Integrative Cancer Therapies
Volume 19: 1–11

DOI: 10.1177/1534735419900927
journals.sagepub.com/home/ict
SAGE

Nitidine Chloride Is a Potential Alternative Therapy for Glioma Through Inducing Endoplasmic Reticulum Stress and Alleviating Epithelial-Mesenchymal Transition

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Abstract

Background: Malignant glioma is a lethal brain tumor that is highly resistant to standard therapy. Our research aims to explore the suppressive effects of nitidine chloride (NC) on gliomas and the mechanisms involved, showing that it is a potential agent for integrative therapy of gliomas. **Methods:** After glioma cells were treated with NC, several experiments were performed to evaluate NC's antitumor effects. CCK-8 assay was used to detect viability. Transwell and 3-dimensional spheroid invasion assays were used to evaluate motility of glioma in vitro, and the sphere-formation assay showed NC's influence on glioma stem cells. Apoptosis and intracellular reactive oxygen species were measured by means of flow cytometry. Subcellular structures were observed through transmission electron microscopy. Western blot analysis reflected expression of endoplasmic reticulum (ER) stress and epithelial-mesenchymal transition (EMT) marker proteins. An orthotopic xenograft model was established to investigate the tumor suppressive effects in vivo. **Results:** Nitidine chloride inhibited glioma cell migration and invasion in vitro, downregulated the EMT proteins, and suppressed sphere formation of glioma stem cells. Furthermore, NC induced persistent ER stress that contributed to apoptosis and reactive oxygen species production. The xenograft model showed that NC effectively restricted glioma growth and invasion in vivo. Furthermore, we confirmed the signaling pathways that ER stress downregulates C/EBPβ and slug, as well as inhibition of the AKT/GSK3β/β-catenin axis caused by NC, in U-87 MG. **Conclusion:** We demonstrated that NC inhibits gliomas in vitro and in vivo by activating ER stress and downregulating EMT, which provides a basis for glioma therapy.

Keywords

glioma, nitidine chloride, endoplasmic reticulum stress, epithelial-mesenchymal transition, orthotopic xenograft

Submitted October 10, 2019; revised December 10, 2019; accepted December 26, 2019

Introduction

Gliomas are the most common primary brain tumor and are very harmful. Furthermore, the malignant tumors (World Health Organization grades III and IV) that have high possibility of recurrence undergo complex molecular mechanisms.[1] Treatments that combine surgery, chemotherapy, and radiotherapy do not prolong overall survival effectively, and the 5-year survival rate of glioblastomas remains at $<5\%$.[2,3] Therefore, there is an urgent need to find alternative avenues for malignant glioma treatment.

Nitidine chloride (NC) is a natural compound extracted from the root of *Zanthoxylum nitidum*. Several recent

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studies have shown that NC displays antitumor functions. In breast cancer, NC inhibits the motility of cancer cells by suppressing the c-Src/FAK pathway,[4] and attenuates both stemness and the epithelial-mesenchymal transition (EMT) by targeting the Hedgehog pathway.[5] In gliomas, NC targets the PI3K/AKT/mTOR signaling pathway, leading to apoptosis as well as the inhibition of motility and proliferation.[6] Furthermore, NC reduces the expression of protein pDok2, which leads to cell cycle arrest at G2/M.[7] Currently, there has been no research on the effect of NC on EMT in glioma cells, and there are limited studies showing homeostasis disturbance by NC.

Many studies have suggested that EMT plays an essential role in glioma cells. EMT is a biological process that allows epithelial cells to change to the mesenchymal cell phenotype with enhanced migration, invasive behavior, and resistance to apoptosis.[8] It was found that knockdown of the EMT transcription factor snail suppressed motility, viability, and proliferation of cancer cells.[9,10] Furthermore, EMT activator ZEB1 has been proven to generate chemoresistance and tumorigenesis in glioblastomas.[11] Therefore, targeting EMT in malignant gliomas could be a meaningful therapeutic strategy.

Endoplasmic reticulum stress (ER stress) is recognized as a regulator of homeostasis regarding the accumulation of misfolded proteins in the ER, which in turn leads to the unfolded protein response (UPR) in cells. This stress can be induced by various conditions, including hypoxia, infection, malnutrition, and chemical agents.[12] Studies have reported ER stress as a seemingly paradoxical process, with both pro-survival and anti-survival effects on cells, including glioma cells.[13] Cancer requires optimal ER stress conditions for survival. Solid tumors challenged by hypoxia and lack of nutrients generally possess high ER stress, and altered ER stress may be a basis of cancer therapy.[14] Additionally, a number of studies have suggested that some antitumor compounds inhibit tumors by activating ER stress.[15,16]

In our study, we sought to indicate that NC inhibits the viability and mobility of glioma cells by alleviating EMT and triggering persistent ER stress. An orthotopic xenograft model was established in order to confirm antitumor effects of NC in vivo. These findings provide evidence that NC has potential as an effective alternative agent for glioma treatment.

Materials and Methods

Reagents and Cell Lines

Human glioma cell lines U-87 MG and U251 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; D6429; Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (10100147; Gibco, Gaithersburg, MD), 100 units/mL penicillin, and 100 ng/mL streptomycin, then incubated at 37°C in 5% CO_2. All cell lines were authenticated by short tandem repeat profiling and proved to be free of mycoplasma contamination.

Nitidine chloride (N117977; Aladdin Biotech, Xi'an, China) was dissolved in 5 mM of dimethyl sulfoxide (D8418; Sigma-Aldrich). N-Acetyl-L-cysteine (NAC; A9165; Sigma-Aldrich) and 4-phenylbutyric acid (4-PBA; P21005; Sigma-Aldrich) were dissolved in DMEM at a concentration of 10 mM and 0.25 M, respectively.

