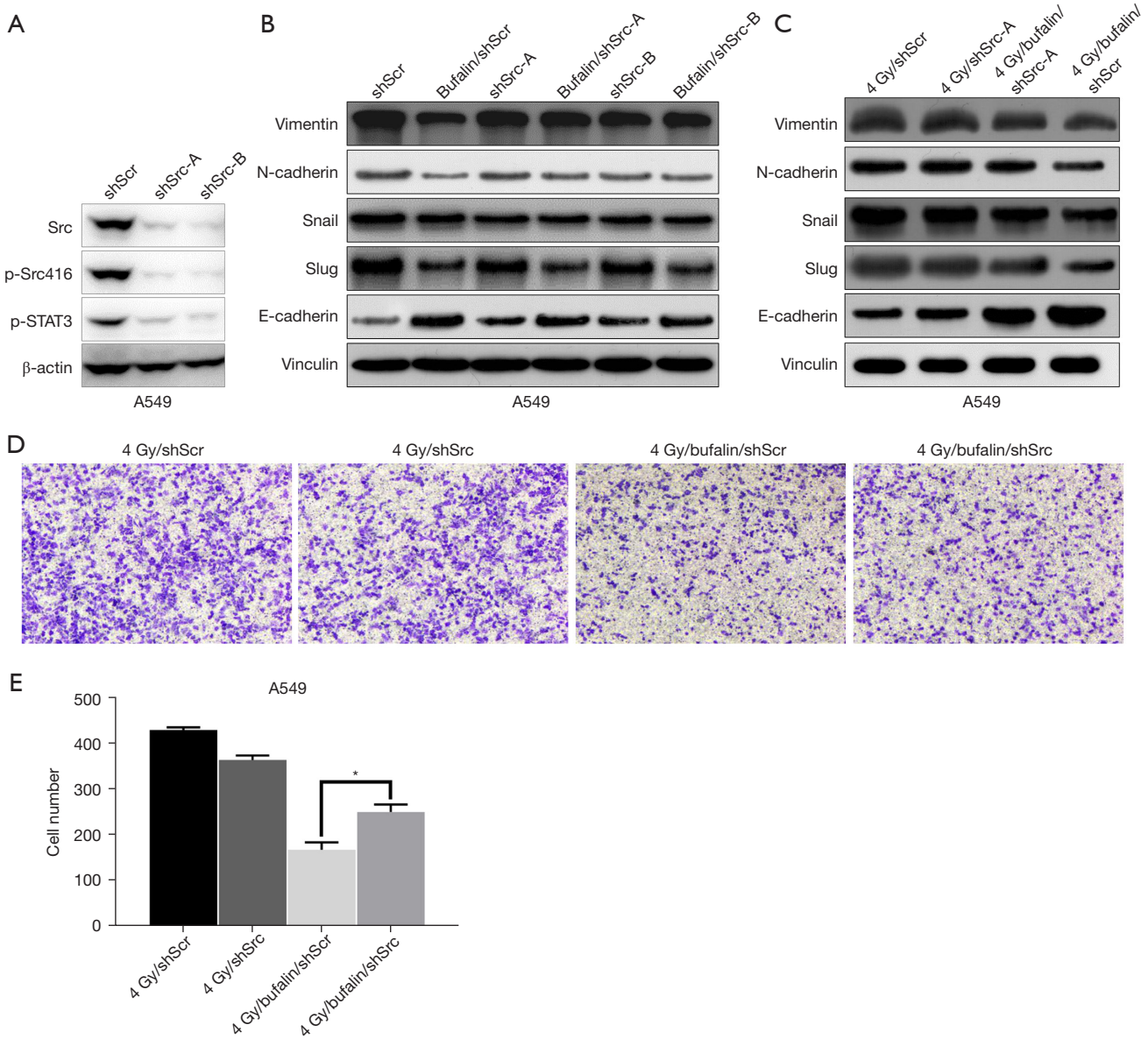


Figure 4 Src mediates bufalin action in cell mobility and EMT. The A549 cells infected with PLKO.1-shScr and pLKO.1-Src-shRNAs were treated with radiation (4 Gy) or 50 nM bufalin. (A,B) A549 cells were infected with pLKO.1-Src-shRNAs (shSrc-A and shSrc-B). At 48 h post-infection, the expression levels of the indicated proteins and EMT marker proteins were assessed by Western blot. The cells infected with pLKO.1-shScr virus was used as controls. (C) The expression levels of the indicated proteins and EMT marker proteins were assessed by Western blot analysis. (D,E) Transwell migration assay was performed in A549 cells. Crystal violet staining; scale bar, 100 μm. The relative migration was calculated by counting the number of stained cells. The data are expressed as the mean ± SD of 3 independent experiments. *, P<0.05. shScr, scramble shRNA; EMT, epithelial-mesenchymal transition; shRNA, short hairpin RNA; SD, standard deviation.

and N-cadherin through Src, but only partially affects Snail, Slug, and E-cadherin (*Figure 4B*). Moreover, depletion of Src largely abrogated bufalin-inhibited cell migration and invasion (Figures S3,S4).

We also examined whether knockdown of Src affects the radiosensitivity enhanced by bufalin. Although depletion of Src had little effect on the occurrence of EMT by simple irradiation, it obviously abrogated the effect of inhibiting the expression of vimentin and N-cadherin when bufalin is combined with radiotherapy, thereby significantly reducing the radiosensitization effect of bufalin (*Figures 4C-4E*).

To sum up, these findings indicate that bufalin inhibition of cell mobility and EMT, and enhancement of radiosensitivity are largely mediated by targeting Src and its signaling pathways.

Discussion

Radiotherapy is commonly used to treat inoperable NSCLC. Nevertheless, radiation resistance remains a major obstacle for successful treatment (28,29). Multiple factors contribute to radiation resistance, including radiation-induced EMT.

