

## Original Article

# Cinobufagin suppresses colorectal cancer growth via STAT3 pathway inhibition

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**Abstract:** Colorectal cancer (CRC) has become one of the most common types of cancer with the highest morbidity and mortality rates globally. Cinobufagin, a natural product extracted from toad venom and a major active ingredient in cinobufotalin, exhibits high antitumor activity. Here, we investigated the *in vitro* and *in vivo* antitumor activities of cinobufagin and explored the underlying mechanisms in CRC. Cinobufagin could inhibit proliferation, migration, invasion and promote apoptosis of HCT116, RKO, and SW480 cells *in vitro*. Mechanistically, cinobufagin simultaneously suppressed the phosphorylation of signal transducer and activator of transcription 3 (STAT3) and blocked the interleukin-6 (IL6)-induced nuclear translocation of STAT3. IL6 activated the STAT3 pathway, subsequently inducing epithelial-mesenchymal transition (EMT). Furthermore, cinobufagin suppressed EMT in CRC by inhibiting the STAT3 pathway. Animal experiments clearly showed that cinobufagin could reduce tumor growth. Cinobufagin may be used clinically as a novel STAT3 inhibitor for CRC adjuvant therapy.

**Keywords:** Cinobufagin, STAT3, EMT, inhibitor, colorectal cancer

## Introduction

Colorectal cancer (CRC) has become one of the most common types of cancer with the highest morbidity and mortality rates globally [1]. The high incidence rate of CRC continues to increase markedly worldwide [2]. CRC is mainly treated by surgical resection. Various target drugs have also been developed, however, the cure rate and postoperative survival quality of patients with CRC have not improved significantly [3]. Therefore, novel therapeutic strategies need to be identified and developed to improve the treatment outcomes of patients with CRC.

Signal transducer and activator of transcription 3 (STAT3), a core regulator of the STAT family, regulates the expression of various genes [4, 5]. STAT3 is associated with the promotion of tumor cell proliferation, immunosuppression, angiogenesis, metastasis, and resistance to

therapies [6, 7]. Studies indicate that STAT3 is overactive in most types of human cancer, including CRC, and is associated with poor prognosis [8-10]. Control of STAT3 activity can effectively inhibit carcinogenesis, tumor progression, and invasion [11-13]. STAT3 is currently an important molecular therapeutic target for CRC.

Epithelial-mesenchymal transition (EMT) is the conversion of epithelial cells to mesenchymal cells [14], which indicates that epithelial cancer cells acquire the ability to migrate and to invade peripheral tissues [15]. Moreover, EMT is regarded as one of the mechanisms that lead to tumor metastasis and recurrence [16]. Accumulating evidence demonstrates that numerous therapeutic strategies suppress migration via EMT downregulation [17]. Thus, the mechanism underlying the role of EMT in migration can provide additional opportunities for drug discovery research. The STAT3 signaling path-

way resides upstream of EMT, and interleukin-6 (IL6) induces cancer cell EMT initiation in a STAT3 activation-dependent manner [18]. More recently, studies on CRC revealed the correlation between P-STAT3 and EMT expression. Meanwhile, STAT3 inhibitors could lead to reductions in tumor growth, invasion, and migration via EMT [19, 20].

Cinobufagin, a primary component found in cinobufotalin, is an effective traditional Chinese medicine extracted monomer from *Bufo bufo gargarizans Cantor* and *Bufo melanostictus Schneider* [21]. Cinobufagin has been suggested to exhibit significant anticancer activity similar to that of cinobufagin venom toad; however, the exact mechanisms for cancer treatment have yet to be elucidated [22]. Thus, cinobufagin shows potential for development as an anticancer drug.

### Materials and methods

#### *Antibodies and reagents*

Cinobufagin was supplied by Baoji Herbest Bio-Tech Co., Ltd. Media were provided by Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies of GAPDH, STAT3, E-Cadherin and Ki67 were supplied by Cell Signaling Technology (Danvers, MA, USA). Antibodies of P-STAT3 and BAX were provided by Abcam Co. (Cambridge, MA, USA). The antibody of BCL-2, Vimentin, Cleaved Caspase 3 and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Total Protein Extraction Kit was supplied by Boster Biological Technology (Wuhan, China). Dimethyl sulfoxide (DMSO) and methylthiazolyl-diphenyl-tetrazolium bromide (MTT) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). Apoptosis kits were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). SiRNAs against STAT3 (Si-STAT3) and negative control SiRNA (Si-NC) were designed and provided by GenePharma (Shanghai, China). Lipofectamine 3000 Transfection Kit was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Caspase 3 Assay Kit was purchased from Abcam Co. (Cambridge, MA, USA). BeyoClick™ Edu-555 was purchased from Beyotime Biotechnology.

#### *Cell lines and cultures*

Human CRC cell lines HCT116, RKO, and SW-480 were provided by the Shanghai Institute of

Biosciences and Cell Resources Center (Chinese Academy of Sciences, Shanghai, China). HCT116 was cultured in McCoy's 5A medium with 10% fetal bovine serum (FBS), and RKO and SW480 were cultured in the 1640 medium with 10% FBS. All cells were maintained in a cell incubator at 37°C and 5% CO<sub>2</sub>.

#### *MTT cytotoxicity assay*

The cultured CRC cells in their log phase were collected, and the cell concentration was adjusted to 4000-5000 per well. Cultures were cultured at 37°C in a humidified incubator under 5% CO<sub>2</sub> and then allowed to attach. The clear supernatant was discarded, and cinobufagin solutions were added. Every concentration was repeated six times. The cells were placed in a cell incubator and further incubated for 48 h. The supernatants were then discarded. Subsequently, 25 µL of MTT was added into each well and then incubated for 4 h. The experiment was terminated by aspirating the medium from the cells. Each well was added with 150 µL of DMSO, shaken for 10 min to allow the crystals to dissolve easily. Sample absorbances were measured at a wavelength of 490 nm. The IC<sub>50</sub> value was calculated using GraphPad Prism (version 7.0).

#### *Clonal assay*

Suspensions of single CRC cells were prepared with conventional digestion passages. Cells were seeded in six-well plates at a density of 500 cells per well, which were maintained at 37°C and 5% CO<sub>2</sub> for 7 d. As single-cell clones become visible, the culture was washed with PBS twice and then fixed with 4% paraformaldehyde for 15 min. The colonies were then stained with crystal violet for 10 min, the dye solution was rinsed off, and the cells were allowed to dry in air.

#### *Edu cell proliferation detection*

Cells were plated in six-well plates to 60%~70%, and treated with cinobufagin (0.1, 0.5, and 1.0 µM). Edu was directly added to the culture medium at a final concentration of 10 µM for 2 h before detection. After removing incubation medium, cells were fixed using 4% paraformaldehyde for 15 minutes. After removal of fix solution and washed 2 times with PBS, cells were permeabilize with 0.1% Triton X-100. Then the reaction system was conducted in accor-

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dance with the BeyoClick™ EdU-555, and incubated at room temperature for 30 minutes and reacted protected from light. Finally, Hoechst 33342 (B2261, Sigma, USA) was used to stain the nuclei for 10 min. After reaction, fluorescence was detected by fluorescence microscopy.

