Anticancer effect of involucrasin A on colorectal cancer cells by modulating the Akt/MDM2/p53 pathway

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Abstract. Colorectal cancer (CRC) is the second leading cause of cancer mortality worldwide; however, there is still a lack of effective clinical anti-CRC agents. Naturally-occurring compounds have been considered a potentially valuable source of new antitumorigenic agents. Involucrasin A, a novel natural molecule, was isolated from *Shuteria involucrata* (Wall.) Wight & Arn by our team. In the present study, the anticancer activity of involucrasin A in HCT-116 CRC cells was evaluated. Firstly, the anti-proliferative effect of involucrasin A on HCT-116 cells was analyzed by sulforhodamine B and colony formation assays. The results revealed that involucrasin A exhibited a potent inhibitory effect on HCT-116 CRC cell proliferation *in vitro*. Subsequently, flow cytometry and western blotting indicated that involucrasin A induced apoptosis and upregulated the expression levels of apoptosis-related proteins, such as cleaved-caspase 6 and cleaved-caspase 9, in a dose-dependent manner. Mechanistically, involucrasin A significantly inhibited the phosphorylation of Akt and murine double minute 2 homologue (MDM2), which resulted in increased intracellular levels of p53. This was reversed by exogenous expression of the constitutively active form of Akt. Similarly, either knocking out p53 or knocking down Bax abrogated involucrasin A-induced proliferation inhibition and apoptosis. Together, the present study indicated that involucrasin A exerts antitumorigenic activities via modulating the Akt/MDM2/p53 pathway in HCT-116 CRC cells, and it is worthy of further exploration in preclinical and clinical trials.

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Introduction

Colorectal cancer (CRC) has been ranked as the second most lethal and the third most prevalent type of cancer throughout the world (1). According to GLOBOCAN statistics in 2020, >935,000 deaths and 1.9 million new cases were registered as CRC, which accounted for ~10% of cancer deaths and new cases in the world (1). Unfortunately, the number of new CRC cases is likely to increase to ~2.5 million by 2035 (2). Currently, the standard treatment for patients with CRC is radical surgery combined with adjuvant chemotherapy, which has positive effects on early cases (2). Benefiting from the rapid development of more effective screening methods and improved treatments, the death rate of CRC declined by ~50% in 2016 compared with that in 1970 according to a clinical study in the United States (2). However, the 5-year survival rate for CRC is only ~64%, which drops to 12% for metastatic CRC (3). Furthermore, current CRC chemotherapy has certain limitations, including systemic toxicity, suboptimal response rate, acquired drug resistance and low tumor-specific selectivity (4,5). Therefore, it is necessary to search for novel efficient compounds with good stability, safety and efficacy for the treatment of CRC.

Natural products are a key source of new antitumorigenic agents, as 80-83% of approved anticancer drugs are natural compounds or their derivatives (6). Flavonoids, an important group of natural molecules, are found in a variety of dietary plants, such as fruits and vegetables. Based on their chemical structure, flavonoids can be classified as isoflavonoids, flavanones, flavanols, flavonols, flavones and anthocyanidins (7). Flavonoids, well known for their chemopreventive and chemotherapeutic activities in multiple cancer types, work by arresting the cell cycle, suppressing cell proliferation, inducing apoptosis, modulating reactive oxygen species (ROS)-scavenging enzyme activities and inhibiting invasiveness (8). Notably, early

epidemiological reports have indicated that flavonoids can reduce the risk of CRC (9,10). Hence, flavonoids have received a great deal of interest in CRC research.

For example, oroxylin A, one of the main bioactive flavonoids of Scutellariae Radix, can suppress the growth of CRC by reprogramming HIF1α-modulated fatty acid metabolism (11). Tectochrysin, one of the major flavonoids of *Alpinia oxyphylla* Miquel, can markedly suppress the proliferation of SW480 and HCT-116 human colon cancer cell lines, and lead to apoptotic cell death through regulating death receptor expression and NF-κB activity (12). Additionally, 6-C-(E-phenylethenyl) naringenin, a type of flavonoid from naringenin-fortified fried beef, has been shown to suppress tumor cell proliferation, and induce necrotic cell death and autophagy in CRC cells (13). However, these flavonoid compounds are still in preclinical trials. Notably, ~90% of drug candidates fail during the various phases of clinical trials and drug approval (14). In order to accelerate the development of new drugs, it is necessary to search for more potential natural flavonoids for CRC treatment.

Shuteria involucrata (Wall.) Wight & Arn, has long been used for the treatment of cold, chronic bronchitis, cough, sore throat, pharyngitis, and tonsillitis in China (15). Recently, the natural flavanone, involucrasin A ($C_{21}H_{22}O_5$), was isolated from *S. involucrata* (Wall.) Wight & Arn by our team (15). However, the anticancer activity of involucrasin A has not been reported in detail. Furthermore, the mechanisms underlying the potential antitumorigenic effects of involucrasin A remain elusive.

The Akt/murine double minute 2 homologue (MDM2)/p53 signaling pathway has significant roles in regulation of the cell cycle, proliferation and apoptosis (16). Akt, a classic intracellular signaling axis, is associated with tumorigenesis through a number of mechanisms (17). The malignancy of numerous types of cancer, such as CRC, lung cancer, breast cancer, endometrial cancer and liver cancer, is induced by increasing abnormal phosphorylation levels of Akt. As an important downstream target of Akt, MDM2 can be phosphorylated and translocated to the nucleus following Akt activation, thereby suppressing the bioactivity of the tumor suppressor protein, p53 (18). Bax, a pro-apoptotic regulator of Bcl2 family members, has been shown to be involved in p53-mediated apoptosis (19). Upon apoptotic stimuli, the expression of Bax is upregulated by p53 (19,20). Furthermore, p53 can regulate the transition of G_2/M phase (21), and p53-dependent G_2 arrest is associated with a decrease in cyclin A2 level (22). Therefore, novel therapeutic compounds inhibiting the Akt/MDM2/p53 signaling pathway are intended as a strategy to eliminate cancerous cells. For example, germacrone and saponin have been reported to induce cancer cell apoptosis and cell cycle arrest by suppressing the Akt/MDM2/p53 signaling pathway, and may be potential therapeutic agents for the treatment of cancer (16,23).