Cell Viability Assay

Cell viability was assessed with Cell Counting Kit-8 (CCK-8; CK04-500; Dojindo, Tokyo, Japan). A total of 3.0×10^3 cells per well of the 96-well plate were treated with various concentrations of NC for 24 or 48 hours. After treatment, the medium was replaced with 100 μL serum-free DMEM with 10 μL CCK-8 solution, then was incubated for 4 hours at 37°C. The optical density was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

Cell Migration and Invasion Assays

Cell migration was assessed by transwell migration assay. After treatment, 2.0×10^4 cells were seeded into the upper chamber of the transwell apparatus (3464; Corning, Corning, NY). DMEM containing 10% fetal bovine serum was then added into the lower chamber. After incubation, cells were fixed with 4% paraformaldehyde and then stained with crystal violet. Cells in the upside of the membrane were scraped. Images were acquired using a Leica inverted microscope. Cell invasion was assessed by 3-dimensional spheroid BME cell invasion assay (3500-096-K; Trevigen, Gaithersburg, MD). A total of 3.0×10^3 cells/well tumor cells were seeded into a 96-well plate with 1× spheroid formation extracellular matrix for glioma spheroids formation, and then the invasion matrix and cell culture media with or without NC were added. The spheroid was photographed every 24 hours using a Leica microscope.

Isolation of Glioma Stem Cell and Sphere Formation Assay

Glioma stem cells (GSC) were derived from human primary glioblastoma tissues, and the culture method is detailed in Gao et al[17] and Bhat et al.[18] Freshly resected glioma tissue was cut into small pieces and digested by trypsin. Then, the dissociated single cells and remaining tissues were cultured with DMEM/F12 media (10565018; Gibco) supplemented with B27 (17504044; Gibco), 20 ng/mL EGF (236-EG; R&D Systems, Minneapolis, MN), and 20 ng/mL bFGF

(233-FB; R&D Systems). One to 2 weeks later, the floating tumor neurospheres were collected and subcultured in the media mentioned above. Regarding the sphere-formation assay, after digesting tumor neurospheres by Accutase (A6964; Sigma-Aldrich), 1000 single GSC cells per well were cultured with or without NC treatment. After 1 week, the spheres were observed with the Leica microscope.

Western Blot

Samples were lysed with RIPA containing 1% protease and phosphate inhibitor cocktail (P8340; Sigma-Aldrich). Proteins were run on 10% SDS-PAGE and then transferred to PVDF membranes, which were incubated with primary antibodies at 4°C overnight and probed by secondary antibodies. Blots were visualized with enhanced chemiluminescence (WBKLS0500; Millipore, Temecula, CA) and the ChemiDoc Touch detection system (Bio-Rad). The following primary antibodies were used: slug (9585; Cell Signaling Technology, Danvers, MA; 1:1000), β-catenin (8480; Cell Signaling Technology; 1:1000), p-eIF2α (3398; Cell Signaling Technology; 1:1000), p-GSK3β (5558; Cell Signaling Technology; 1:1000), GAPDH (ab8245; Abcam, Cambridge, England; 1:1000), C/EBPβ (ab32358; Abcam; 1:1000), N-cadherin (ab76057; Abcam; 1:1000), MMP-2 (ab92536; Abcam; 1:1000), AKT (ab8805; Abcam; 1:1000), p-AKT (ab81283; Abcam; 1:1000), vimentin (ab92547; Abcam; 1:5000), and cleaved caspase-3 (19677-1-AP; Proteintech, Rosemont, IL; 1:500).

Immunohistochemistry

Tissue sections were boiled in sodium citrate buffer (pH 6.0) for antigen retrieval, with H_2O_2 to eliminate endogenous horse radish peroxidase activity. The sections were blocked using 10% goat serum and incubated with primary antibodies overnight at 4°C. The slides were incubated with secondary antibodies, then stained by diaminobenzidine counterstaining mixed with hematoxylin. Images were obtained using a Leica microscope.

Intracellular Reactive Oxygen Species Detection

After treatment, glioma cells were loaded with 10-μM H2DCFDA (D399; ThermoFisher, Waltham, MA) at 37°C for 30 minutes. Uncombined probes were rinsed by phosphate-buffered saline (PBS). Then the intracellular reactive oxygen species (ROS) was immediately measured by BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA).

Annexin V/Propidium Iodide Staining for Apoptosis

Cell apoptosis was detected using a FITC Annexin V Apoptosis detection kit (556547; BD Biosciences). Cells were washed with PBS and then resuspended in binding buffer at a concentration of 1×10^6 cells/mL. Five microliters of FITC-annexin V and propidium iodide staining were added into a 100 μL suspension. After incubating at room temperature for 15 minutes, 400 μL 1× binding buffer was added. Samples were analyzed by BD Accuri C6 flow cytometer.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed following Wang et al.[19] After treatment with either NC or 4-PBA, the morphology of organelles was observed by TEM.

Xenograft Model

The animal model was established conforming to the national guidelines of the Institutional Animal Care and Use Committee. A total of 3×10^5 U-87 MG-luciferase cells were intracranially injected in the right frontal lobe of 4-week-old male nude mice. After implantation, the mice were intraperitoneally injected with either 10 mg/kg/day NC or PBS containing the same dose of dimethyl sulfoxide. Administration was performed at 8 PM each day until the animals were euthanized. Every 4 days, the mice were injected intraperitoneally with 150 mg/kg luciferin, taking bioluminescence images through ex vivo Imaging System IVIS Lumina series III (PerkinElmer, Waltham, MA). Magnetic resonance images were obtained at the Department of Radiology, Qilu Hospital of Shandong University, on day 12.

