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3-*epi*-bufotalin suppresses the proliferation in colorectal cancer cells through the inhibition of the JAK1/STAT3 signaling pathway

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Abstract: Traditional Chinese medicine (TCM) has been increasingly employed in the last decades in China for both preventing and treating a variety of cancers. 3-*epi*-bufotalin is an active ingredient of TCM "Chanpi" with anti-tumor potential. However, the effect and mechanism of 3-*epi*-bufotalin on colorectal cancers were not well disclosed. The present study demonstrated that 3-*epi*-bufotalin could reduce viability, trigger apoptosis, and block the cell cycle at the G_2/M stage in colorectal cancer cell lines HT29, RKO, and COLO205 *in vitro*. Moreover, 3-*epi*-bufotalin inhibited the JAK1/STAT3 signaling pathway. These results indicated the anti-proliferation ability of 3-*epi*-bufotalin in colorectal cancer cells.

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors with high mortality (Feng *et al.*, 2019; Sung *et al.*, 2021; Zakaria *et al.*, 2021). A large number of studies have focused on the pathogenic mechanism, effective prevention, and treatment of colorectal cancer. So far, while chemotherapy remains one of the important treatments for CRC, resistance to chemotherapeutic agents such as 5-fluorouracil (Vodenkova *et al.*, 2020) and oxaliplatin (Martinez-Balibrea *et al.*, 2015) remains a major problem. Thus, it is particularly important to discover more effective chemotherapeutic drugs.

Owing to its action on multiple signal pathways, only a few adverse effects, and systematic regulatory function, traditional Chinese medicine (TCM) has attracted growing appreciation in the clinical treatment of a variety of cancers (Wang *et al.*, 2011; Wang *et al.*, 2018). "Chanpi," the skin of *Bufo bufo gargarizans* Cantor or *Bufo melanostrictus* Schneider, is a commonly used TCM in China for carbuncles, swelling, heatstroke coma, vomiting, diarrhea, and cancer (College, 1986). Furthermore, Cinobufacini (brand name: Huachansu), a water-soluble drug extracted from Chanpi, has been approved by the State of Food and Drug Administration of China to be applied to cancer patients in the clinical treatment and has shown promising efficacy in the treatment of hepatocellular carcinoma, lung cancer, colorectal carcinoma, and pancreatic cancer (Wang *et al.*, 2018). However, the anti-tumor mechanisms of Chanpi and its processed products have not been studied exhaustively.

Bufadienolides are the predominant constituents of Chanpi and carry out anti-cancer activity through different mechanisms (Wang *et al.*, 2011; Zhan *et al.*, 2020). As a bufadienolide, bufotalin is rich in Chanpi and shows noticeable growth inhibition of hepatocellular carcinoma HepG2 cells (Zhang *et al.*, 2012) and malignant melanoma A375 cells (Pan *et al.*, 2019). In addition, 3-*epi*-bufotalin, with an opposite configuration of -OH at C-3bufotalin, is easily found in Chanpi or obtained via biotransformation from bufotalin. Bufotalin led to growth inhibition of HepG2 and breast cancer MCF-7cell lines (Zhang *et al.*, 2011). However, the anti-tumor effects and mechanisms of 3-*epi*-bufotalin are unclear.

Here, we investigated the anti-proliferative effect of 3-*epi*-bufotalin on CRC cells. Our data indicated that 3-*epi*-bufotalin induced apoptosis and cell cycle arrest at the G2/M phase in CRC cells. Furthermore, 3-*epi*-bufotalin suppressed the growth of CRC cells via the Janus kinase 1/signal transducer and activator of transcription 3 (JAK1/STAT3) signaling pathway. These findings suggest that 3-*epi*-bufotalin could be a potential growth inhibitor for CRC cells.