As a broad-spectrum anti-tumor agent, cinobufosin can inhibit cell proliferation and angiogenesis and induce cell apoptosis (16,30,31). Bufalin, a single extract of cinobufagin, can act as a potential radiosensitizer. In this research, we explored the inhibitory effect of bufalin on the migration and invasion of NSCLC cells and its effect on radiotherapy sensitivity.

The aberrant expression of Src is responsible for the disintegration of tight junctions and adhesion junctions and connected with the decrease of epithelial markers and the increase of mesenchymal markers in tumors (32-34). Consistently, our data revealed that an alteration of EMT marker expression induced by radiation could be partially or completely reversed by bufalin in NSCLC cells, and bufalin notably reduces the expressions of Src and downstream proteins, which indicates that bufalin inhibits the initiation of EMT by regulating the activities of Src and STAT3, thus achieving the radiosensitization effect.

Our results also manifest that knockdown of Src reverses the expressions of N-cadherin and vimentin, but does not completely reverse the levels of all EMT markers. Hence, we speculate that inhibition of EMT is only one aspect of bufalin against tumor migration. Several studies have shown that H1299 cells have mesenchymal characteristics (35,36) and that A549 cells have more interstitial properties than epithelial features (37). Moreover, compared with complete EMT, partial EMT is more related to the invasive progression of cancer cells (38). In view of these discoveries, it might be of crucial importance to investigate whether bufalin thoroughly interrupts EMT to generate an epithelial phenotype or it merely inhibits EMT to produce a mixed E/M phenotype in part. The interaction between Src and STAT3 in the pharmacological process of bufalin has not yet been clarified. The STAT3 signaling activation relies on the phosphorylation at tyrosine 705 (Y705) triggered by a number of upstream tyrosine-specific kinases, including receptor tyrosine kinases (RTKs) and cytoplasmic tyrosine kinases (CTKs) containing Src (39,40). Based on previous studies, we elucidated that the inhibitory effect of bufalin on STAT3 activation can be enhanced by the inhibition of bufalin on Src. However, the interaction of Src and STAT3 with bufalin should be addressed by future research.