Statistical Analysis

Data analyses were performed using the SPSS 20.0 and the GraphPad Prism 6. Results are presented as the means ± standard deviations. The Student's *t* test was used to analyze the statistical difference between the 2 groups. The log-rank test was used to evaluate the probability of mice survival. For all statistical analyses, $P < .05$ was considered statistically significant. *P* values are indicated as follows: $*P < .05$, $**P < .01$, and $***P < .001$.

Results

Nitidine Chloride Inhibits the Viability of Glioma Cells

The chemical structure of NC is shown in Figure 1A. To determine the effect of NC on glioma cells, CCK-8 cell viability assay was used. U-87 MG and U251 were individually treated with different concentrations of NC for 24 and 48 hours, respectively (Figure 1B). As demonstrated in the curve, NC treatment inhibited the viability of glioma

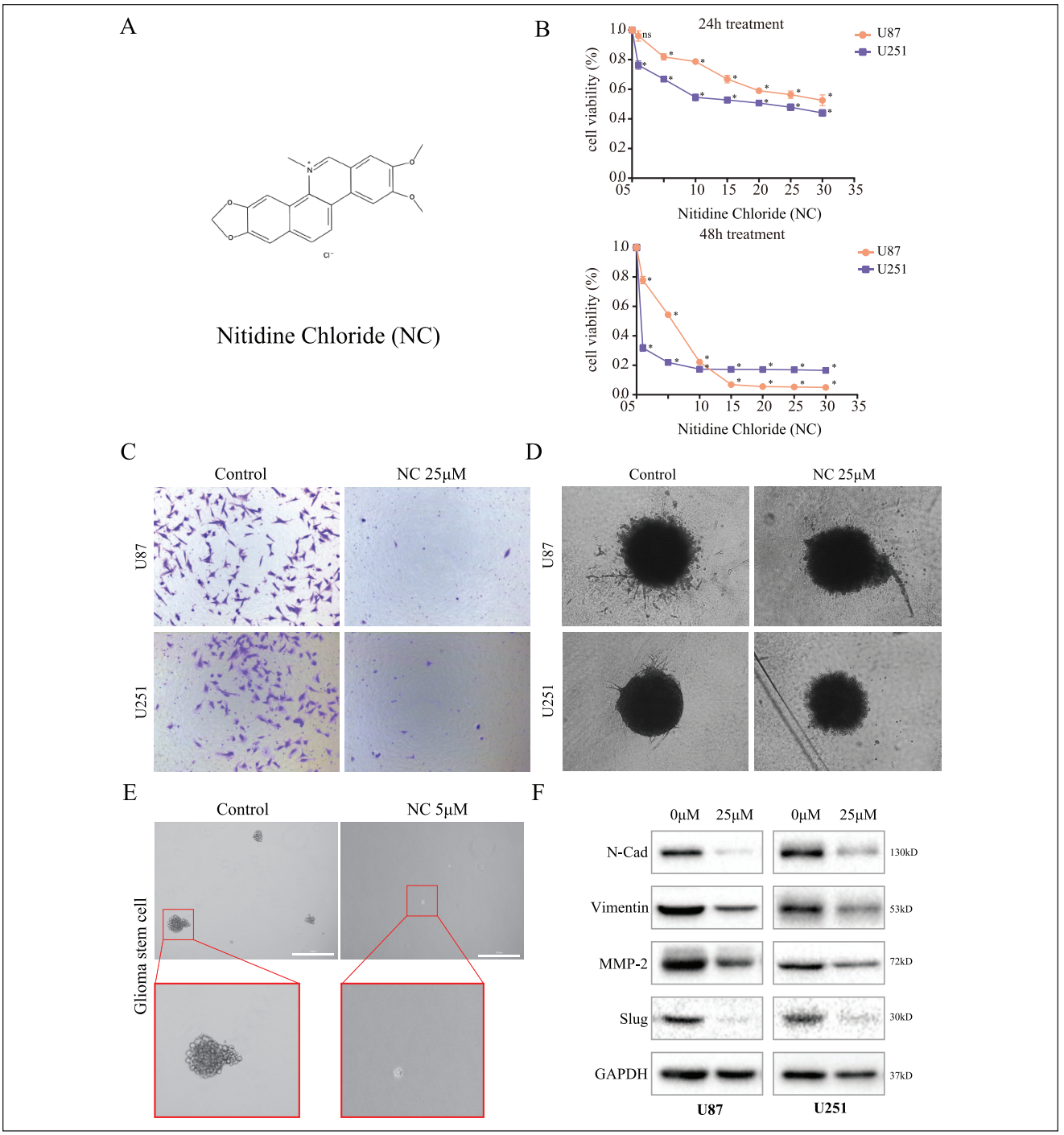

Figure 1. Nitidine chloride (NC) inhibits viability and motility of glioma cells. (A) The chemical structure of NC. (B) Viability of U-87 MG and U251 treated with different concentration of NC were determined by CCK-8 for 24 and 48 hours (*$P < .05$). (C) Transwell assay for migration and (D) 3-dimensional spheroid cell invasion assay indicated that 25 μM NC treatment for 24 hours suppress motility of glioma significantly in vitro. (E) Five micromole NC treatment inhibited sphere formation of glioma stem cell significantly. (F) Western blot for N-cadherin, vimentin, MMP-2, slug, and GAPDH in U-87 MG and U251 treated with 25 μM NC for 24 hours.

cells in a time-dependent and concentration-dependent manner. For U-87 MG and U251, treatment with 25 μM NC for 24 hours eliminated approximately 50% viability. The statistical data are given in the supplementary material (Supplemental Tables S1 and S2, available online).