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Materials and Methods

Materials

Human CRC cell lines HT29, RKO, and COLO205, were purchased from Genechem (Shanghai, China). 3-*epi*-bufotalin was isolated from the aqueous extract of Chanpi, refined to 95% of purity by high-performance liquid chromatography, dissolved in DMSO as a stock concentration of 10 mM and stored at −20°C. Fetal bovine serum (BSA) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Sulforhodamine B (SRB) was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). The phosphatase inhibitor was obtained from Roche (Mannheim, Germany). Phenylmethanesulfonyl fluoride (PMSF), FITC-Annexin V Apoptosis Detection Kit, and Cell Cycle Analysis Kit were obtained from Beyotime (Shanghai, China). Antibodies of JAK1 (#ET1705-84), STAT3 (#ET1605-45), p-STAT3 (S727) (#ET1607-39), B-cell lymphoma-2 (BCL-2; #ET1702-53), goat anti-Rabbit IgG-HRP antibody (#HA1001) and goat anti-Rat IgG (H+L)-HRP antibody (#HA1023) were purchased from Huabio (Hangzhou, China); antibodies for p-JAK1 (Y1022+Y1023) (#ab130085) and Tubulin (#ab6106) were purchased from Abcam (Cambridge, UK); antibodies for CyclinB1 (#12231), p-cell division control2 (p-CDC2; Tyr15) (#4529) and Bcl-2-associated X protein (BAX; #2772) were purchased from Cell Signal Technology (Boston, Massachusetts, USA); antibody for GAPDH (#10494-1-AP) was purchased from Proteintech (Rosemont, Minnesota, USA). PVDF transfer membranes were obtained from Millipore (Darmstadt, Germany).

Cell culture

The CRC cell lines HT29, RKO, and COLO205 were cultured in RPMI-1640 medium supplied with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified incubator containing 5% CO_2.

Cell viability assay

Cells were seeded in 96-well plates at 5×10^3 cells/well and cultured for 24 h to allow adherence. Then, cells were exposed to a series of concentrations of 3-*epi*-bufotalin while cells of the control group were added with a 1‰ volume of DMSO. After 24 to 72 h of incubation, cells were subjected to SRB colorimetry (Skehan *et al.*, 1990), and the absorbance of each well was measured at 530 nm using SpectraMax i3X plate reader (Molecular Devices, USA).

Cell cycle and apoptosis analysis

Cells were plated into 6-well plates at 5×10^4 cells/well for 24 h and then incubated with 1.5 to 6 μM of 3-*epi*-bufotalin for 24 h. Cells were washed with cold PBS, collected in 500 μL binding buffer, then incubated with 10 μL RNase A (10 μg/mL) at 37°C in the dark for 30 min and combined with 20 μL propidium iodide (50 μg/mL) at 4°C in the dark for 20 min. Then the cell cycle phase was analyzed using a flow cytometer C6 (BD Bioscience, USA). For apoptosis analyses, cells were gently collected in cold PBS and resuspended in binding buffer, followed by treatment with 10 μL FITC-Annexin-V and 10 μL propidium iodide at room temperature in the dark for 15 min. Finally, cells were analyzed by flow cytometer C6.

Western blot analysis

Cells were plated into 6-well plates for culturing and then exposed to 1.5 to 6 μM 3-*epi*-bufotalinfor 24 h. The total protein of cells was extracted by RIPA buffer with proteinase inhibitor (PMSF) and phosphatase inhibitor. Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% or 12% gel and transferred to PVDF membranes. After blocking with 5% BSA for 2 h at room temperature, membranes were incubated with primary antibodies (1:500 or 1:1000 dilution) at 4°C overnight and goat anti-Rat IgG-HRP antibody or goat anti-Rabbit IgG-HRP antibody (1:5000 dilution) for 2 h at room temperature. Protein bands were developed by an ECL chemiluminescence kit and visualized by ChemiDoc Imaging System (Bio-Rad, USA).

Statistical analysis

For statistical analyses, cells cultured at different passages were treated with drugs, and all of the experiments were performed three times independently. Differences between each 3-*epi*-bufotalin group and the control group were determined by ANOVA analysis using SPSS 17.0 (IBM, Armonk, New York, USA). P-value < 0.05 and P-value < 0.01 were considered statistically significant.

Results

Effect of 3-epi-bufotalin on colorectal cancer cells proliferation

The structure of 3-*epi*-bufotalin is presented in Fig. 1A. To test the effect of 3-*epi*-bufotalin on human CRC cells, human CRC HT29, RKO, and COLO205 cell lines were treated with different concentrations of 3-*epi*-bufotalin (0, 1.25, 2.5, 5, 7.5, and 10 μM) for 24 to72 h. SRB assay showed that 3-*epi*-bufotalin significantly suppressed the viability of these cell lines in a dose and time dependent manner (Fig. 1), suggesting the anti-proliferation effect of 3-*epi*-bufotalin on CRC cell lines.