In the present study, the antitumorigenic activities of involucrasin A in HCT-116 CRC cells were investigated and its regulatory effects on the Akt/MDM2/p53 pathway were examined.

Materials and methods

Reagents and antibodies. The chemical structure of involucrasin A ($C_{21}H_{22}O_5$) is shown in Fig. 1A. Involucrasin A was isolated as a white amorphous powder from *S. involucrata* (Wall.) Wight & Arn by our team (15). The whole plant of *S. involucrata* (Wall.) Wight & Arn was identified and collected from Pu'er district (Yunnan, China) by Professor Zi-Gang Qian (Yunnan University of Chinese Medicine, Kunming, China). Involucrasin A was dissolved in dimethyl sulfoxide (Thermo Fisher Scientific, Inc.) and stored at -40˚C. Sulforhodamine B (SRB), trichloroacetic acid (TCA), puromycin, Tris base and crystal violet were purchased from MilliporeSigma. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco; Thermo Fisher Scientific, Inc. The Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Biosciences.

In this study, all primary antibodies were used at a dilution of 1:1,000. Furthermore, the secondary antibodies were used at a dilution of 1:10,000. Primary antibodies against human proteins included anti-β-actin (cat. no. A5441; mouse monoclonal; MilliporeSigma); anti-cyclin A2 (cat. no. AF6624; rabbit polyclonal; Beyotime Institute of Biotechnology); anti-Bax (cat. no. 2772; rabbit polyclonal), anti-cleaved caspase-9 (cat. no. 9505; rabbit polyclonal), anti-cleaved caspase-6 (cat. no. 9761; rabbit polyclonal), anti-phosphorylated (p)-Akt (cat. no. 2965; rabbit monoclonal) and anti-Akt (cat. no. 9272; rabbit polyclonal) (all from Cell Signaling Technology, Inc.); anti-p53 (cat. no. sc-126; mouse monoclonal; Santa Cruz Biotechnology, Inc.); anti-p-MDM2 (cat. no. ab170880; rabbit monoclonal) and anti-MDM2 (cat. no. ab16895; mouse monoclonal) (both from Abcam). The anti-rabbit (cat. no. 211-035-109) and anti-mouse (cat. no. 715-035-150) secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc.

Cell lines and culture. Colorectal cancer cell lines (HCT-116, Caco-2 and CT26), breast cancer cell lines (BT-549, SKBR3, HS578T, MDA-MB-436 and MDA-MB-453) and a liver cancer cell line (HepG2) were obtained from the American Type Culture Collection and grown in DMEM supplemented with 10% FBS and 1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin), and incubated at 5% CO_2 and 37˚C. CT26 is a murine CRC cell line from a BALB/c mouse. The HCT-116 p53 KO cell line was provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA) and was cultured in DMEM supplemented with 10% FBS and 1% antibiotics, and incubated at 5% CO_2 and 37˚C (24). To disrupt the two p53 alleles in HCT116 cells, two promoterless targeting vectors were used, each containing a hygromycin- or geneticin-resistance gene in place of the p53 genomic sequences (24).

Colony formation assay. To assess the effects of involucrasin A on the clonogenic capacity of HCT-116 cells, cells were plated into 12-well plates ($1x10^3$ cells/well) or 24-well plates (500 cells/well) and allowed to adhere overnight. Cells were then treated with three concentrations of involucrasin A (12.5, 25 and 50 μM) for 10 days, and incubated at 5% CO_2 and 37˚C. Colonies were washed with PBS and stained with 0.2% (w/v) crystal violet in buffered formalin for 20 min at room temperature. A colony was defined as consisting of at least 50 cells and was counted manually. Colony images were captured using an Epson Perfection V700 Photo (Epson Corporation).

Cell viability assay. Cell viability was determined using the SRB assay. Briefly, HCT-116 cells were seeded in 96-well

plates at a density of $5x10^3$ cells/well overnight. After incubation, fresh media containing different concentrations (0, 3.12, 6.25, 12.5, 25, 50, 100, and 200 μM) of involucrasin A was added, and further incubated at 5% CO_2 and 37˚C for 48 or 72 h. The cells were fixed with 50 μl ice-cold 50% (w/v) TCA at 4˚C for 1 h. Then, wells were carefully rinsed with deionized water five times. After air drying, cells were subsequently stained with 0.4% (w/v) SRB for 30 min and rinsed with 1% acetic acid at room temperature. Finally, adhered cells were solubilized with 200 μl 10 mM Tris base solution (pH 10.5), and plates were agitated for 5 min before absorbance was measured using a SpectraMax paradigm microplate reader (Molecular Devices, LLC) at a wavelength of 515 nm.

Cell cycle analysis. Cell cycle analysis was performed by flow cytometry. Briefly, HCT-116 cells were plated in 6-well plates at a density of $2x10^5$ cells/well and treated with involucrasin A (0, 25, 50 and 100 μM) in DMEM supplemented with 10% FBS and 1% antibiotics, and incubated at 5% CO_2 and 37˚C for 72 h. Subsequently, cells were washed with 1X PBS before trypsinization (3 min), centrifugation (300 x g for 5 min at room temperature), and fixation with 70% ethanol at -20˚C for 2 h. Then, cells were stained with 500 μl propidium iodide (PI) (50 μg/ml)/RNase A (200 μg/ml; Nanjing KeyGen Biotech, Co., Ltd.) for 30 min at room temperature. Finally, cells were washed with 1X PBS, centrifuged at room temperature (300 x g for 5 min), and resuspended in 1X PBS, and their fluorescence was detected using a FACS Aria II flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software (V.10.4.1; FlowJo LLC).