Nitidine Chloride Inhibits Migration and Invasion of Glioma Cells

The transwell assay was used to investigate the effects of NC on glioma cell migration. As shown in Figure 1C, U-87 MG and U251 cell lines were treated with 25 μM NC for 24 hours; cell migration was attenuated significantly with NC treatment. Three-dimensional spheroid invasion assay was applied to measure invasion. After 25 μM NC exposure, the protrusion and invasion areas of glioma cells decreased significantly (Figure 1D); Figures show the invasion procedure at 48 hours after spheroid formation. Having considered the correlation between the EMT and cell motility, several EMT markers were determined by western blot. We found that NC decreased expression of the EMT markers N-cadherin, vimentin, MMP2, and slug.

Nitidine Chloride Inhibits Sphere Formation in Glioma Stem Cells

A total of 1×10^3 single cells of GSC (with or without 5 μM NC treatment) were seeded into 6-well plates and cultured in suitable conditions for 1 week; GSC without NC treatment showed normal sphere formation, whereas cells cultured with NC did not (Figure 1E). Thus, it appears that NC suppresses the viability of GSC.

Nitidine Chloride Activates Endoplasmic Reticulum Stress and Elevates Intracellular ROS

As shown in Figure 2A, we applied TEM to observe organelle morphology, with the red arrow indicating the ER. The ER was narrow and regular in control U-87 MG cells. However, after 25 μM NC treatment for 24 hours, the ER had become dilated and swollen. This change could be rescued by co-treatment with 2.5 mM 4-PBA, a chemical chaperone that acts as an inhibitor of ER stress. We further determined the ER stress signaling pathway using western blot analysis. Figure 2B shows that the expression of phospho-eIF2α increased significantly, while co-treating with 4-PBA lowered phospho-eIF2α. These results demonstrate that ER stress in glioma cells was activated in response to NC. Moreover, we detected intracellular ROS levels of glioma cells when treated with NC by using H2DCFDA. Twenty-five micromole NC treatment for 24 hours elevates intracellular ROS significantly (Figure 2C); Figure 2D is the quantified ROS generation. We had anticipated to discover the function of elevated ROS, but instead discovered that ROS generation cannot be effectively suppressed by NAC.

ER Stress Triggered by Nitidine Chloride Has a Close Relationship With Apoptosis and ROS Overload

We then explored what part ER stress plays in glioma cells treated with NC. We used 4-PBA to inhibit ER stress activated by NC. Figure 3A and B shows that inhibiting ER stress caused by NC reversed downregulation of slug but showed no change in N-cadherin, vimentin, or MMP-2. Five micromole 4-PBA treatment shows the same outcome. Due to the close relationship of ROS and ER stress, we measured ROS levels after treatment.

Figure 3C shows that using 4-PBA significantly reduced ROS level elevated by NC. As NC can cause cell apoptosis, we then investigated whether ER stress influences glioma cell apoptosis under NC treatment. Figure 3D and E shows a clear trend that apoptotic cells decreased significantly after 4-PBA co-treatment compared with the NC group. As cleaved caspase3 (c-casp3) is a key protein involved in apoptosis execution, Figure 3F shows that NC treatment increased c-casp3, whereas 4-PBA co-treatment decreased its expression. Interestingly, cells treated with only 4-PBA reduced apoptosis and c-casp3.

Nitidine Chloride Inhibits Glioma Progressing In Vivo

To investigate the effect of NC on gliomas in vivo, we established an orthotopic xenograft model (control vs NC treatment, $n = 3$ animals per group). Figure 4A indicates that intraperitoneal administration of NC (10 mg/kg/day) restricted tumor growth, which is reflected in the bioluminescence value. T2WI-MRI in Figure 4B shows that the NC-treated animal has less tumor volume. As shown in Figure 4C, mice had longer median survival rates after NC treatment when compared with the control group (21 days vs 15 days). Hematoxylin-eosin staining in Figure 4D shows a lower tumor volume with NC treatment. Immunohistochemistry shows that NC downregulated expression of vimentin, MMP-2, and Ki-67 in orthotopic xenografts (Figure 4E). These results suggest that NC effectively inhibits glioma progression in vivo.

Nitidine Chloride Downregulates C/EBPβ and β-Catenin Involved in EMT Pathways

We have demonstrated that suppressed ER stress caused by NC partially reversed downregulation of slug. According to Wu et al,[20] activated ER stress downregulates C/EBPβ in gastric cancer through the calpain cascade, thus decreasing slug. As shown in Figure 5A, decreased C/EBPβ and slug with NC