3-epi-bufotalin induced apoptosis in colorectal cancer cells

To explore whether 3-*epi*-bufotalin induces the apoptosis in CRC cells, we detected the proportion of apoptotic cells stained with FITC-Annexin-V and propidium iodide by flow cytometry. The proportion of apoptotic cells (cells in the right upper quadrant are undergoing apoptosis at later stage and cells in the right lower quadrant are apoptosis at an early stage) markedly increased after treatment with 6 μM 3-*epi*-bufotalin in CRC cell lines (Figs. 2A–2C). Furthermore, we examined the expression of mitochondrial apoptosis-associated proteins BAX and BCL-2 after 3-*epi*-bufotalin treatment. Western blot analysis indicated an increase in the pro-apoptotic protein BAX after 3-*epi*-bufotalin treatment, while the anti-apoptotic protein BCL-2 decreased in CRC cells (Figs. 2D–2F). Overall, these results indicate the activation of intrinsic apoptosis pathway by 3-*epi*-bufotalin in CRC cells.

3-epi-bufotalin induced cell cycle arrest in colorectal cancer cells

As continuous and precisely regulated progression of cell cycle ensures cell growth and proliferation, cell cycle arrest is a major indicator of cytotoxic effects of various chemical agents and always intimately coupled with apoptotic induction (Pucci *et al.*, 2000). We detected the effect of

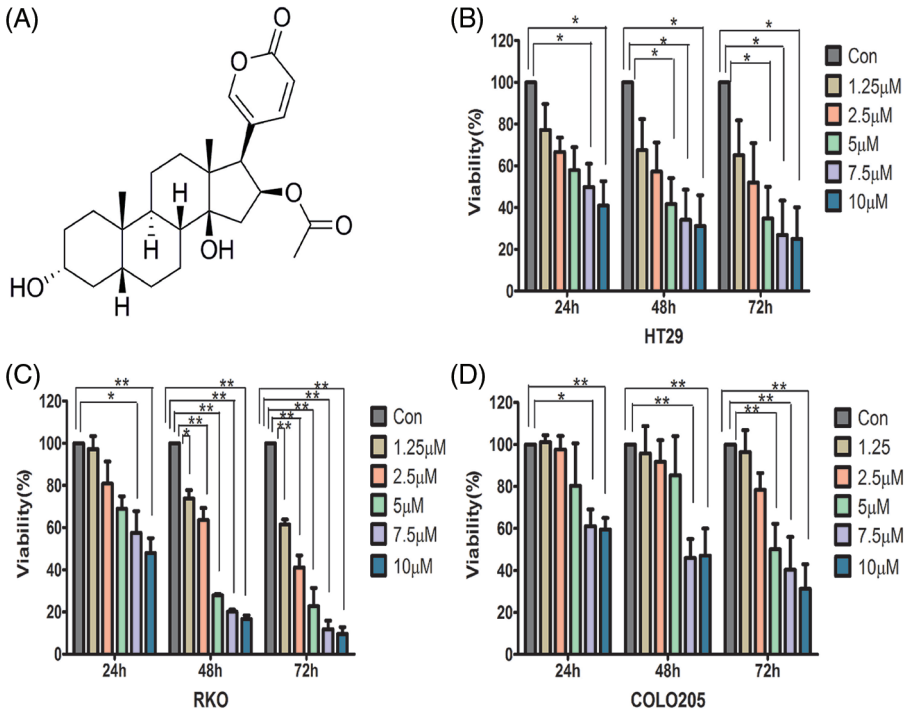

FIGURE 1. Inhibitory effect of different doses of 3-*epi*-bufotalin on colorectal cancer cells for different durations. Structure of 3-*epi*-bufotalin. (A), viability of HT29 cells (B), RKO cells (C), and COLO205 cells (D) (*$P < 0.05$ *vs.* control, **$P < 0.01$ *vs.* control).