### *Hoechst 33342 staining*

A treated coverslip was placed into six-well plates. Cells were cultured on coverslips and grown overnight, and the stimulation was evaluated. The CRC cells were allowed to undergo apoptosis with cinobufagin. The cell culture medium was subsequently removed and fixed with paraformaldehyde for 10 min. After the cells were washed twice with PBS, they were stained with Hoechst 33258 for 20 min and then rinsed. Blue nuclei were ultimately detected by fluorescence microscopy.

### *Cell apoptosis assay*

CRC cells were plated into six-well plates for overnight attachment and then treated with cinobufagin along a gradient of concentration to continue culturing. The effect of cinobufagin on CRC apoptosis was determined. The cells were then collected, centrifuged for 5 min to discard the supernatant and washed in PBS. The cells were resuspended in a binding buffer solution with the Annexin V-FITC apoptosis kit. The CRC cells were incubated with fluorescein-labeled Annexin V and propidium iodide in darkness for 15 min and then cell apoptosis was measured on the BD FACSCalibur platform (BD Biosciences, Baltimore, MD, USA). Data were analyzed using the software FlowJo v10.

### *Determination of caspase 3 activity*

Cells ( $1 \times 10^6$  to  $5 \times 10^6$ ) were spread in Petri dishes. Cinobufagin (0.1, 0.5, and 1.0  $\mu\text{M}$ ) was added to induce apoptosis in CRC cells. The cells were resuspended and lysed with 50  $\mu\text{L}$  of precooled lysis buffer for 10 min on ice and then centrifuged for 1 min. The supernatant fraction was immediately transferred into a fresh tube. The reaction system was subsequently configured as specified in the Caspase 3 binding assay kit and maintained at 37°C for 2 h. The absorbance was determined at 405 nm by using a SpectraMax iD3 (Molecular Devices, San Jose, CA, USA).

### *Western blot analysis*

After overnight attachment, the cells were processed with a gradient concentration of cinobufagin (0.1, 0.5, and 1.0  $\mu\text{M}$ ). The cells were then washed gently with PBS 2 times. The CRC cells were lysed using cell lysates for 20 min on ice, which were transferred to new tubes and then centrifuged. Protein concentration was determined by Coomassie Brilliant Blue staining to configure the reaction system. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 5% skim milk for 1.5 h and then washed with Tris Buffered Saline with Tween 20. The blots were ultimately incubated with specific primary antibodies and incubated with secondary antibodies on the following day. The software Image J was used to analyze the reactive bands.

### *Invasion assay*

Cell suspensions were collected using a serum-free medium, and 100-200  $\mu\text{L}$  of the suspension was seeded to the transwell chamber. 500  $\mu\text{L}$  of the medium supplemented with 10% FBS was added to the 24-well plate and contacted with the bottom of the chamber. Air bubbles were thus prevented. Treatment was initiated for 8-15 h in an incubator at 37°C. The chambers were subsequently removed from the 24-well plate by using tweezers. Before fixation, excess liquid was removed using a small-range pipetting gun. After crystal violet staining and washing, the non-invading cells were carefully wiped off with a cotton swab and then dried.

### *Immunofluorescence staining*

RKO cells were inoculated into a fluorescence cuvette. The cells were fixed with 4% paraformaldehyde, permeabilized using 0.5% Triton X-100 (in PBS), and washed four times with PBS. They were then blocked with 1% bovine serum albumin for 1 h. Cell slides were incubated with a primary antibody (P-STAT3, E-Cadherin, Vimentin and Ki67) in appropriate proportions. The cells were incubated with an anti-rabbit secondary antibody (Alexa Fluor® 488) or anti-mouse secondary antibody (Alexa Fluor® 488) on the following day and then with the DAPI stain protected from light. The cell slides were washed with PBS again and then

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sealed with anti-fluorescence quenching sealing tablets to prevent fluorescence quenching until they were captured by confocal microscopy.

## *Cytoplasmic and nuclear protein extraction*

HCT116 cells were grown at 80%-90% confluency in a 10 cm dish and treated with cinobufagin (0.1, 0.5, and 1.0  $\mu\text{M}$ ). After IL6 stimulation for 1 h, the cells were immediately placed on ice. Nuclear and cytoplasmic proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit. Western blot analysis was conducted to detect the related protein expression.

## *Transient transfection of small interfering RNA (SiRNA)*

Synthetic Si-RNAs, specifically STAT3 small interfering RNA (Si-RNAs) and negative-control SiRNAs (Si-NC), were custom-designed. The Si-RNAs were transiently transfected into HCT116 and RKO. The STAT3 Si-RNAs were as follows: STAT3 Si-RNA1 STAT3-Homo-978 5-GCAACA-GAUUGCCUGCAUUTT-3; STAT3 Si-RNA2 STAT3-Homo-398 5-CCACUUUGGUGUUUCAUAATT-3; and STAT3 Si-RNA3 STAT3-Homo-1070 5-CCC-GUCAACAAUUAAGAATT-3.

## *Animal model*

The female BALB/c athymic nude mice used in this experiment were raised and bred in the animal experimental center of Wenzhou Medical University with the approval of The Animal Policy and Welfare Committee of Wenzhou Medical University. HCT116 cells were subcutaneously injected into nude mice ( $5 \times 10^6$ ), cells were diluted by an appropriate amount of Matrigel and PBS. After 5-7 d, the tumor measured  $\sim 100 \text{ mm}^3$ . The mice were grouped for the experiments (control group, negative controls treated with napabucasin 10 mg/kg, treatment group A treated with cinobufagin 0.5 mg/kg, and treatment group B treated with cinobufagin 1.0 mg/kg). Each group consisted of more than 7 mice, and treatment was administered intraperitoneally. Tumor volumes were measured, and weight changes every other day for 14 d were recorded. After the mice were killed, tumors and organs such as the heart, liver, kidneys, and lungs were excised for subsequent Western blot analysis and H&E staining experiments.

## *Statistical analysis*

Each experiment was independently repeated at least three times to ensure the reproducibility and reliability of the experiments. The data obtained were statistically analyzed using GraphPad Prism 7.0 (GraphPad Software, CA, USA). Differences between experimental groups were analyzed using the t-test. For all analyses, if the *P*-values were less than 0.05, cinobufagin showed evidence of good therapy outcomes (significant difference).