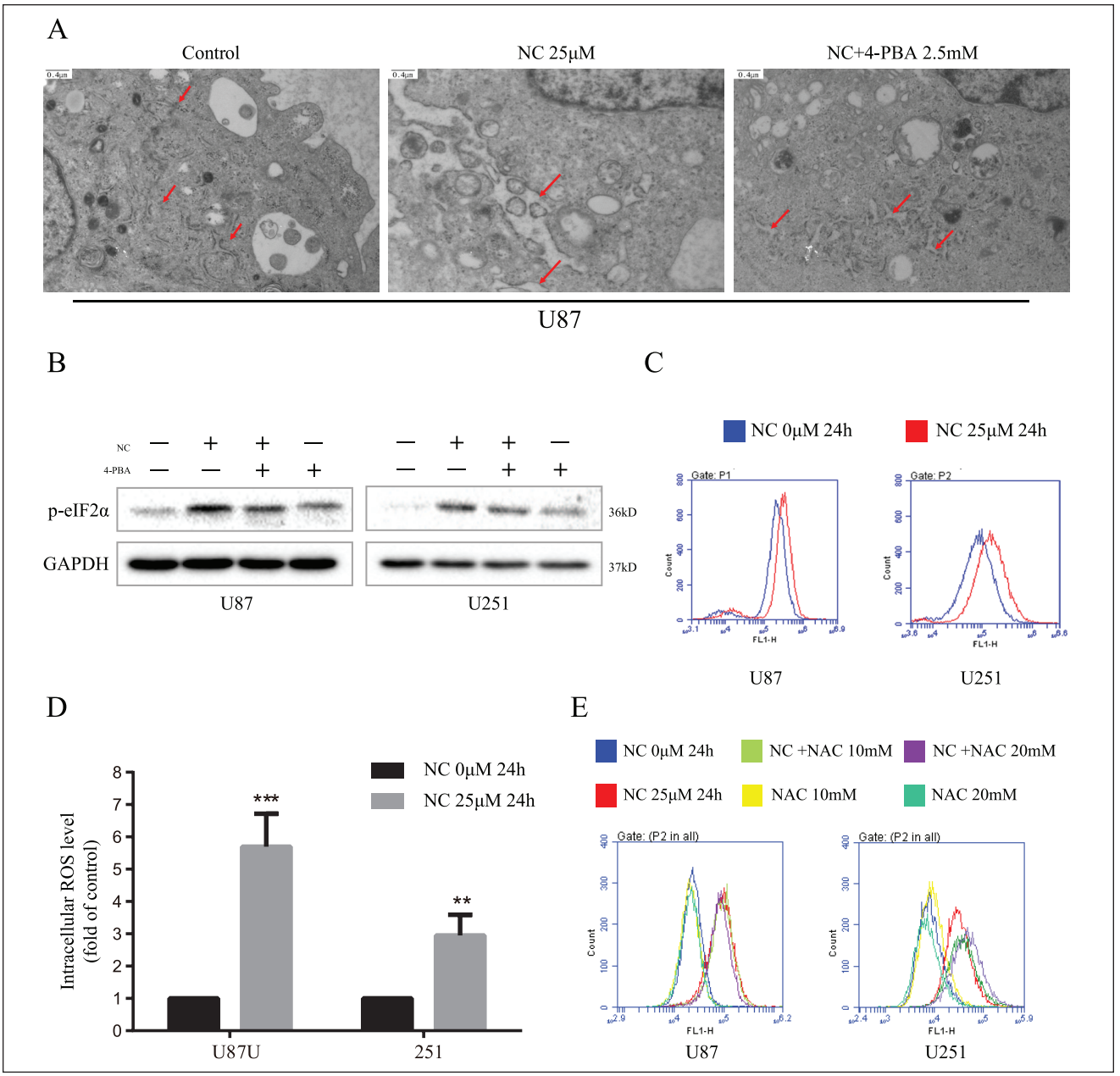

Figure 2. Nitidine chloride (NC) activates endoplasmic reticulum (ER) stress and elevates intracellular reactive oxygen species (ROS). (A) U-87 MG cells were treated with 25 μM NC solely or co-treated with 2.5-mM 4-PBA for 24 hours. Morphology of ER organelles was observed by transmission electron microscopy (red arrows indicate ER lumen). (B) U-87 MG and U251 treated with NC or co-treated with 4-PBA for 24 hours. Western blot for p-eIF2α and protein loading control GAPDH. (C) Treatment of 25 μM NC for 24 hours elevated intracellular ROS in U-87 MG and U251 with the quantification and statistical analysis (D). (E) U-87 MG and U251 were pretreated with NAC (10 mM or 20 mM) for 2 hours before exposure of 25 μM NC for 24 hours. NAC failed to reduce intracellular ROS production in glioma cells. Data are represented as the mean ± standard deviation of results from 3 independent experiments. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

treatment was rescued by 4-PBA. However, inhibition of ER stress had no distinct influence on other EMT markers, which suggests that there may be other mechanisms involved.

In a previous study by Cheng et al,[21] NC suppressed AKT phosphorylation to maintain GSK3β in osteosarcoma cells. Furthermore, activated GSK3β degraded β-catenin,[22] which is vital in EMT.[23,24] In our study, NC downregulated p-AKT, p-GSK3β, and β-catenin in glioma cells (Figure 5B). Thus, we hypothesized that NC inhibits EMT by means of ER stress–mediated C/EBPβ degradation and the AKT/GSK3β/β-catenin axis. Figure 5C summarizes the mechanisms involved in this process.[20]