Flow cytometric analysis of apoptosis. The evaluation of apoptosis induction was performed using a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instructions. HCT-116 cells ($2x10^5$ cells/well) in a 6-well plate were treated with involucrasin A (0, 25, 50 and 100 μM) and incubated at 5% CO_2 and 37˚C for 48 or 72 h. Then, cells were washed with cold 1X PBS (pH 7.4) before trypsinization (3 min), centrifugation (300 x g for 5 min at room temperature), resuspension in 100 μl 1X binding buffer, staining with 2.5 μl FITC Annexin V and 2.5 μl PI, and incubation for 15 min in the dark at room temperature. The cellular analysis was performed using a flow cytometer (Beckman Coulter, Inc.).

Lentivirus transduction for gene overexpression and knockdown. Three different genes encode the isoforms of Akt, including Akt1, Akt2 and Akt3. Akt3 is expressed mainly in the testes and brain, Akt2 is highly expressed in insulin-responsive tissue, and Akt1 is ubiquitously expressed (25). Notably, Akt1 is involved in the cellular survival pathway through suppressing the apoptotic process, and has an important role in numerous types of cancer (26). Therefore, the constitutively active Akt (pCDH-puro-myr-HA-Akt1; CA-Akt) was exogenously expressed in the present study. The CA-Akt plasmid was obtained from Addgene, Inc. (cat. no. 46969). Empty plasmid was used as the control. The short hairpin RNA (shRNA) control plasmid (sh007) was from MilliporeSigma (cat. no. SHC007; insert sequence, 5'-CCGGCGCTGAGT ACTTCGAAATGTCCTCGAGGACATTTCGAAGTA CTCAGCGTTTTT-3'). For Bax knockdown, the following double-strand oligonucleotides were cloned into a pLKO.1 plasmid (cat. no. 10878; Addgene, Inc.): shBax, F-5'-CCG GGACGAACTGGACAGTAACATGCTCGAGCATGTTAC TGTCCAGTTCGTCTTTTTG-3', R-5'-AATTCAAAAAGA CGAACTGGACAGTAACATGCTCGAGCATGTTACTGT CCAGTTCGTC-3'. Lentivirus was produced using the 293T cells transduced with 0.5 μg of the aforementioned construct, 0.375 μg psPAX2 (cat. no. 12260; Addgene, Inc.) and 0.125 μg pMD2.G (cat. no. 12259; Addgene, Inc.) in 50 μl Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) with 3 μl FuGENE HD (Promega Corporation). The cells were incubated in a humidified incubator at 5% CO_2, 37˚C for 16 h, and the medium containing the transfection mixture was replaced with fresh medium. After an additional 48 h, the medium was collected and filtered using a 0.45-μM filter unit, and directly added to target HCT116 cells. HCT-116 cells were incubated at 5% CO_2 and 37˚C with lentivirus for 24 h before selection with puromycin (cat. no. A1113803; Thermo Fisher Scientific, Inc.) for 3 days. The concentrations of puromycin used for selection and maintenance were 10 and 1 μg/ml, respectively.

Western blot analysis. Western blotting was performed to detect the protein expression levels extracted from HCT-116 cells. Firstly, the protein samples were collected in RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) with phosphatase inhibitor (Roche Diagnostics GmbH) and protease inhibitor cocktail (Roche Diagnostics GmbH). Next, a BCA Protein Assay Kit (Beyotime Institute of Biotechnology) was used to determine the concentration of the protein samples. Samples containing 20 μg of protein were separated by SDS-PAGE on 10-15% gels and transferred to PVDF membranes. Membranes were blocked with 3% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blocked membranes were then incubated with primary antibodies diluted in TBST containing 5% bovine serum albumin (Beyotime Institute of Biotechnology) overnight at 4˚C. Membranes were washed three times with TBST for 1 h at room temperature and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies diluted in 5% skimmed milk in TBST for 1 h at room temperature. Membranes were washed three times with TBST and visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) via an Amersham Imager 800 (Cytiva). ImageJ software (version 1.48; National Institutes of Health) was used to semi-quantify protein levels after western blotting.

Statistical analysis. SPSS version 20.0 (IBM Corp.) was used for statistical analysis. All data are from at least three independent experiments. Data are presented as the mean ± standard deviation. Comparisons between two groups were performed using an unpaired Student's t-test; for comparisons of three or more groups, one-way ANOVA followed by Tukey's multiple comparison test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Involucrasin A effectively inhibits HCT-116 cell proliferation. To determine the effects of involucrasin A on CRC viability,