A recent study has pointed out that the root bark of *Morus alba* affects cancer cell migration and EMT markers by inhibiting STAT3 (41). Flavonoids compounds luteolin and quercetin can repress the migration and invasion of squamous cell carcinoma by reducing the Src/STAT3/S100A7 pathway (42). Although some natural products have been shown to be relatively effective inhibitors of EMT both *in vivo* and *in vitro*, meticulous studies are still warranted to verify their effects in clinical trials. These natural products have huge therapeutic potential in traditional chemotherapy and combined radiotherapy and chemotherapy.

Conclusions

To sum up, our findings provide novel insights into the mechanism of bufalin inhibiting NSCLC cell migration and invasion. We demonstrated that EMT mediated by Src and STAT3 has a vital role in the pharmacological activity of bufalin. Further investigations are warranted to assess the efficacy of bufalin in preclinical and clinical context and we prudently indicate that bufalin may be employed as a radiosensitizer in clinical practice.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1859/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1859/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1859/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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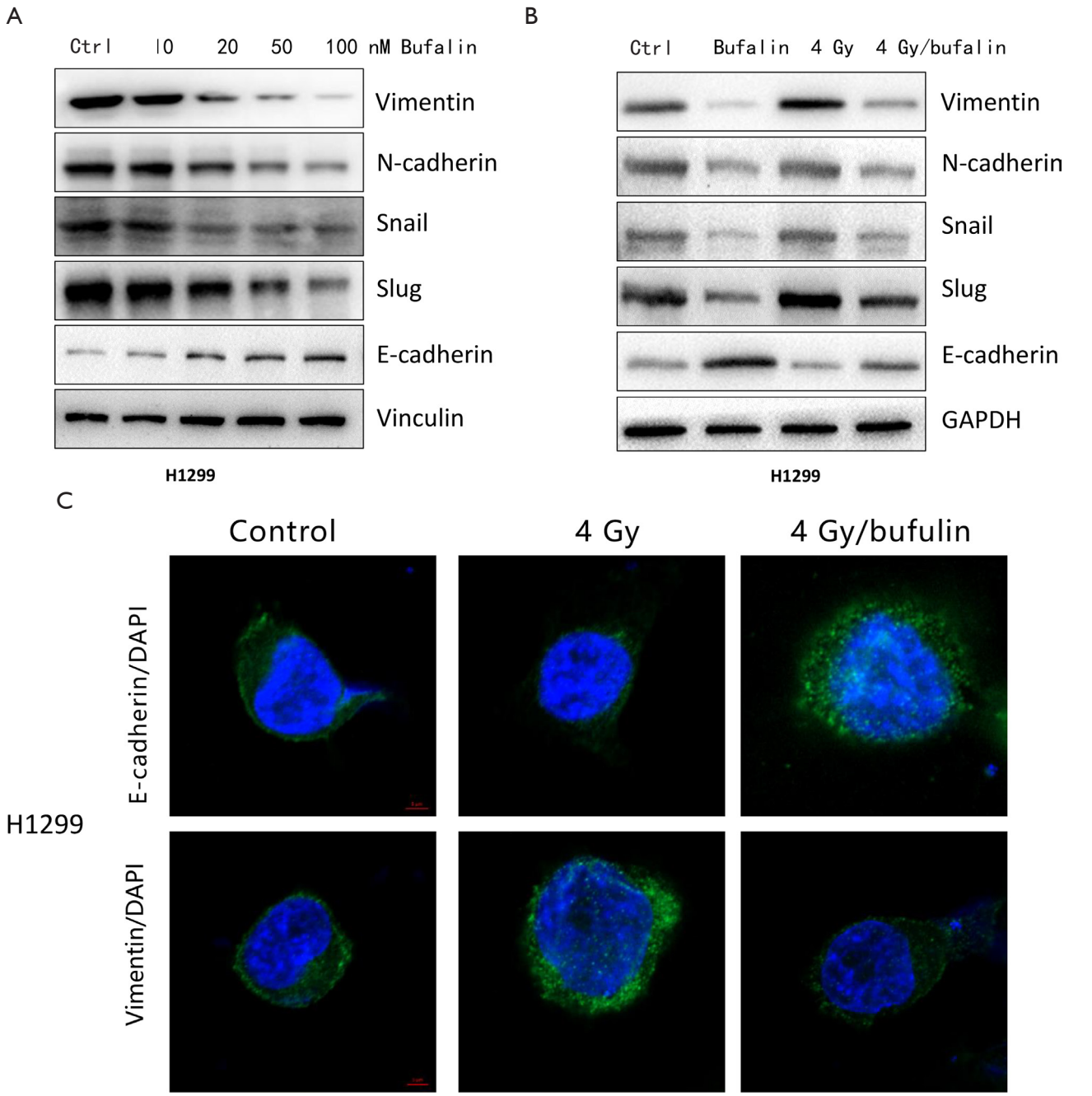