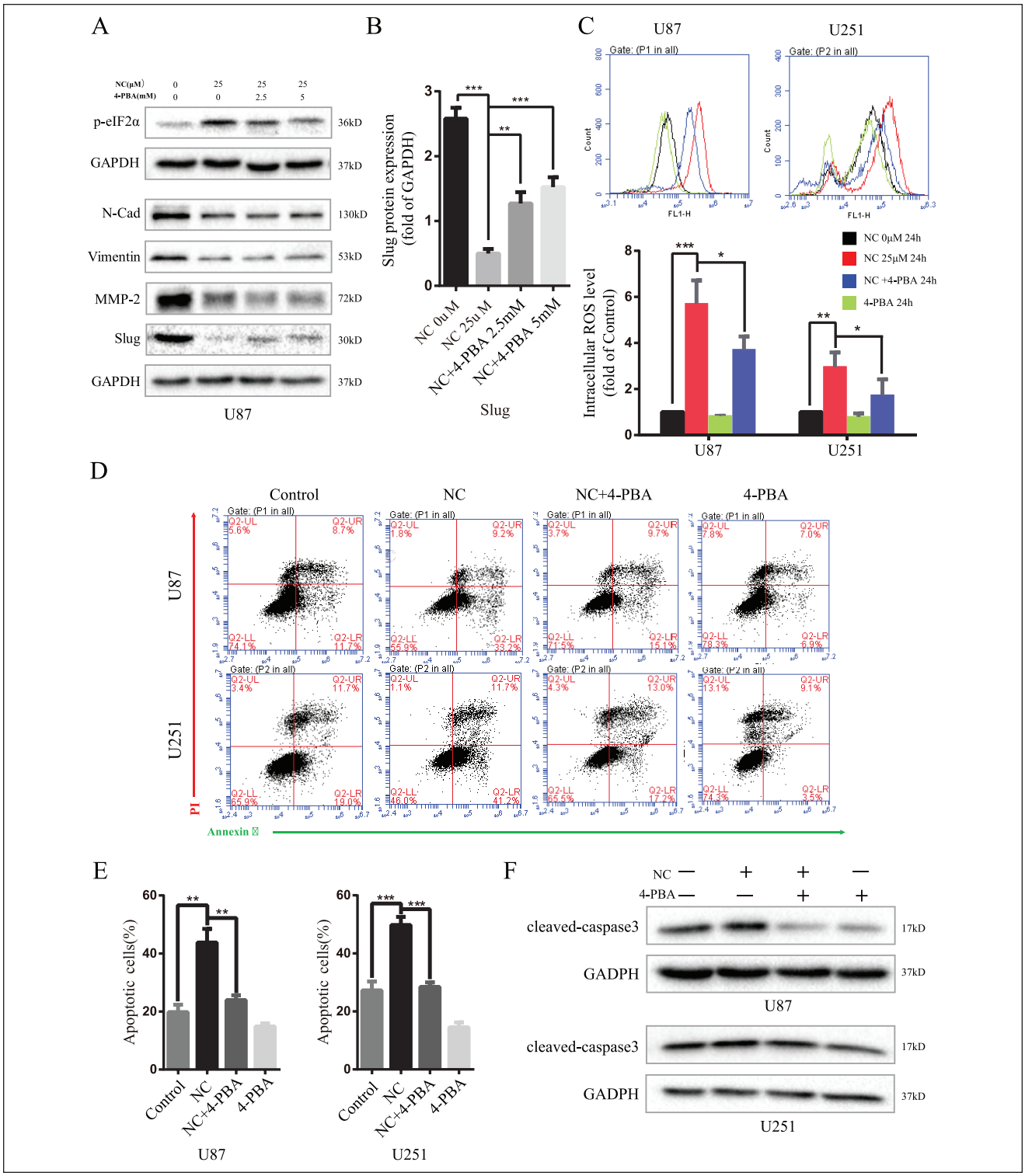

Figure 3. The effect of using 4-PBA to attenuate endoplasmic reticulum stress on epithelial-mesenchymal transition (EMT) and apoptosis. (A) Twenty-five micromole nitidine chloride (NC) treated U-87 MG solely or co-treated with 4-PBA (2.5 mM and 5 mM) for 24 hours. Western blot for p-eIF2α and EMT associated protein. (B) Relative intensity for slug expression performed by ImageJ. (C) A total of 2.5 mM 4-PBA treatment rescued reactive oxygen species (ROS) production in U-87 MG and U251 treated with NC for 24 hours. The histogram is the quantification and statistical analysis for ROS production. (D) Annexin V/propidium iodide staining indicated that 2.5 mM 4-PBA treatment rescued apoptosis induced by NC in U-87 MG and U251, and right histogram (E) shows statistical analysis. (F) Western blot for cleaved-caspase 3 and GAPDH. Data are represented as the mean ± standard deviation of results from 3 independent experiments. *$P < .05$, **$P < .01$, and ***$P < .001$.