## **Results**

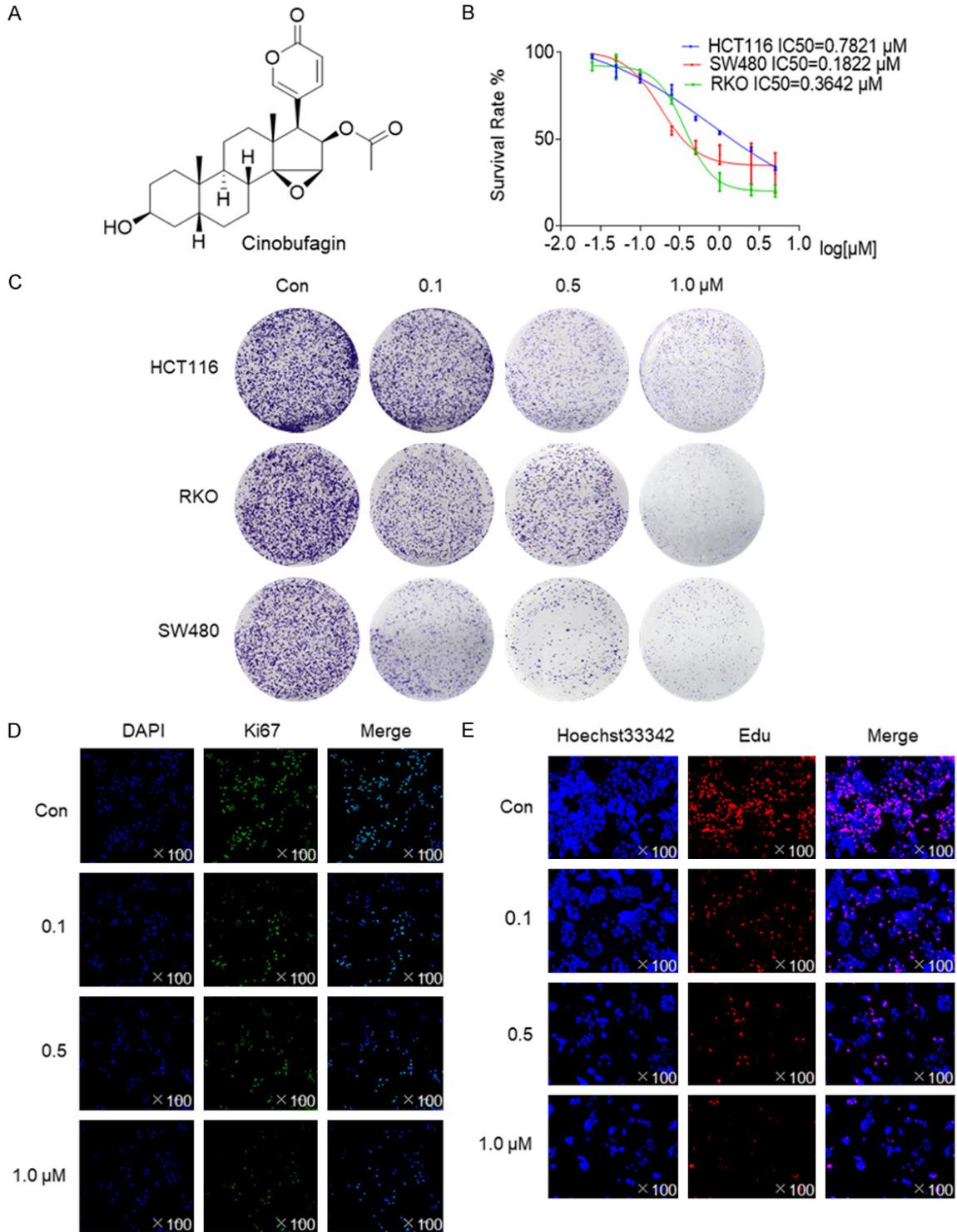
### *Cinobufagin inhibits the viability and proliferation of CRC cells*

To assess the inhibitory effect of cinobufagin (**Figure 1A**), cell viability was determined using the MTT assay. The results showed that half maximal inhibitory concentration values (IC<sub>50</sub>) of CRC cells (HCT116, RKO, and SW480) were 0.7821  $\mu\text{M}$ , 0.3642  $\mu\text{M}$ , and 0.1822  $\mu\text{M}$ , respectively (**Figure 1B**). As a concentration gradient was created, the viability of the cells was markedly inhibited in CRC cells. Moreover, cinobufagin significantly reduced the colony formation capacity of the CRC cells (**Figure 1C**). These results showed that cinobufagin effectively reduced the viability of the CRC cells. To examine the effects of cinobufagin on cancer cell proliferation, we performed the expression of Ki67 in HCT116 and SW480 cells by immunofluorescence. Our results showed the fluorescence intensity of Ki67 was notably weakened (**Figure 1D**, [Supplementary Figure 1A](#)). Furthermore, we also performed cell proliferation assay for three cells using an Edu cell proliferation assay kit. **Figure 1E** and [Supplementary Figure 1B](#) showed CRC cells were successfully labeled with Edu fluorescent dye (red). Cinobufagin effectively reduced the fluorescence intensity with increased drug concentration. Together, these results showed that cinobufagin inhibited cell growth and proliferation dose-dependently.

### *Cinobufagin promotes apoptosis in human CRC*

To determine the effect of cinobufagin on apoptosis in CRC, three cell lines HCT116, RKO, and SW480 were treated with different concentrations of cinobufagin (0, 0.1, 0.5, and 1.0  $\mu\text{M}$ ). Hoechst staining was used to stain the nuclei

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**Figure 1.** Cinobufagin inhibits the proliferation of CRC cells. **A.** Chemical structure of cinobufagin. **B.** Cells treated with the drug at different concentrations for 48 h; formation of blue formazan crystals (solubilized with dimethyl sulfoxide) by cells incubated with the MTT solution; absorbance values at OD490 nm. **C.** CRC cells at three concentrations (0.1, 0.5, and 1.0  $\mu\text{M}$ ) incubated using cinobufagin for 12-20 h until the appearance of colonies; cells stained with crystal violet; and determination of statistical differences. **D.** Changes in the signals received by proteins (Ki67) in HCT116 were verified by immunofluorescence staining. **E.** Proliferation of HCT116 were detected using BeyoClick™ Edu-555 (red) immunofluorescence staining kit. Capacity of cinobufagin to suppress CRC cells, as determined from the four aforementioned experiments.

of the cells, and the results showed that cells treated with cinobufagin displayed strong blue fluorescence and revealed signs of substantial apoptosis (**Figure 2A**). Similar apoptotic effects were obtained by flow cytometry, three cell lines were treated with cinobufagin for 24 h. The results showed that cinobufagin significantly increased the proportion of apoptotic cells (**Figure 2B**). Moreover, the expression of apoptosis-related proteins BAX was increased, whereas that of BCL-2 was decreased dose-dependently (**Figure 2C**). Here, we also found the activity of caspase 3 significantly increased, as determined using the caspase activity assay, and the pro-apoptotic effect of cinobufagin was confirmed (**Figure 2D**). Overall, cinobufagin markedly promoted apoptosis in CRC.

### *Cinobufagin inhibits the invasion and metastases of CRC cells*

Abnormal differentiation, invasion, and metastases are typical biological features of tumor cells [23]. To analyze the role of cinobufagin treatment in the biological properties of CRC, the invasion and metastases of the indicated cells were measured using transwell assays. Assay demonstrated that cinobufagin inhibited the invasion and metastases of CRC cells with increasing concentration (**Figure 3A**). In addition, E-Cadherin and Vimentin were key proteins of EMT [24]. Immunofluorescence staining indicated that the fluorescence of E-Cadherin increased, whereas that of Vimentin decreased (**Figure 3B**). Subsequently, a similar change was observed by western blot analysis. Compared with the control group, E-Cadherin expression increased, whereas Vimentin expression decreased (**Figure 3C**). Thus, we concluded that cinobufagin inhibited not only invasion and metastases but also changes in the protein levels of cell invasion and metastases.