Figure S1 Bufalin inhibits EMT and abrogates radiation-induced EMT in H1299 cells. (A) Western blot analysis for protein levels of vimentin, N-cadherin, Snail, Slug, and E-cadherin after treatment of H1299 cells with increasing concentrations of bufalin. (B) Western blot to assess protein levels of vimentin, N-cadherin, Snail, Slug, and E-cadherin after treatment of H1299 cells with DMSO, bufalin, radiation, and combined treatment (50 nM bufalin + radiation 4 Gy). (C) Cells were treated with radiation (4 Gy) or radiation plus bufalin, the changes of vimentin and E-cadherin in H1299 cells were visualized by immunofluorescence microscopy (magnification ×200). Ctrl, control; DMSO, dimethyl sulfoxide; EMT, epithelial-mesenchymal transition.

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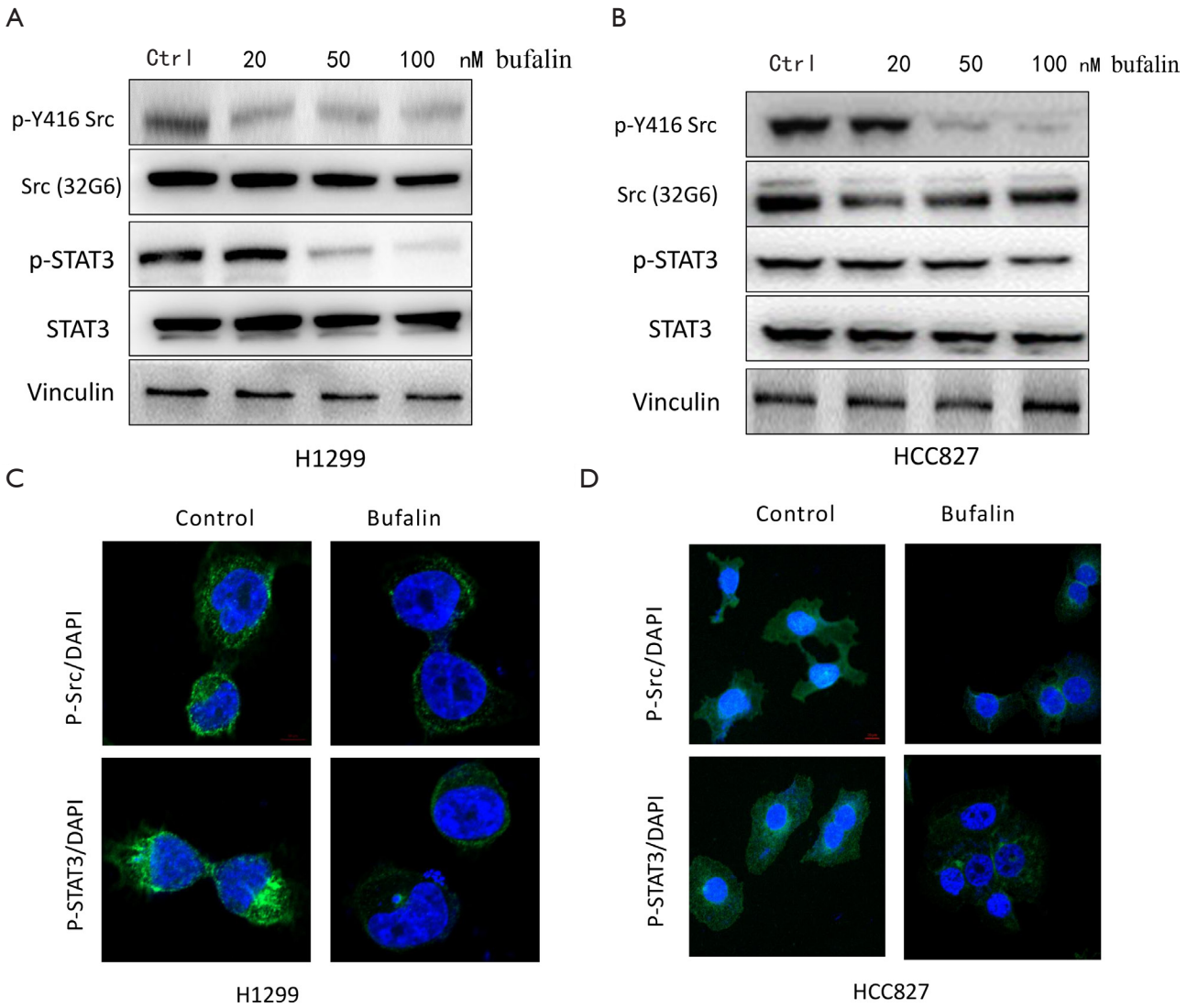