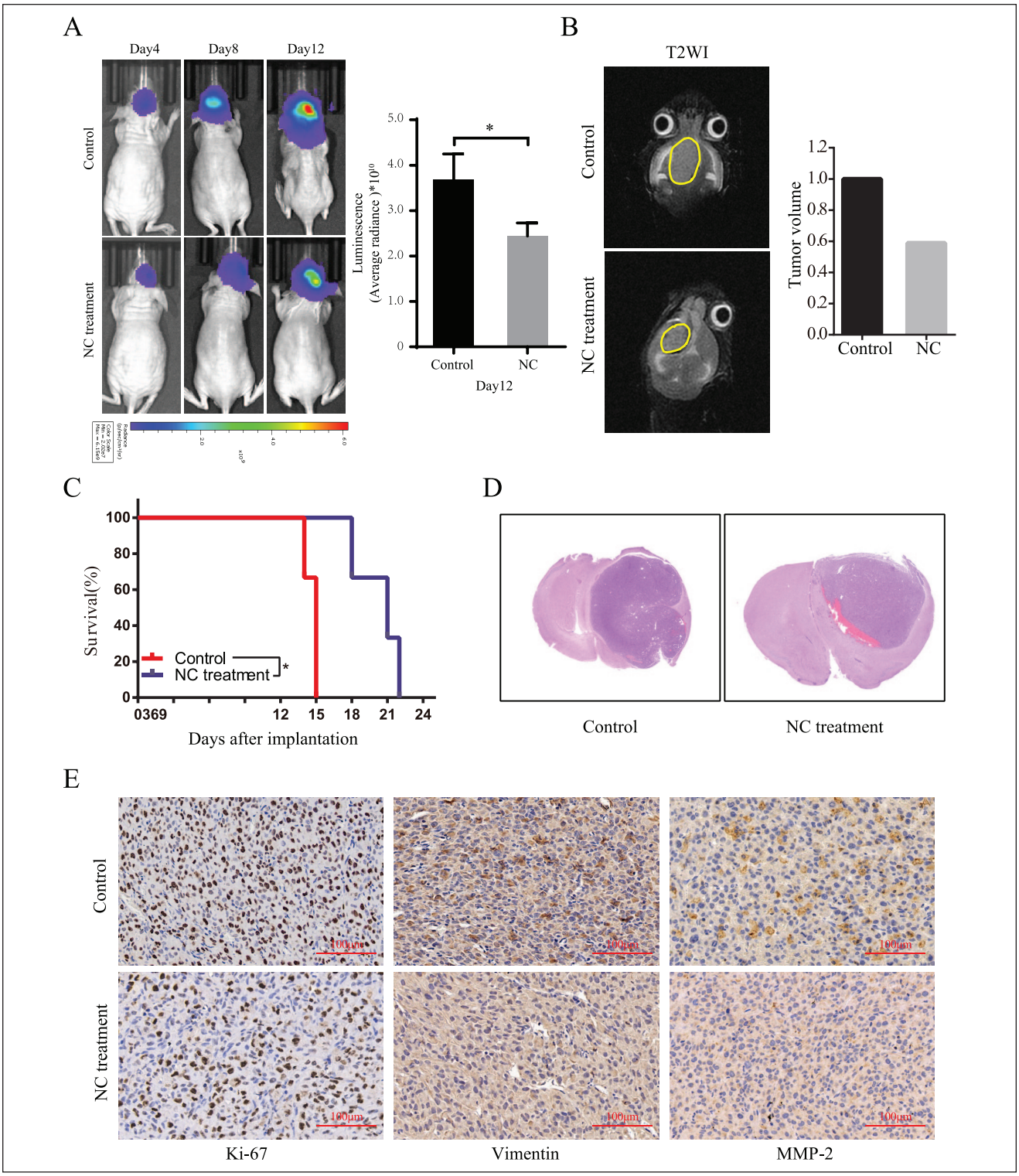

Figure 4. Nitidine chloride (NC) inhibits glioma progression in vivo. (A) A total of 3×10^5 U-87 MG cells with luciferase were injected into frontal lobe of brain to establish a xenograft model in mice. Mice were injected with 10 mg/kg/day NC or phosphate-buffered saline vehicle (n = 3) intraperitoneally. The tumor volume was detected by bioluminescence at days 4, 8, and 12. (B) T2-weighted magnetic resonance imaging showed tumor size in mice brains (yellow line) and quantification for tumor size comparison. (C) Survival analysis for xenograft control mice and NC-treated mice ($P < .05$ by log-rank test, 3 animals per group). (D) Hematoxylin-eosin staining of brain sections indicated tumor volume and margin. (E) Immunohistochemistry showed that NC downregulates expression of vimentin, MMP-2, and Ki-67 in vivo. *$P < .05$, **$P < .01$, and ***$P < .001$.