### *Cinobufagin inhibits EMT via STAT3 signaling*

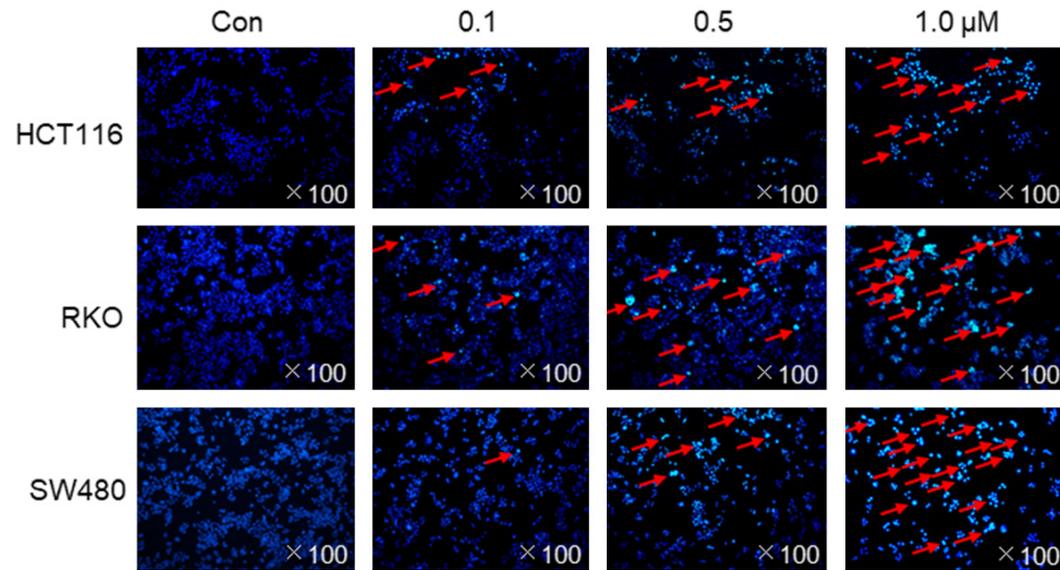
Bufalin exerts inhibitory effects on tumors by repressing the STAT3 signaling pathway [25, 26]. The structure of cinobufagin is analog to that of bufalin, and a small body of literature demonstrates that cinobufagin can also inhibit STAT3 signaling pathway [27]. Moreover, cinobufagin prevents the proliferation of cancer cells and promote cell apoptosis. Therefore, we con-

ducted western blot analysis to determine whether cinobufagin was involved in the suppression of cell growth in CRC via the STAT3 signaling pathway. The results demonstrated that total STAT3 expression remained relatively stable with drug treatment (0, 0.1, 0.5, and 1.0  $\mu\text{M}$ ), whereas STAT3 phosphorylation considerably decreased. This result suggested that cinobufagin suppressed STAT3 activation (**Figure 4A**). Furthermore, immunofluorescence staining showed that STAT3 was localized to the cytoplasm and then translocated to the nucleus in response to IL6 stimulation, as indicated by the enhanced green fluorescent protein in the nucleus. This occurrence could be reversed by adding cinobufagin into the culture (**Figure 4B**). The above results strongly indicated that IL6-induced STAT3 nuclear translocation was inhibited by cinobufagin. Moreover, we then isolated STAT3 proteins from the nucleus and the cytosol. After stimulation for 0.5 h with IL6, the STAT3 protein expression in the nucleus was higher than that in non-stimulated cells. Conversely, STAT3 protein expression in the cytoplasm was reduced. It was also found that cinobufagin inhibited IL6-induced STAT3 nuclear translocation dose-dependently (**Figure 4C**). These results strongly suggested that cinobufagin inhibits STAT3 signaling and disrupts the nuclear translocation of STAT3.

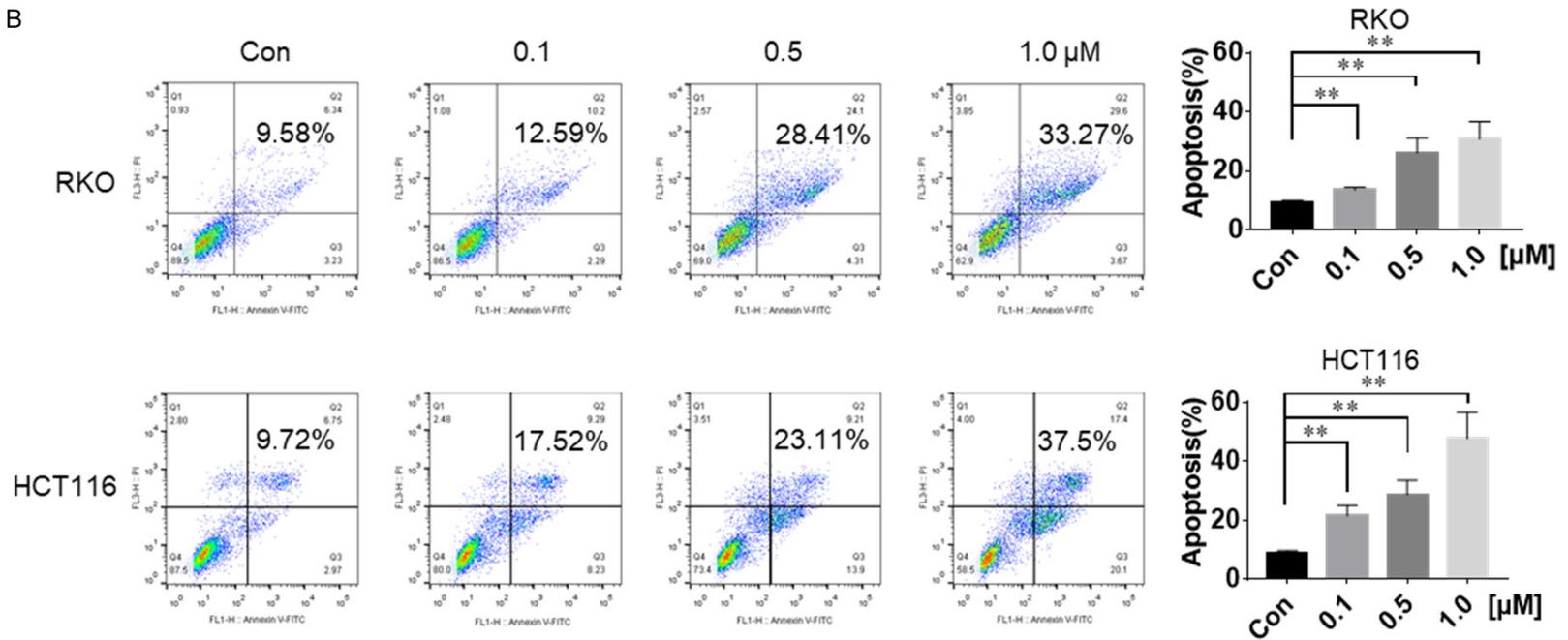
Previous reports have indicated that EMT occurs downstream in the STAT3 pathway [28]. To further determine whether cinobufagin inhibits the function of EMT as tumor suppressors by inhibiting the STAT3 signaling pathway, CRC cells were treated with IL6 (20 ng/mL) for 0.5 h. STAT3 phosphorylation in the cells exhibited a significant increase relative to that in the untreated controls. E-Cadherin expression decreased, whereas Vimentin expression increased. This occurrence can be inhibited by cinobufagin, which can decrease STAT3 phosphorylation and EMT (**Figure 4D**). Furthermore, we attempted to screen 1 Si-STAT3 with maximum inhibitory activity by western blot analysis. Western blot analysis showed that Si-1 showed the maximum inhibitory activity of STAT3 (**Supplementary Figure 1C**). As shown in **Figure 4E**, the results showed that no significant effect on STAT3 pathways and EMT were found after knocking down STAT3 using Si-RNA against it. To further validate the results above, STAT3