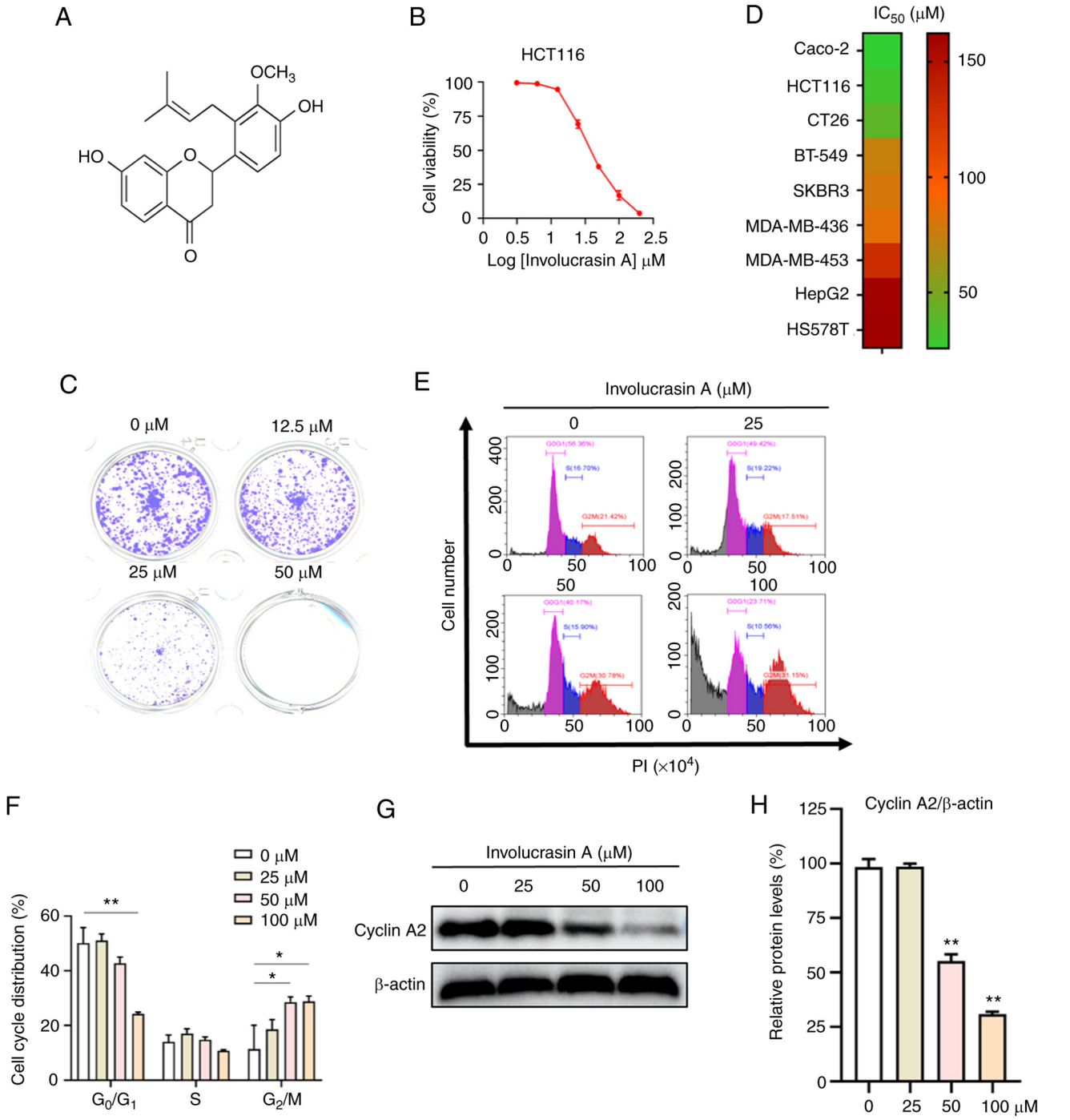

Figure 1. Involucrasin A effectively inhibits HCT-116 cell proliferation. (A) Chemical structure of involucrasin A. (B) Dose-dependent effect of involucrasin A on the viability of HCT-116 cells at 72 h. (C) Colony formation of HCT-116 cells treated with involucrasin A (0, 12.5, 25 and 50 μM) for 10 days. (D) Dose-dependent effect of involucrasin A on the viability of different cancer cell lines at 72 h. Colorectal cancer cell lines include HCT116, Caco-2 and CT26. (E and F) HCT-116 cells were treated with involucrasin A (0, 25, 50 and 100 μM) for 72 h. The cell cycle assay was analyzed by flow cytometry. (G and H) Protein expression levels of cyclin A2 were analyzed using western blotting. β-actin served as a loading control. All data are from at least three independent experiments. *P<0.05, **P<0.01 vs. control (0 μM). PI, propidium iodide.

HCT-116 cells were treated with involucrasin A at various doses for 72 h, and cell viability was analyzed by SRB assay. The results suggested that involucrasin A effectively inhibited the viability of the CRC cell line HCT-116 in a dose-dependent manner with an IC_{50} value of 37.92 mM (Fig. 1B). This was confirmed by a long-time colony formation assay (Fig. 1C). Furthermore, involucrasin A exhibited the most potent anti-proliferative activity in the colorectal cancer cell lines (HCT-116, Caco-2 and CT26) compared with other types of cancer cell lines (Fig. 1D). Therefore, the anticancer activities and mechanisms of involucrasin A in CRC cells became the focus of the present study.

In cancer, cancer cell proliferation is generally induced by a dysregulated cell cycle (27,28). Thus, the cell cycle distribution of HCT-116 cells treated with involucrasin A was analyzed. After 72 h, a significant increase in the G_2/M fraction and a decrease in the G_0/G_1 fraction was observed in cells treated with 50 and