Figure S2 Bufalin reduced the phosphorylation of Src-Y416 and p-STAT3 in H1299 and HCC827 cells. (A,B) Western blot analysis for protein levels of p-Y416 Src, Src (32G6), p-STAT3, and STAT3 after treatment of H1299 and HCC827 cells with increasing concentrations of bufalin. (C,D) Representative immunofluorescence micrographs of H1299 and HCC827 cells stained with antibodies against p-Y416 SRC and p-STAT3 (magnification ×200). Ctrl, control; DMSO, dimethyl sulfoxide.

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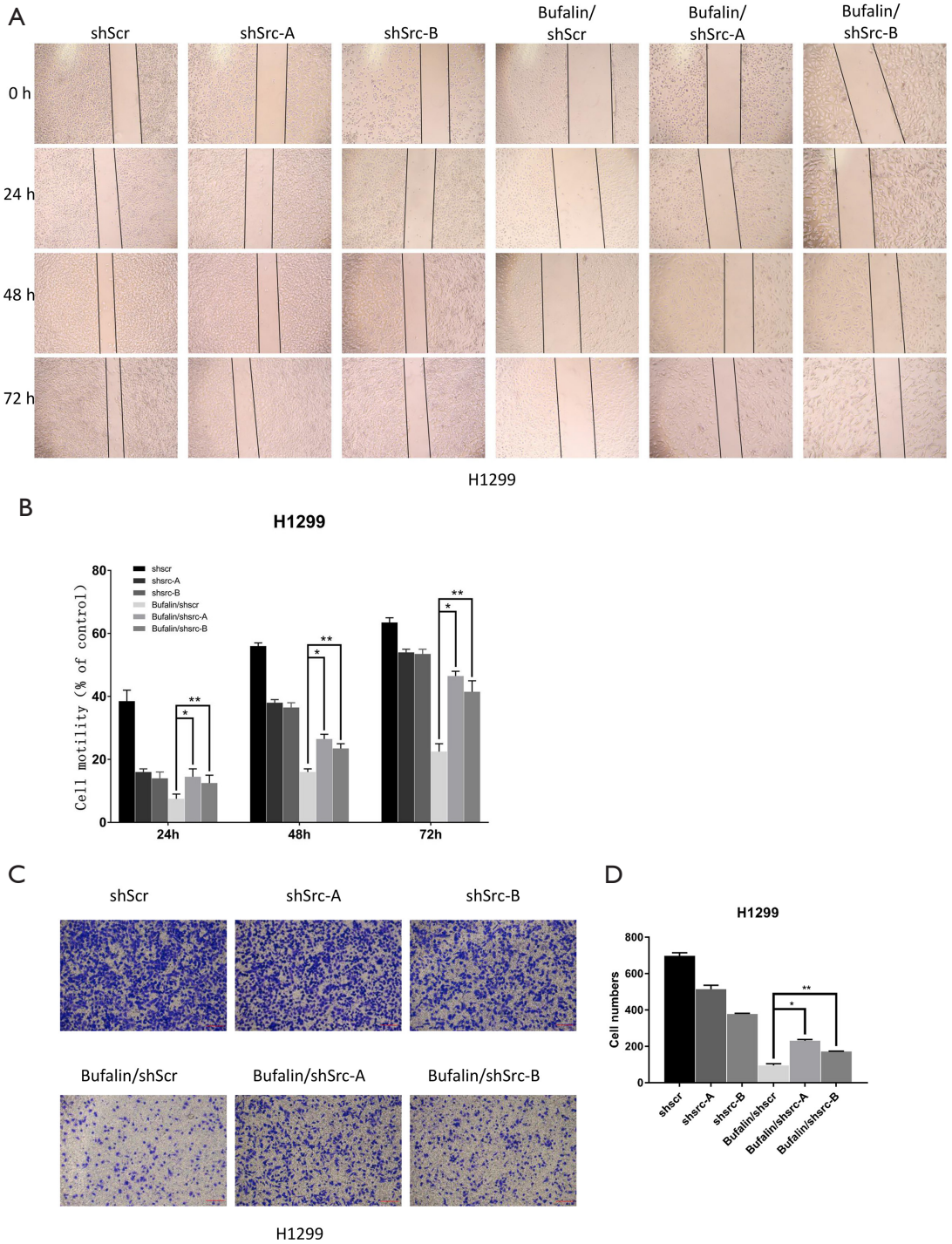

Figure S3 Depletion of Src abrogated bufalin-inhibited cell migration and invasion in H1299 cells. Wound healing assay and transwell migration assay were performed in H1299 cells. (A,B) The cells infected with PLKO.1-shScr and pLKO.1-Src-shRNAs (shSrc-A and shSrc-B) were treated with 50 nM Bufalin. The wound closure was monitored under a microscope for 72 h and photographs were taken at 0, 24, 48 and 72 h after wound generation (50× magnification), and the cell motility (% of control) was calculated. (C,D) The relative migration was calculated by counting the number of stained cells. Crystal violet staining; scale bar, 100 μm. The data are expressed as the mean ± SD of three independent experiments. *, P<0.05 *vs.* the cells infected with pLKO.1-Src-A-shRNAs virus; **, P<0.05 *vs.* the cells infected with pLKO.1-Src-B-shRNAs virus. shScr, scramble shRNA; SD, standard deviation.