3-*epi*-bufotalin on cell cycle progression via flow cytometry, and found well-maintained at the G_2/M stage after 3-*epi*-bufotalin treatment (Figs. 3A–3C). Furthermore, 3-*epi*-bufotalin also decreased the levels of G_2/M phase-associated regulatory proteins, CyclinB1 and phosphorylated CDC2/CDK1 (Figs. 3D–3E). These results suggest that 3-*epi*-bufotalin could arrest the cell cycle at the G_2/M stage in CRC cells.

3-epi-bufotalin inhibited the JAK1/STAT3 signaling pathway

The JAK/STAT signaling pathway is important for cell growth, proliferation, and differentiation, and excessive activation of the STAT3 pathway is considered a hallmark of many malignancies (Huynh *et al.*, 2017). In view of this, we investigated whether 3-*epi*-bufotalin suppresses cell proliferation via inhibiting the JAK1/STAT3 signaling pathway. Western blotting revealed that total JAK1 and STAT3, as well as phosphorylated JAK1 and STAT3 were reduced after 3-*epi*-bufotalin treatment (Fig. 4). Furthermore, treatment of cells with 5 μM of AG490, a STAT3 inhibitor, indeed inhibited the activation of STAT3. Then, the effects of 3-*epi*-bufotalin (6 μM) on CRC cells after 24 h treatment revealed that AG490 decreased the levels of p-STAT3, and 3-*epi*-bufotalin did not further inhibit the proliferation or induce more apoptosis in CRC cells (Figs. S1A and S1B). Meanwhile, the expression of BAX and BCL-2 did not change noticeably after pre-treating with AG490 (Fig. S1C). These results suggested that the JAK1-STAT3 signal pathway participates in the inhibition effect of 3-*epi*-bufotalin on CRC cell proliferation. Thus, 3-*epi*-bufotalin inhibited the proliferation of colorectal cancer cells via down-regulation of the JAK1/STAT3 signal pathway.

Discussion

Bufotalin is a bufadienolide with wide-spectrum anti-cancer activities (Qi *et al.*, 2011; Wang *et al.*, 2011; Wei *et al.*, 2017). Toad compounds are highly toxic, and bufotalin is no exception. Bufotalin suppresses the proliferation of various cancer cells, such as hepatocellular carcinoma HepG2 cells (Zhang *et al.*, 2012), osteoblastoma U2OS, SaOs-2 and MG-63 cells (Zhu *et al.*, 2014), esophageal squamous cell carcinoma Eca-109 and EC9706 cells (Lin *et al.*, 2018), and malignant melanoma A375 cells (Pan *et al.*, 2019), via blocking cell cycle at G_2/M phase and inducing apoptosis. Apoptosis was activated via the intrinsic pathway marked by the increase in BAX, and the decrease in BCL-2 expressions. Although the anti-cancer activities of bufotalin have been widely studied, the bioactivity of its epimer 3-*epi*-bufotalin remains unknown. This study also showed that 3-*epi*-bufotalin arrests the cell cycle at the G_2/M phase and induces apoptosis in CRC cells.

The JAK/STAT signaling pathway plays a crucial role in the multi-step development of tumors, while excessive activation of this pathway results in tumor genesis, promoting metastatic ability and resistance to chemotherapy agents. JAK1 is widely expressed in various cells, and STAT3 is associated with the promotion of oncogenesis and immune suppression (Huynh *et al.*, 2017; Yu *et al.*, 2009). JAK/STAT3 is hyper-activated in many kinds of solid tumors, including colorectal cancer, and is associated with poor prognosis, invasion, metastasis, and multidrug resistance (Jin, 2020; Johnson *et al.*, 2018). Therefore, JAK/STAT3 pathway is a promising target for the treatment of cancer. Several compounds of bufadienolides have been shown to inhibit JAK/STAT3 in cancer cells. For example, bufalin down-regulated anti-apoptotic protein myeloid leukemia 1 and B-cell lymphoma-Xl via inhibition of STAT3 in breast cancer cells (Dong *et al.*, 2011). Likewise, BF211, a derivative of bufalin, induced apoptosis via inhibition of the IL-6/JAK2/STAT3 signaling pathway in myeloma cells (Wu *et al.*, 2018). Cinobufagin suppressed the viability of osteosarcoma cancer U2OS/MG-63 cells by