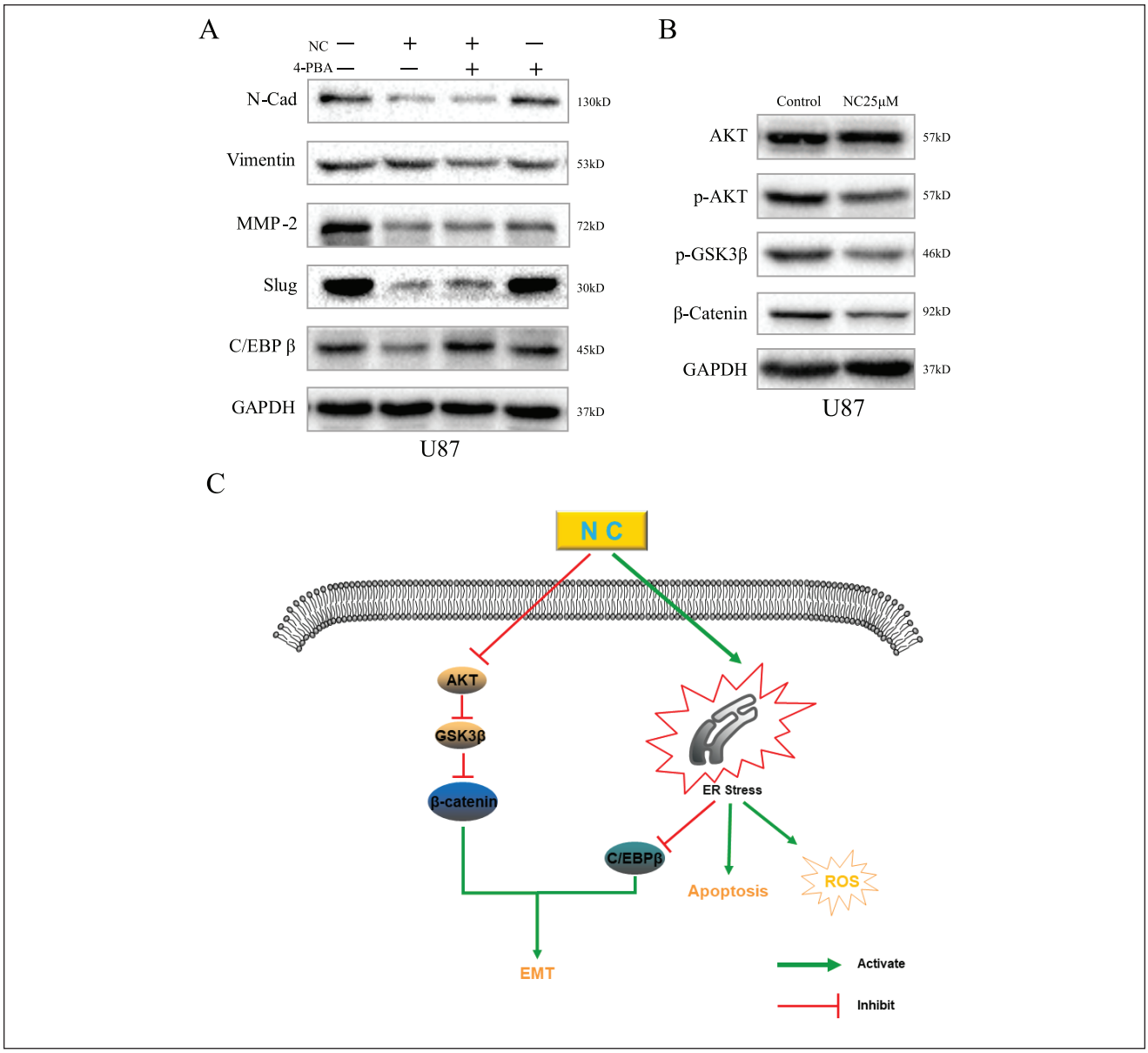

Figure 5. The pathways participating in downregulating EMT (epithelial-mesenchymal transition) and summary diagram. (A) Nitidine chloride (NC) downregulated slug via ER stress-C/EBP β pathway. Twenty-five micromole NC-treated U-87 MG cells downregulated N-cad, vimentin, MMP-2, and slug. Pretreatment with 2.5-mM 4-PBA reversed slug expression, but had no apparent effect on other proteins. (B) Treatment with 25 μM NC U-87 MG cells for 24 hours inhibited the AKT/GSK3β/β-catenin pathway. (C) Summary diagram for mechanisms of glioma cell suppression by NC.

Discussion

Prior studies have noted the antitumor effect of NC in various malignant tumors.[7,25,26] In our study, we have demonstrated that NC inhibits viability and mobility of malignant gliomas in vitro. We have focused on how NC alleviates EMT and induces apoptosis in glioma cells, thus attenuating the progression of cancer. A xenograft model was established to determine that NC inhibits glioma progression in vivo. These results indicate that NC acts as an effective therapeutic strategy for gliomas.

Endoplasmic reticulum stress plays a vital role as a homeostasis regulator for the survival and expansion of malignant cancer. In our study, we investigated the involvement of ER stress in anti-glioma effects of NC. As shown in Figure 2A and B, NC treatment altered the morphology of the ER and elevated the phosphorylation of eIF2α, while using 4-PBA, an ER stress inhibitor, tended to reverse these changes. As the double-edged sword, ER stress and consequent UPR seem to have contradictory functions in cancer progression: the signal that activates ER stress can be either protective or pro-apoptotic.[27]

PERK/eIF2α/ATF4 is a main branch of ER stress signaling. The ER stress/UPR and subsequent eIF2α phosphorylation has been proven to be an essential part in immune modulation and hypoxia adaption.[28,29] However, ER stress can also cause apoptosis. This means that if UPR cannot resolve misfolded protein stress, persistent and severe stress leads to cell death.[14] Considering our results, glioma cells treated with 4-PBA reduced apoptosis induced by NC (Figure 3D), corresponding to western blot for cleaved-caspase3 (Figure 3F). These results suggest that NC causes severe and persistent ER stress that mediates apoptosis in glioma cells.

Considering that ER stress and oxidative stress are closely linked, excessive ROS can induce cancer cell death.[30] Thus, we detected ROS production in our study. Interestingly, intracellular ROS production increased significantly after NC treatment, while 4-PBA prevented that ROS elevation (Figures 2C and 3C). We suggest that this result may be in accordance with an earlier study stating that misfolded protein accumulation is sufficient to generate ROS, and both ROS and misfolding proteins are required to activate UPR.[31] However, it was unanticipated that ROS scavenger NAC failed to decrease ROS production mediated by NC.

Another important finding was found as we explored EMT-related signaling pathways in U-87 MG treated with NC. As demonstrated in Figure 5A and B, we had determined that ER stress induced by NC downregulates C/EBPβ and its downstream slug in U-87 MG cells. This finding supports previous work done by Wu et al,[20] revealing the possibility of alleviating EMT via activating ER stress. Moreover, the effect in NC treatment on the AKT/GSK3β/β-catenin axis was investigated in U-87 MG. This study has provided sufficient evidence that β-catenin is essential for EMT progression.[22,32] Thus, NC acts as an efficient antitumor drug, which inhibits EMT by targeting multiple pathways.

In conclusion, we have investigated NC, a Chinese herb extract, which inhibits glioma progression by activating ER stress and downregulating EMT. Its antitumor effect has been confirmed in a xenograft model. These findings demonstrate that NC could be an effective therapeutic strategy as an alternative treatment of glioma. Further research is necessary in order to investigate ROS induced by NC.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by grants from the National Natural Science Foundation of China (81571284; 91542115; 81702468; 81874083; and 81802966), National Natural Science Foundation of Shandong Province of China (Nos. 2017CXGC1203; 2017G006012; and 2013GGE27006), and Taishan Scholars of Shandong Province of China (No. ts201511093). We appreciate the support from Dr Anning Li and the Department of Radiology, Qilu Hospital of Shandong University.

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Supplemental Material

Supplemental material for this article is available online.

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