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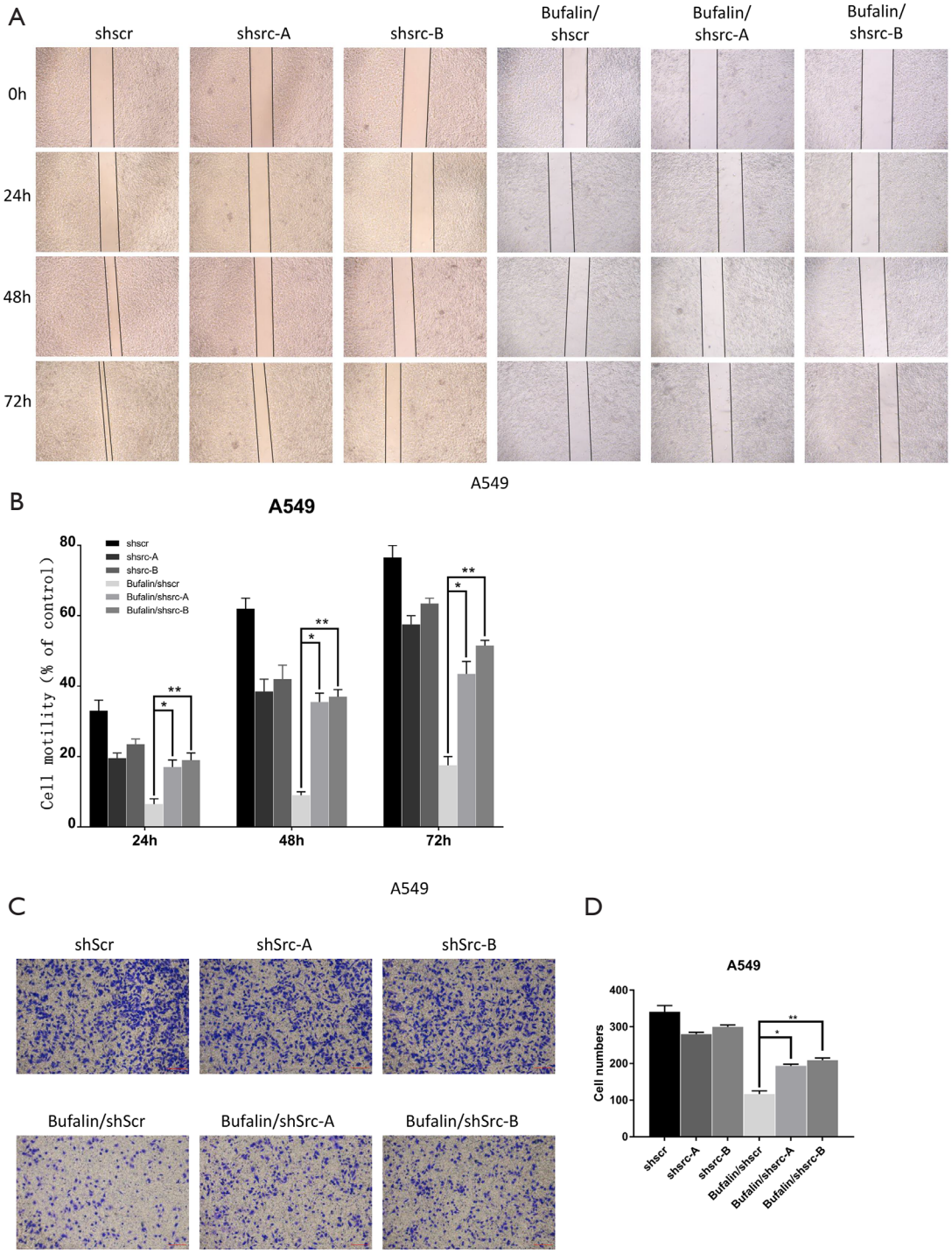

Figure S4 Depletion of Src abrogated bufalin-inhibited cell migration and invasion in A549 cells. (A,B) Wound healing assay and transwell migration assay were performed in A549 cells. The cells infected with PLKO.1-shScr and pLKO.1-Src-shRNAs (shSrc-A and shSrc-B) were treated with 50 nM bufalin. The wound closure was monitored under a microscope for 72 h, and photographs were taken at 0, 24, 48, and 72 h after wound generation (50× magnification), and the cell motility (% of control) was calculated. (C,D) The relative migration was calculated by counting the number of stained cells. Crystal violet staining; scale bar, 100 μm. The data are expressed as the mean ± SD of three independent experiments. *, P<0.05 *vs.* the cells infected with pLKO.1-Src-A-shRNAs virus; **, P<0.05 *vs.* the cells infected with pLKO.1-Src-B-shRNAs virus. shScr, scramble shRNA; SD, standard deviation.

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