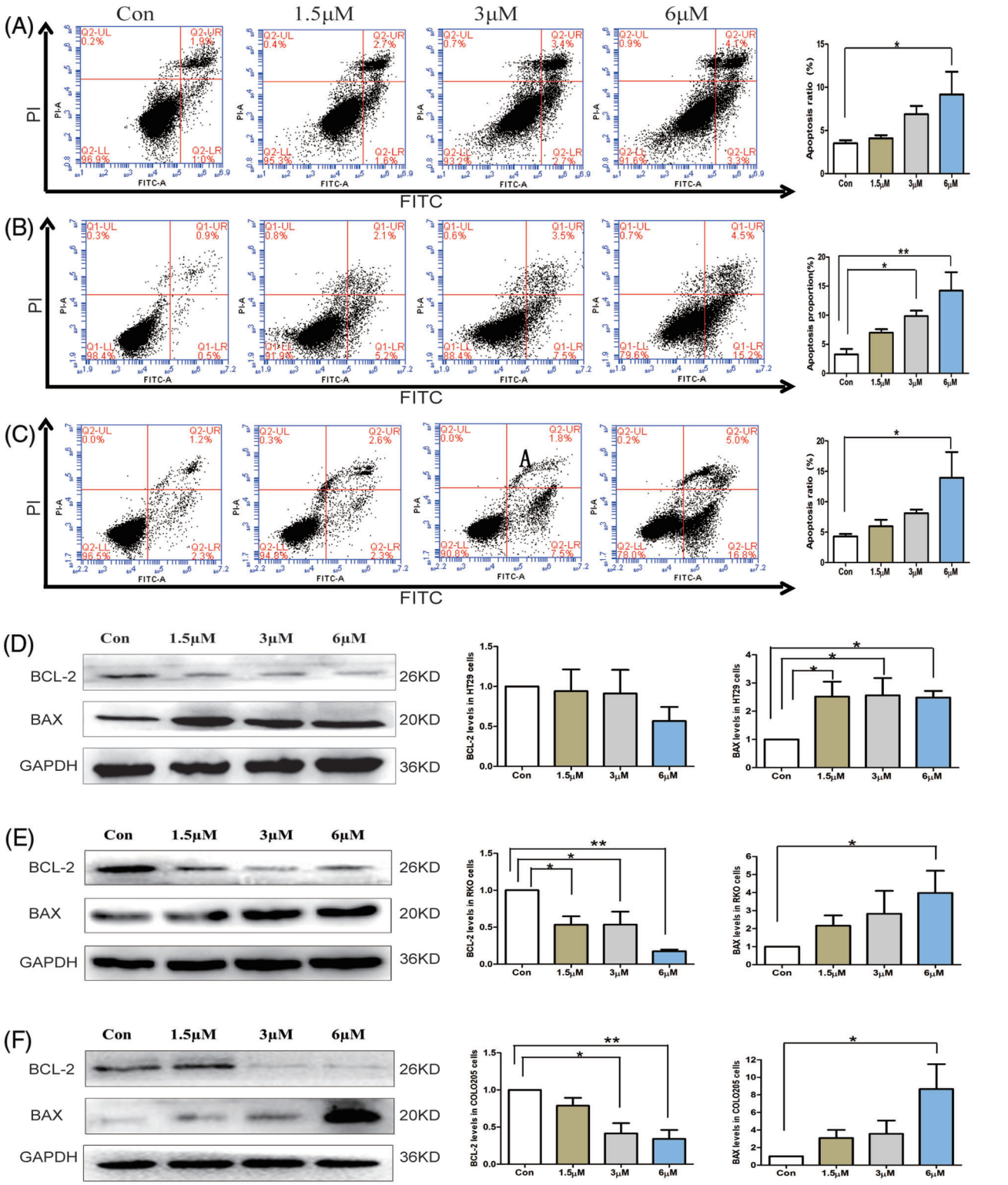

FIGURE 2. Apoptosis induced in colorectal cancer cells by 3-*epi*-bufotalin treating for 24 h. Apoptosis ratio of HT29 cells (A), RKO cells (B), and COLO205 cells (C). Mitochondrial apoptosis-associated protein BAX and BCL-2 were detected in HT29 cells (D), RKO cells (E), and COLO205 cells (F) (*$P < 0.05$ *vs.* control, **$P < 0.01$ *vs.* control).

inhibiting the IL-6-OPN-STAT3 signaling pathway (Zhang *et al.*, 2019). In this study, we found that 3-*epi*-bufotalin reduced the levels of total JAK1 and STAT3, as well as phosphorylated JAK1 and STAT3 in CRC cells. Our work indicates that 3-*epi*-bufotalin could be a new JAK/STAT3 inhibitor for treating CRC.