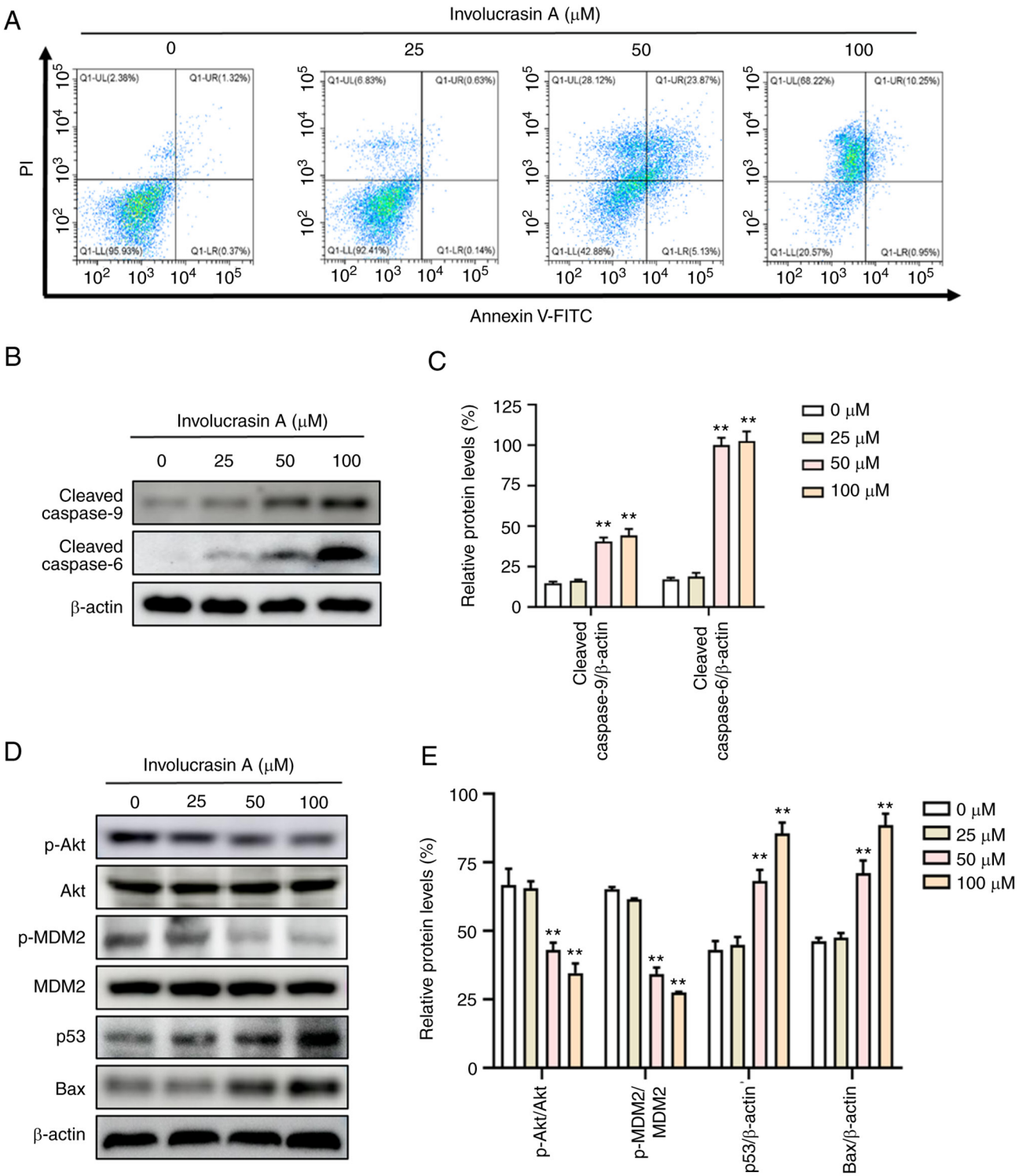

Figure 2. Involucrasin A effectively induces HCT-116 cell apoptosis. HCT-116 cells were treated with involucrasin A (0, 25, 50 and 100 μM) for 72 h. (A) HCT-116 cells were stained with Annexin V-FITC/PI, and apoptosis was quantified by flow cytometry. (B-E) Protein expression levels of cleaved-caspase 9, cleaved-caspase 6, p-Akt, Akt, p-MDM2, MDM2, p53 and Bax were examined via western blotting. β-actin served as a loading control. All data are from at least three independent experiments. **P<0.01 vs. control (0 μM). PI, propidium iodide; p, phosphorylated; MDM2, murine double minute 2 homologue.

100 μM involucrasin A compared with in the untreated cells (Fig. 1E and F). These results indicated that involucrasin A could induce HCT-116 cell cycle arrest at G_2/M phase. Cell cycle progression is regulated by several key proteins, such as cyclin A2 and cyclin D1 (28). Cyclin A2 is a crucial enzyme that promotes the start of cell mitosis, and depletion of cyclin A2 leads to an arrest in G_2 phase (29). In line with its effect on cell cycle distribution, involucrasin A significantly downregulated the expression levels of cyclin A2 in HCT-116 cells (Fig. 1G and H).

Involucrasin A effectively induces HCT-116 cell apoptosis. A close relationship exists between cell cycle progression, proliferation and apoptosis in cancer cells (27). Therefore, whether involucrasin A can induce apoptosis was analyzed.