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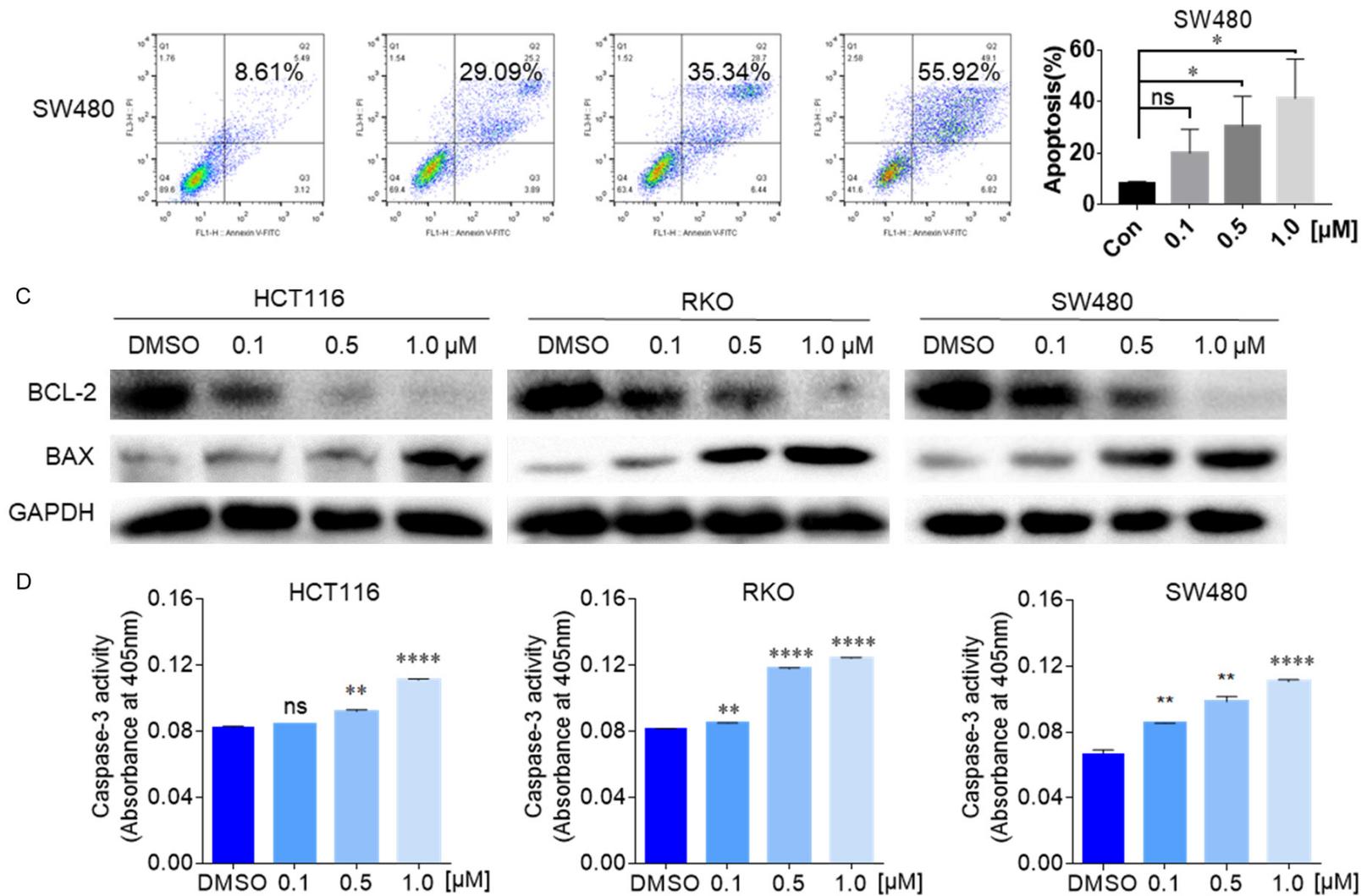
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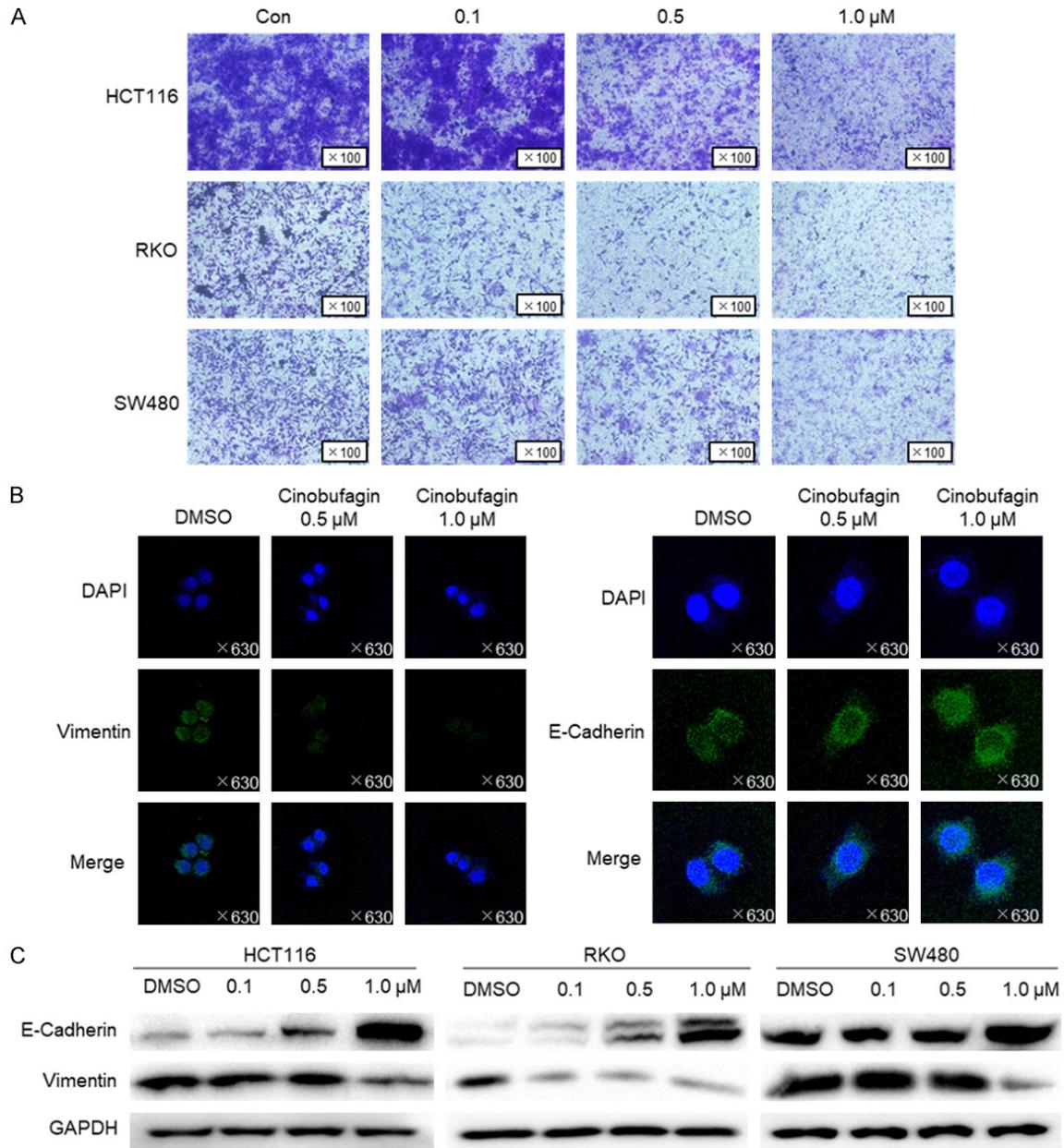