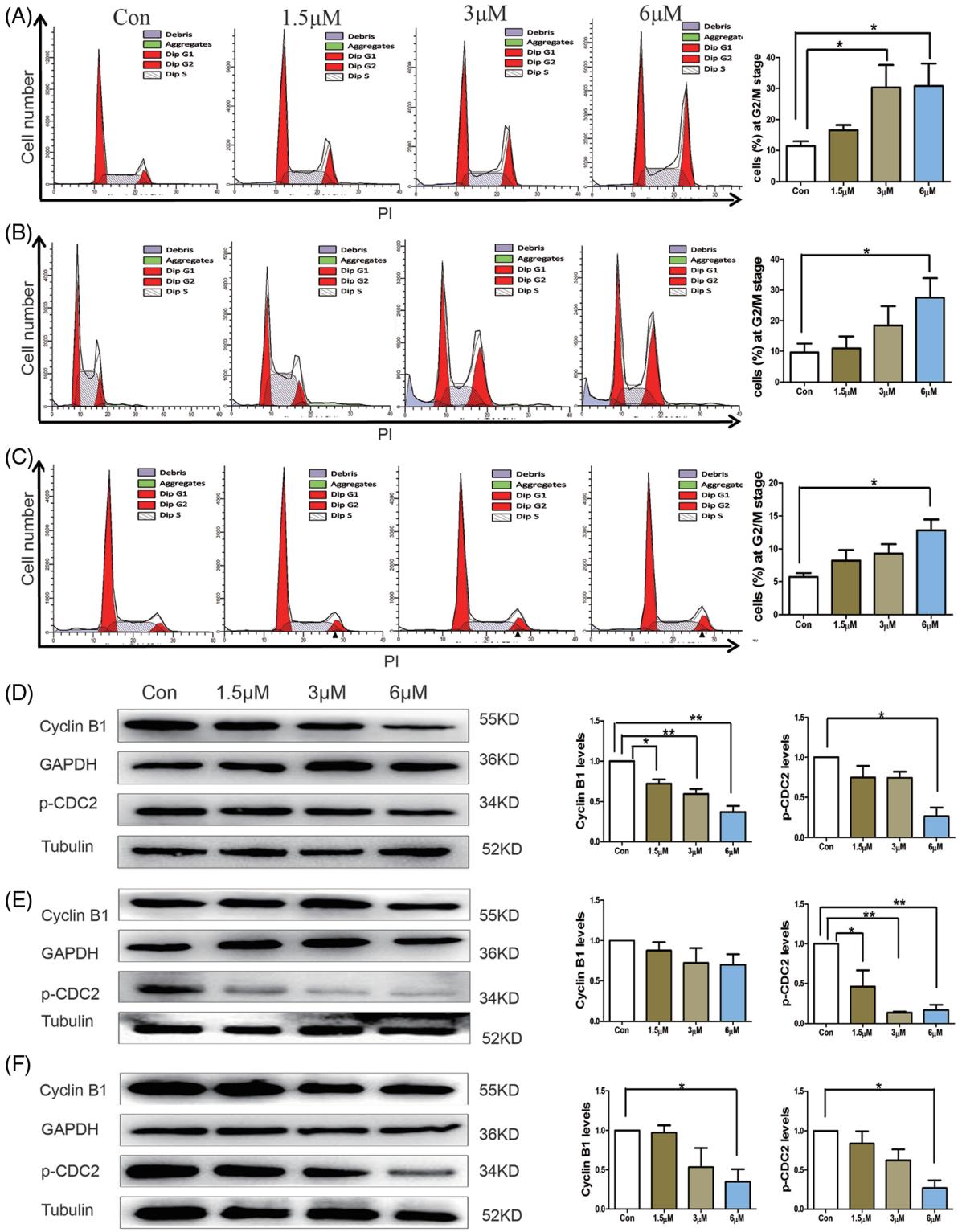

FIGURE 3. Cell cycle arrest in colorectal cancer cells after 24 h of 3-*epi*-bufotalin treatment. Cell cycle distribution of HT29 cells. (A), RKO cells (B), and COLO205 cells (C). Relative expression levels of cyclin B1 and p-CDC2 in HT29 cells (D), RKO cells (E), and COLO205 cells (F) (*$P < 0.05$ *vs.* control, **$P < 0.01$ *vs.* control).