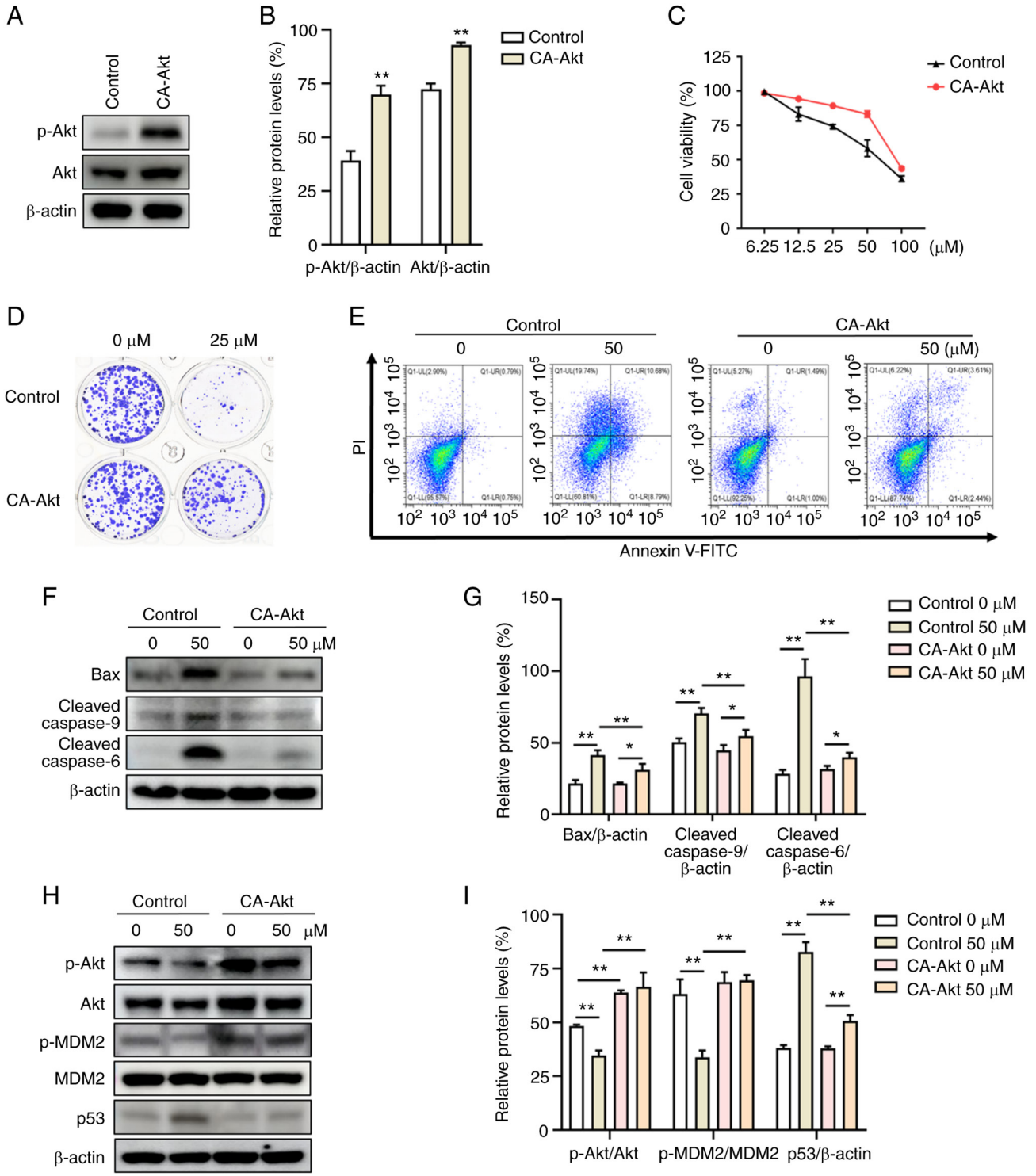

Figure 3. CA-Akt attenuates involucrasin A-induced inhibition of proliferation and apoptosis. (A and B) In cells with CA-Akt, the protein expression levels of p-Akt and Akt were significantly increased. HCT116 cells transduced with the empty vector alone as the negative control. (C) HCT-116 cells were treated with involucrasin A at the indicated concentrations for 48 h. Cell viability was analyzed by sulforhodamine B assay. (D) Colony formation of control and CA-Akt HCT-116 cells treated with involucrasin A (0 or 25 μM) for 10 days. Cells were treated with involucrasin A (0 or 50 μM) for 48 h. (E) Apoptosis was quantified by flow cytometry assay. (F) Western blotting was used to analyze the protein expression levels of cleaved-caspase 9, cleaved-caspase 6 and Bax. β-actin served as a loading control. (G) Relative protein expression levels of Bax, cleaved-caspase 9, cleaved-caspase 6 were semi-quantified. (H and I) The protein levels of p53, p-Akt, Akt, p-MDM2 and MDM2 were analyzed by western blotting. All data are from at least three independent experiments. *P<0.05, **P<0.01 as indicated or vs. control. CA-Akt, constitutively active Akt; PI, propidium iodide; p, phosphorylated; MDM2, murine double minute 2 homologue.

After treatment for 72 h, 50 and 100 μM involucrasin A markedly induced HCT-116 cell apoptosis compared with the control group (Fig. 2A). Induced by the apoptotic signal, Bax promotes the release of cytochrome *c* from mitochondria, and cytochrome *c* subsequently activates the caspase-9 apoptotic pathway (30,31). Once activated, caspase 9 activates downstream 'effector caspases' (i.e., caspase 6), and upregulates the levels of cleaved-caspase 6 (32). In the present study, the protein