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**Figure 2.** Cinobufagin promotes the apoptosis of human CRC cells. A. CRC cells at three different concentrations treated with cinobufagin for 12-20 h; Hoechst staining (images taken with ultraviolet light). B. CRC cells treated with cinobufagin for 20-24 h; apoptotic cells were treated using the Annexin V-FITC apoptosis kit, and the percentage of cell apoptosis as analyzed by flow cytometry. C. BCL-2 and BAX proteins extracted from cells lysed with protease inhibitors after treatment for 20-24 h, using GAPDH as the internal reference. D. Caspase 3 activity evaluated using the Caspase Assay Kit, and absorbance at 405 nm. (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001).

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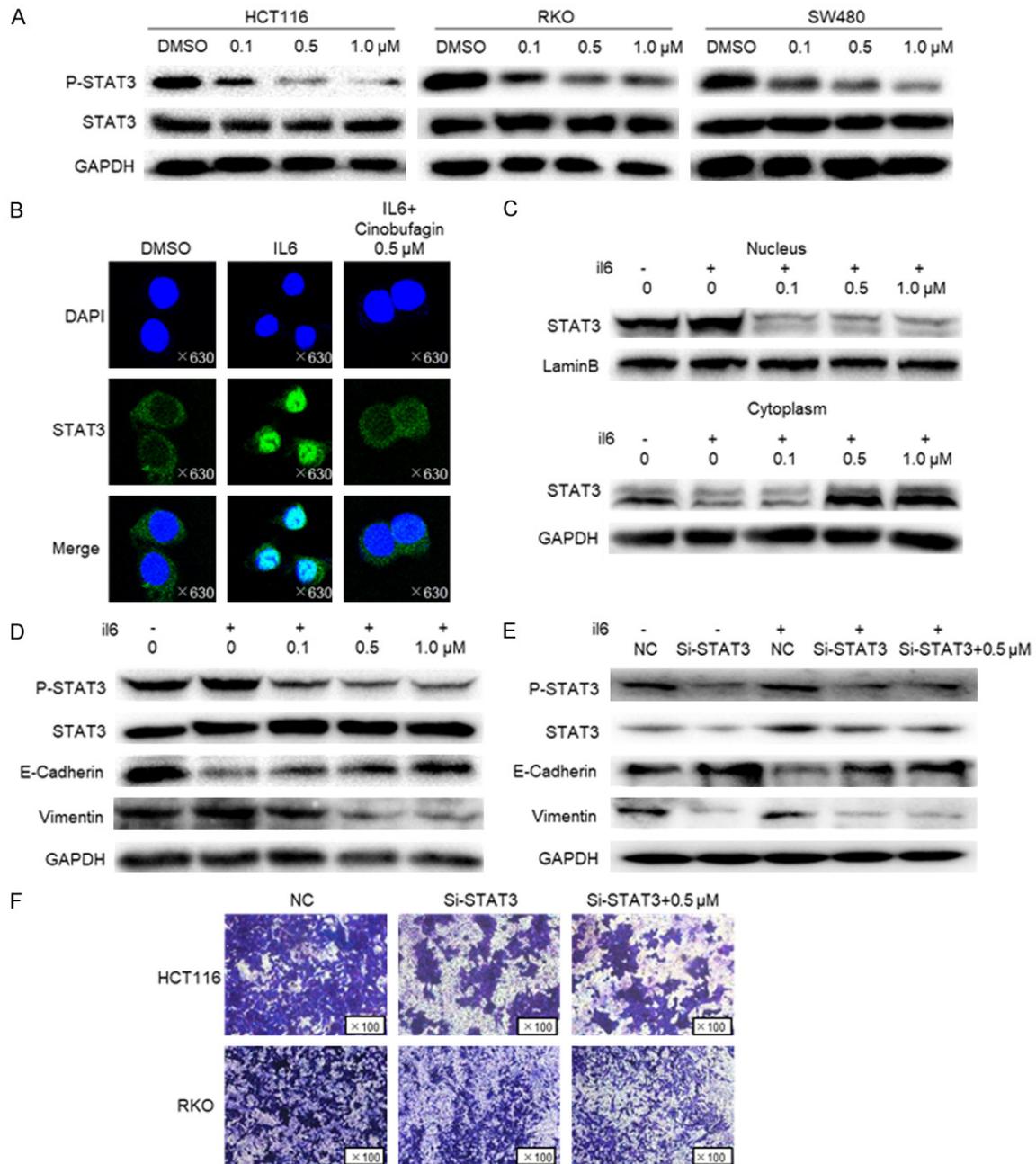
**Figure 3.** Cinobufagin inhibits the invasion and metastasis of CRC cells. A. CRC cells incubated on the upper side of the chamber and treated with cinobufagin for 10-20 h; cells stained with crystal violet to estimate the invasion ability of CRC cells. B. Changes in the signals received by proteins (E-Cadherin and Vimentin) in RKO were verified by immunofluorescence staining. C. Expression of proteins related to EMT signaling pathways as detected by western blot analysis.

was knocked down in CRC cells, followed by treatment with 0.5  $\mu\text{M}$  cinobufagin. As shown in **Figure 4F**, no significant effect on invasion and metastases was found. In summary, cinobufagin significantly exerted an anticancer effect by inhibiting EMT in CRC via the STAT3 pathway.