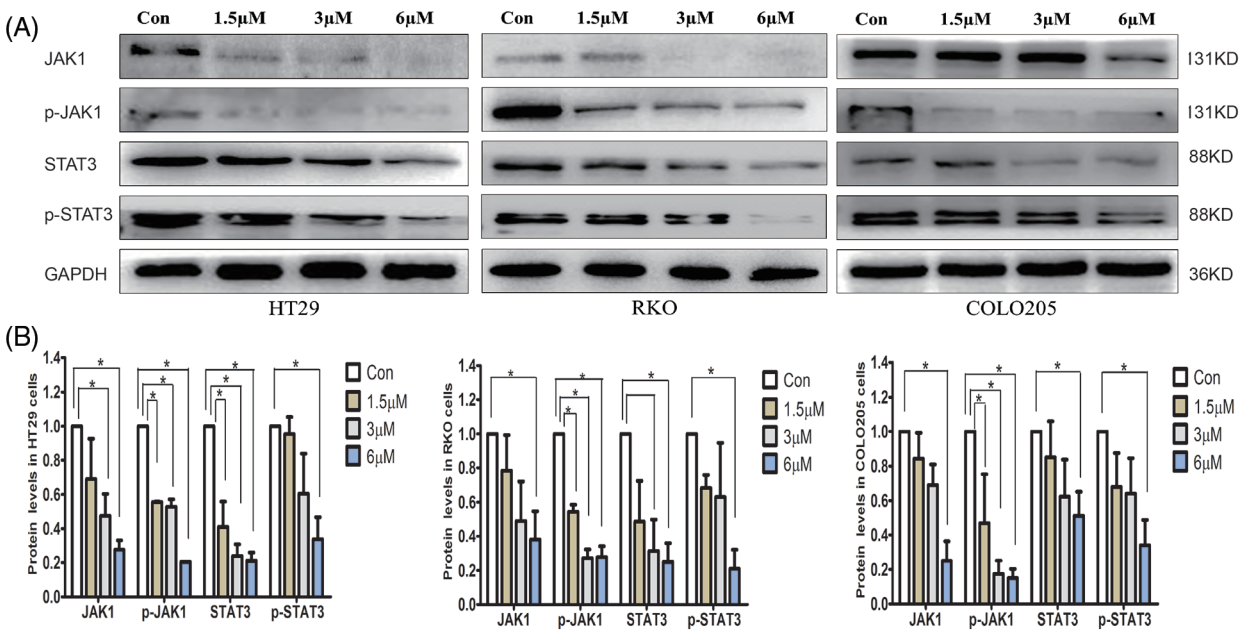

FIGURE 4. Treatment with 3-*epi*-bufotalin for 24 h led to down-regulation of the JAK1/STAT3 signaling pathway in colorectal cancer cells. The expression levels of JAK1, STAT3, phosphorylated-JAK1, and phosphorylated-STAT3(A, B) (*$P < 0.05$ *vs*. control,**$P < 0.01$ *vs*. control).

Conclusion

To conclude, our results demonstrated that 3-*epi*-bufotalin inhibited the growth of CRC cells via suppressing the JAK1/STAT3 signaling pathway and arrested the cell cycle at the G_2/M stage to induce intrinsic apoptosis in CRC cells.

Availability of Data and Materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contribution: Yun Liu, Lingjie Meng, and Sanhua Li conceptualized and designed the study; Sanhua Li, Qinghong Kong, Xiaoke Zhang, Xinting Zhu, Chunbo Yu, Changyan Yu, Nian Jiang, and Jing Hui performed the experiments. Yun Liu, Lingjie Meng, and Sanhua Li revised the manuscript. All authors have read and approved the final manuscripts.