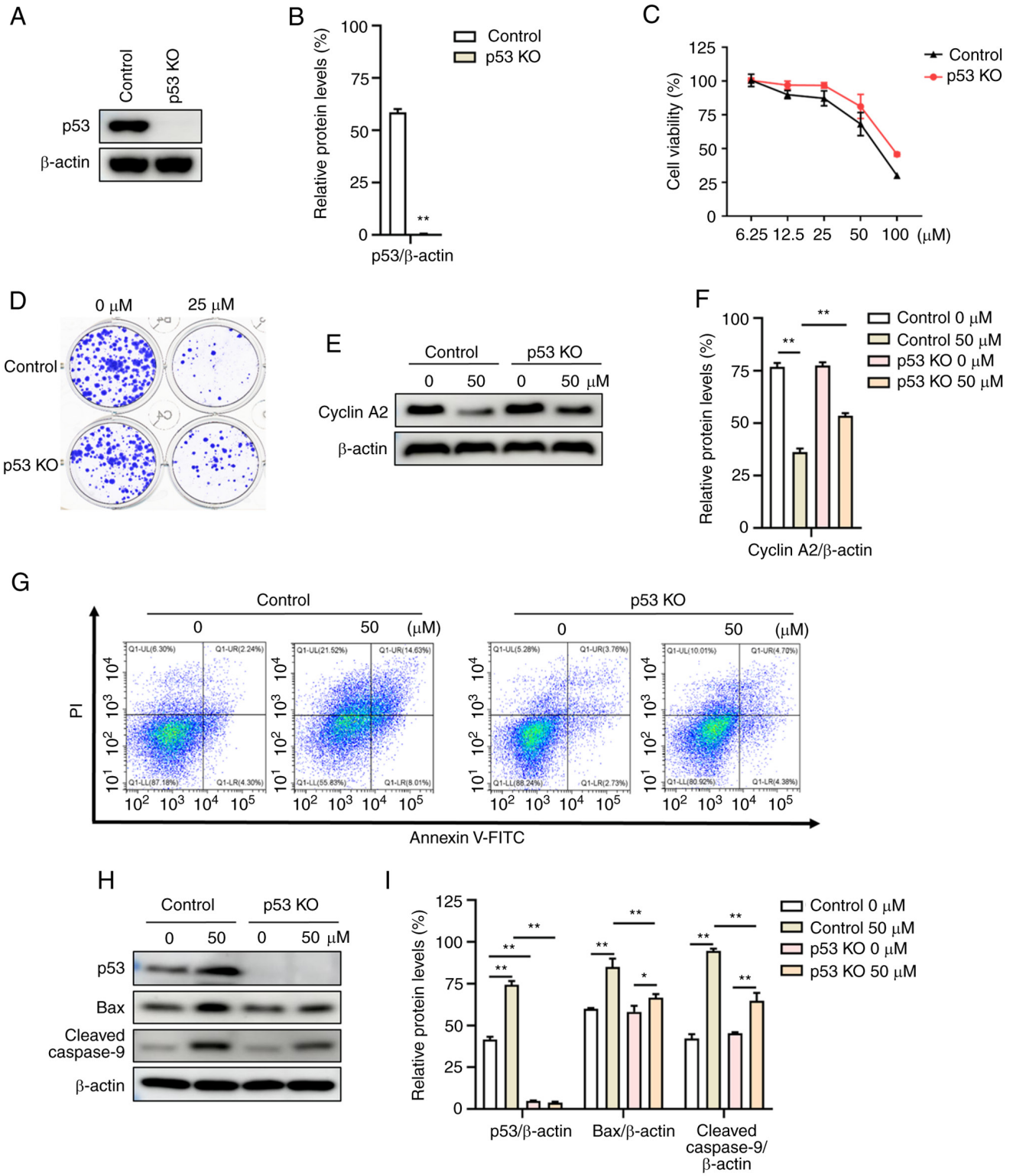

Figure 4. p53 KO attenuates the anticancer functions of involucrasin A. Parental HCT-116 cells were used as the control group. (A and B) In the cells with p53 KO, the protein expression levels of p53 were significantly decreased. (C) Cells were treated with involucrasin A at the indicated concentrations for 48 h. Cell viability was analyzed by sulforhodamine B. (D) Control and p53 KO HCT-116 cells were treated with involucrasin A (0 or 25 μM) for 10 days, and colony formation was determined. (E and F) Protein expression levels of cyclin A2 were analyzed using western blotting. Cells were treated with 0 or 50 μM involucrasin A for 48 h. (G) Apoptosis was quantified by flow cytometry. (H and I) The protein expression levels of cleaved-caspase 9 and Bax were examined via western blotting. β-actin served as a loading control. All data are from at least three independent experiments. *P<0.05, **P<0.01 as indicated or vs. control. KO, knockout; PI, propidium iodide.

expression levels of cleaved-caspase 9 and cleaved-caspase 6 were significantly upregulated with involucrasin A treatment (Fig. 2B and C).

Involucrasin A exerts anticancer activities through modulating the Akt/MDM2/p53 pathway. The Akt/MDM2/p53 signaling pathway is a well-known modulator of cell proliferation and

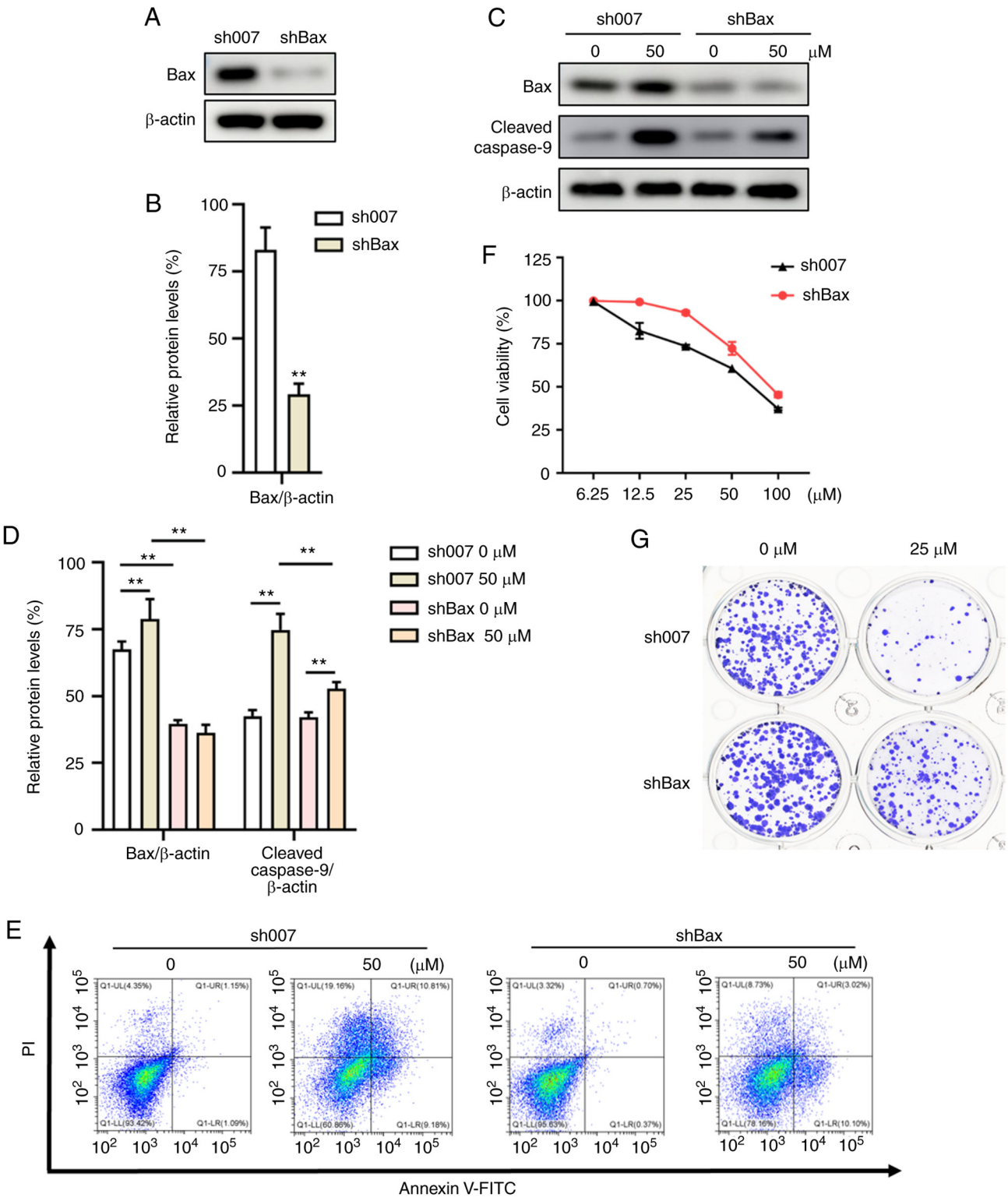

Figure 5. Bax knockdown attenuates the anticancer functions of involucrasin A. (A and B) In cells transduced with shBax, protein expression levels of Bax were significantly decreased. (C-E) Cells were treated with involucrasin A at the indicated concentrations (0 or 50 μM) for 48 h. The expression levels of cleaved-caspase 9 and Bax were examined using western blotting, and apoptosis was quantified by flow cytometry. (F) Cells were cultured with 0, 6.25, 12.5 25, 50 and 100 μM involucrasin A for 48 h. Cell viability was analyzed by sulforhodamine B. (G) For colony formation assay, cells were treated with 0 or 25 μM involucrasin A for 10 days. All data are from at least three independent experiments. **P<0.01 as indicated or vs. sh007. sh, short hairpin; PI, propidium iodide.