### *Cinobufagin inhibits the growth of the CRC xenograft model*

We further explored the mechanism by which cinobufagin inhibits STAT3 and EMT *in vivo*. A suspension of HCT116 cells was injected into the backs of the nude mice to build a xenograft

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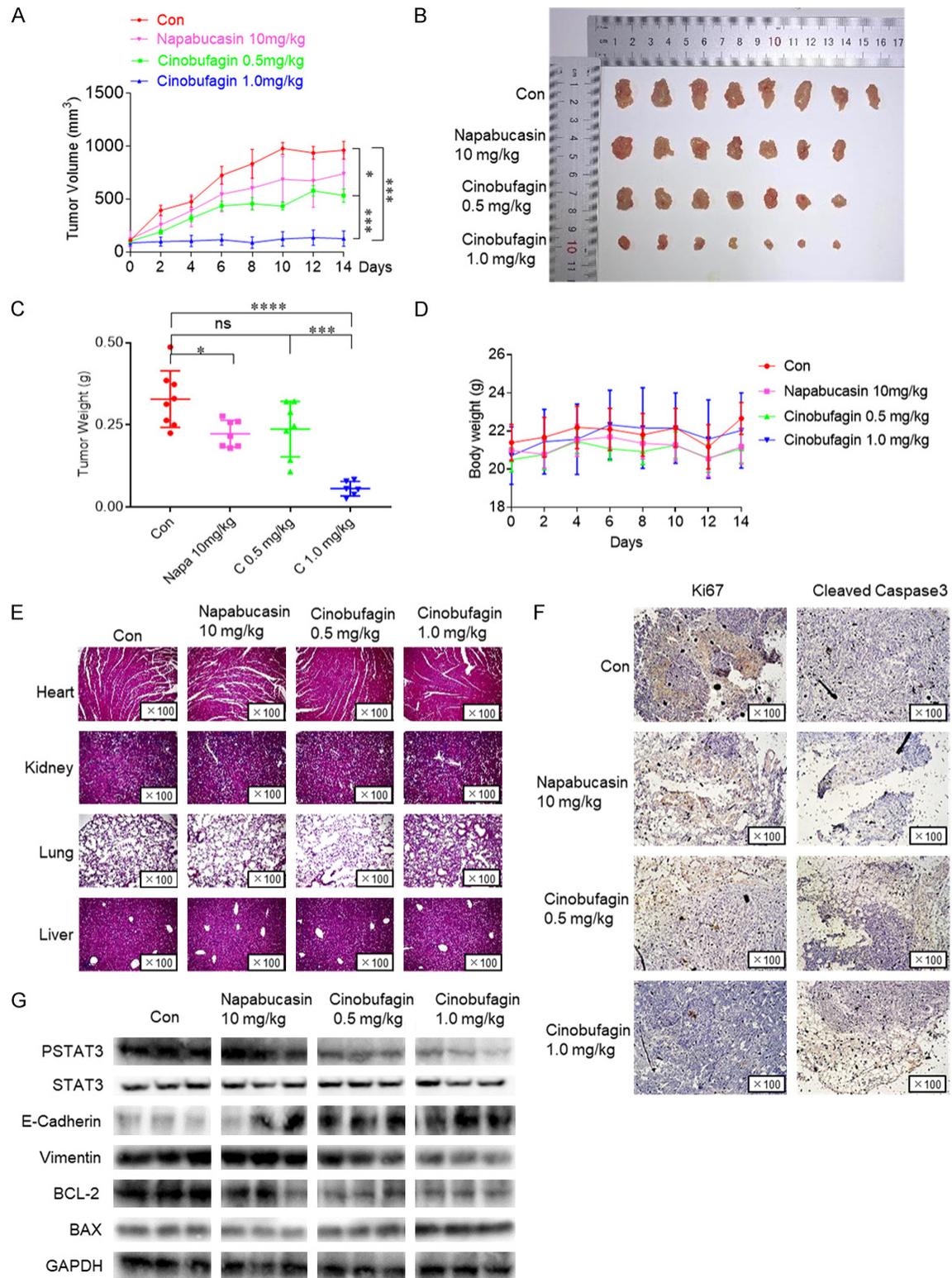


**Figure 4.** Cinobufagin inhibits EMT via STAT3 signaling. A. Expression of proteins related to STAT3 signaling pathways as detected by Western blot analysis. B. Cellular localization of proteins as visualized by immunofluorescence staining in RKO; analysis of changes in the intracellular location of STAT3. C. Nuclear and cytosolic proteins extracted from HCT116 after treatment; distribution of STAT3 in cells as determined by western blot analysis. D. RKO exposed to IL6 for 30 min after treatment with cinobufagin for 10-20 h; expression levels of proteins related to STAT3 and EMT signaling pathways as detected by western blot analysis. E. STAT3 knockdown RKO treated with control, IL6 or IL6 + cinobufagin (0.5  $\mu$ M), expression levels of proteins related to STAT3 and EMT signaling pathways as detected by western blot analysis. F. Invasive abilities of HCT116 and RKO silenced of STAT3 evaluated, using Si-NC as the control.

model and thereby confirm the suppressive effects of cinobufagin on CRC cells *in vivo*. Napabucasin (10 mg/kg, positive control) and

different doses of cinobufagin (0.5 mg/kg, 1.0 mg/kg) were administered intraperitoneally every other day. The growth curve of the xeno-

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**Figure 5.** Cinobufagin inhibits the growth of CRC xenograft models. A. Nude mice injected with napabucasin (10 mg/kg) or cinobufagin (0.5 or 1.0 mg/kg) via the intraperitoneal route every other day for 7 doses, and measured the tumor volumes. B. Size of the xenograft tumor (by direct observation). C. Comparison of the tumor weights of the four groups. D. Weight changes in mice during 14 d. E. Hematoxylin-eosin staining of the heart, liver, lungs, and kidneys to check for toxicity. F. Immunohistochemical staining images of cell proliferation marker Ki67 and Cleaved Caspase 3 in tumor tissues. G. Homogenized fresh tumor tissues; Expression of STAT3- and EMT-related proteins detected by western blot analysis. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ).

graft tumor in the nude mice revealed a sharp decrease in tumor growth in the treated mice (**Figure 5A**) and the size of the excised tumors was reduced, particularly for cinobufagin (**Figure 5B**). Meanwhile, the tumor weight decreased (**Figure 5C**). No significant difference in body weight was observed during therapy (**Figure 5D**). H&E staining of the major organs (heart, liver, lung, and kidneys) exhibiting structure morphogenesis showed that no change occurred (**Figure 5E**). The above results strongly indicated that no apparent cytotoxicity in cinobufagin was observed, confirming its safety *in vivo*. In order to further research the impact of cinobufagin on tumor growth, the Ki67 and Cleaved Caspase 3 expression was conducted by immunohistochemistry. Our results showed a decrease in Ki67 and an increase in Cleaved Caspase 3 (**Figure 5F**). These findings proved that cinobufagin significantly inhibited proliferation and induced apoptosis *in vivo*. Western blot analysis also confirmed that cinobufagin inhibited STAT3 signaling and EMT (**Figure 5G**). These results strongly indicated that cinobufagin can potentially be used for the treatment of CRC. This study provides insight into cancer treatment by inhibiting the migration and invasion of CRC by STAT3 regulation.