Ethics Approval: Not applicable.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

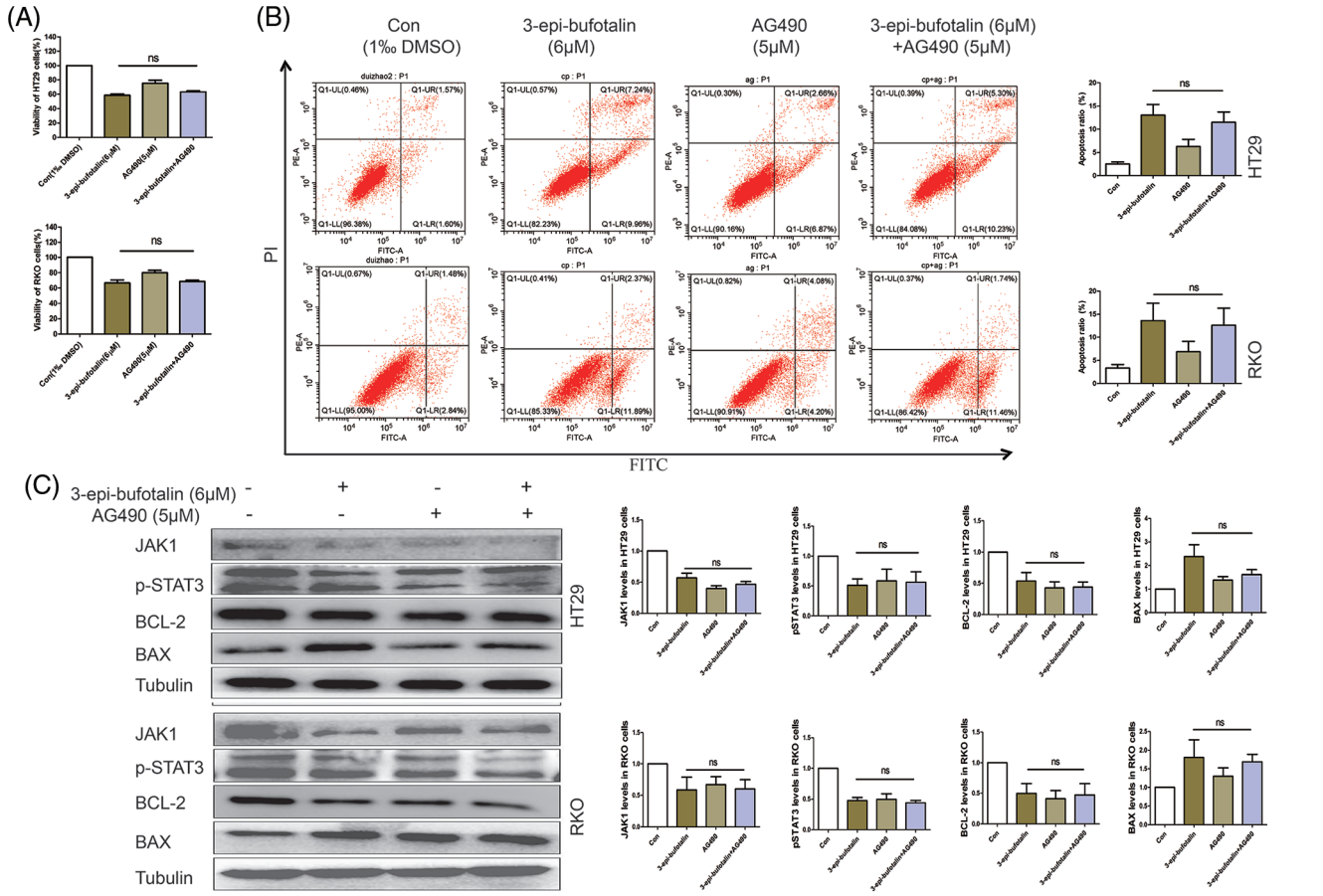

FIGURE S1. HT29 and RKO cells were treated with AG490 (5 μM) for 2 h and followed with treating with 3-*epi*-bufotalin (6 μM) for 24 h. Viability of cells (A). Apoptosis ratios of cells (B). Changes of JAK1 protein, p-STAT3 protein, apoptosis relative proteins BAX and BCL-2 (C) (ns: no statistical significance).