### Discussion

As knowledge of the molecular mechanisms of tumorigenesis and tumor development improved, the discovery of active pharmaceutical ingredients from natural products for the treatment of various malignant tumors has become a major goal for modern healthcare workers [29-32]. Cinobufagin is a natural product extracted from toad venom, one of the major active ingredients in cinobufotalin injection, which is mainly used to treat intermediate and advanced tumors. Therefore, an in-depth study of the mechanism of cinobufagin is significant for monomer development. In addition, a large number of preclinical and clinical data suggested that the growth of CRC cells was significantly inhibited by the small molecule inhibitors of STAT3 [33-35]. For this experiment, we confirmed a functional role of cinobufagin in 3 colon cancer cell lines. The lowest IC<sub>50</sub> reached the 0.1 micromolar level. Furthermore, cinobufagin suppression of the proliferation of CRC cells and promoted cell apoptosis. We also verified the distinct anticancer mechanism, as well as stable efficacy and low toxicity of cinobufa-

gin. Altogether, cinobufagin exerted an anticancer effect.

EMT has been strongly associated with tumor progression in various cancer types [36]. It interacts with multiple signaling pathways (JAK/STAT signal pathway, transcription factors, and signaling factors) [37, 38]. After EMT in cancer cells, CRC cells lose not only cell-cell adhesion but also tight junction with the basement membrane and significantly enhance the migratory and invasive abilities of the cells [39]. In the current study, E-Cadherin was increased, whereas Vimentin was decreased. Cinobufagin controlled the invasion and metastasis of CRC cells via EMT inhibition and thus could achieve the purpose of CRC treatment.

Studies have shown that the JAK/STAT3 pathway would be overactivated by high levels of IL6 [40]. After Tyr705 phosphorylation, STAT3 would form dimers and be translocated to the nucleus, specifically binding to particular DNA sequences and inducing the transcription of downstream target genes and EMT [41]. Several studies have indicated that Twist-1 can be induced by STAT3 activation and enables further activation of the EMT program [42]. An active IL6R/STAT3/miR-34a loop affects EMT [43], which exists in primary CRC. By inhibiting STAT3 phosphorylation and nuclear translocation, cinobufagin could transcriptionally regulate STAT3 activity, which could also transform motile mesenchymal cells into epithelial cells. Cinobufagin did not reduce the migratory and invasive abilities of the STAT3 silencing cells. Overall, we speculate that cinobufagin is a natural cancer treatment, inhibiting EMT in colorectal cancer by targeting the STAT3 pathway. According to the previous research of our group [26], aberrant STAT3 activation promoted abnormal proliferation and inhibited apoptosis of cells. When STAT3 was inhibited, the proliferation and apoptosis of cells were affected. In addition, cinobufagin exhibited therapeutic effects on transplanted tumors without damaging the organ. This result suggests the low toxicity of cinobufagin and its potential as a drug. Thus, the biological mechanisms involving STAT3 and EMT need to be elucidated for their important implications, which should further be explored in subsequent studies.

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**Disclosure of conflict of interest**

None.

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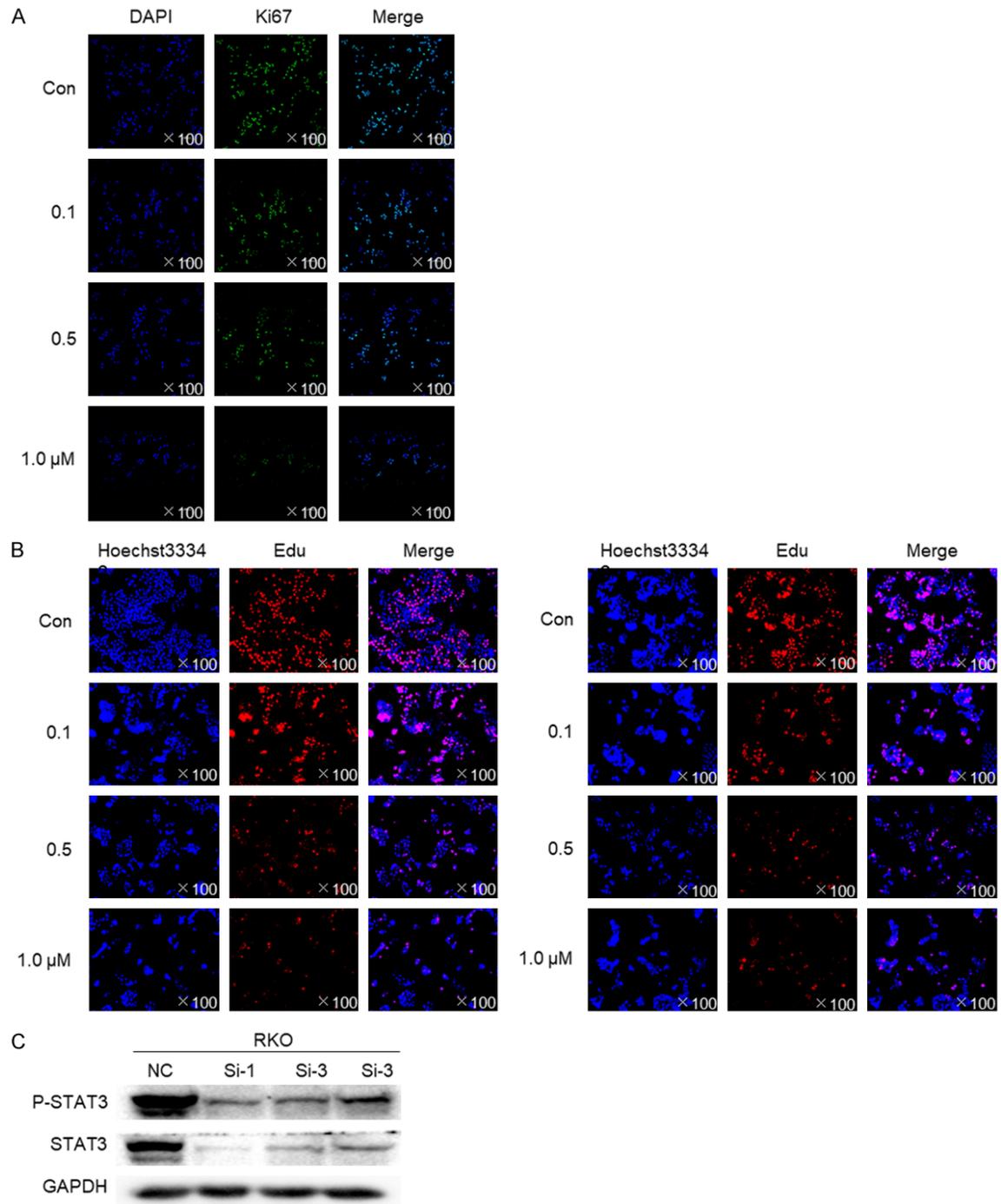
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**Supplementary Figure 1.** A. Changes in the signals received by proteins (Ki67) in SW480 were verified by immunofluorescence staining. B. Proliferation of RKO and SW480 were detected using BeyoClick™ Edu-555 (red) immunofluorescence staining kit. C. Sifting out the Si-STAT3 with maximum inhibitory activity by western blot in RKO.