apoptosis (16). To investigate whether this signaling pathway was affected by involucrasin A, the present study examined the related protein expression levels by western blotting. Notably, the phosphorylation levels of Akt and MDM2 were significantly decreased with involucrasin A treatment in a dose-dependent manner (Fig. 2D and E). MDM2 is a key downstream protein of Akt, which can be phosphorylated by p-Akt (33). p-MDM2 translocates to the nucleus, promoting ubiquitin-dependent degradation of p53 (34). As expected, p53 was upregulated by involucrasin A in HCT-116 cells. Notably,

p53 can upregulate the expression levels of Bax, leading to programmed cell death (20). Therefore, the protein expression levels of Bax were also analyzed via western blotting. In the present study, Bax was shown to be upregulated by involucrasin A.

CA-Akt attenuates involucrasin A-induced inhibition of proliferation and apoptosis. To confirm that involucrasin A exerts antitumorigenic functions by modulating the Akt/MDM2/p53 pathway, CA-Akt was exogenously expressed in HCT-116 cells. As shown in Fig. 3A and B, the protein expression levels of p-Akt and Akt were significantly increased in cells transfected with CA-Akt. The anti-proliferative and pro-apoptotic effects of involucrasin A on HCT-116 cells were reversed by CA-Akt (Fig. 3C-E). Consequently, the involucrasin A-induced expression levels of apoptotic proteins, cleaved-caspase 9 and cleaved-caspase 6, were attenuated in CA-Akt-expressing cells (Fig. 3F and G). Accordingly, the involucrasin A-induced changes in the protein expression levels of p-Akt, p-MDM2, p53 and Bax were blunted in CA-Akt cells compared with in the control cells (Fig. 3H and I). All results indicated that CA-Akt reversed involucrasin A-induced anti-proliferative and pro-apoptotic effects.

p53 KO attenuates the anticancer functions of involucrasin A. To further investigate the dependency of p53 in involucrasin A-induced anti-proliferative and pro-apoptotic effects, the present study took advantage of the isogenic p53 KO HCT-116 cell line. In cells with p53 KO, the protein expression levels of p53 were significantly decreased compared with those of the control group (parental HCT-116 cells) (Fig. 4A and B). Depletion of p53 led to the reversal of involucrasin A-induced proliferation inhibition (Fig. 4C and D). Additionally, the survival and proliferation of p53 KO and control HCT116 cells were almost the same when not treated with involucrasin A (Fig. 4D). Activation of p53 can induce G_2 arrest, which is associated with transcriptional downregulation of cell cycle genes, such as cyclin A2 (22). After treatment with involucrasin A, the protein expression levels of cyclin A2 in p53 KO cells were increased significantly compared with those of the control cells (Fig. 4E and F). Furthermore, involucrasin A-induced apoptosis was attenuated in p53 KO cells (Fig. 4G). The induction of cleaved-caspase 9 and Bax were also reversed in involucrasin A-treated p53 KO cells, compared with control cells (Fig. 4H and I). These data suggested that involucrasin A-induced anti-proliferation and pro-apoptotic effects were p53 dependent.

Bax knockdown attenuates the anticancer functions of involucrasin A. To confirm the involvement of Bax in involucrasin A-induced cytotoxic effects on HCT-116 cells, the present study knocked down Bax using lentivirus-mediated RNA interference. In cells transduced with shBax, the protein expression levels of Bax were significantly decreased (Fig. 5A and B). Notably, Bax knockdown significantly attenuated the induction of cleaved-caspase 9 following treatment with involucrasin A (Fig. 5C and D). As expected, Bax knockdown markedly inhibited involucrasin A-induced apoptosis (Fig. 5E). Consequently, the SRB assay and the colony formation assay revealed that Bax knockdown markedly decreased involucrasin A-induced proliferation inhibition (Fig. 5F and G).

Discussion

Although chemotherapy is the preferred treatment method for human CRC, adverse side effects and chemoresistance remain major hurdles to successful treatment. Natural flavonoids are a rich repository for colon cancer drug discovery (35). Involucrasin A, a novel natural flavonoid isolated from *S. involucrata* (Wall.) Wight & Arn, exhibited anti-proliferative activities on cancer cells (15). This finding stimulated our interest in further investigating the effects and mechanisms of involucrasin A on CRC cells.

Proliferation is a crucial part of cancer development and progression. Cancer therapy involving cytotoxic drugs kills cells that have a high basal level of proliferation. Therefore, excellent anticancer agents, such as vinblastine, homoharringtonine, etoposide, teniposide, docetaxel and camptothecin derivatives, can block proliferation, resulting in cell cycle arrest and apoptosis (36). To determine the effects of involucrasin A on CRC cell proliferation, SRB and colony formation assays were performed. The results suggested that involucrasin A could efficiently inhibit the proliferation of human CRC cells. Furthermore, cell cycle analysis showed that involucrasin A significantly increased G_2/M arrest and downregulated the expression levels of cyclin A2 in HCT-116 cells. The present study also analyzed whether apoptosis was affected by involucrasin A. After treatment for 48 h, involucrasin A markedly induced apoptotic cell death. In addition, involucrasin A significantly upregulated apoptotic protein expression levels of cleaved-caspase 9 and cleaved-caspase 6.

According to a previous report, flavonoids induce apoptosis by increasing levels of ROS in cancer cells (37). However, ROS levels were not assessed in the present study. The Akt/MDM2/p53 signaling pathway in cancer has been studied by our team for a long time. Importantly, a preliminary experiment by our team found that the protein expression levels of p53 and Bax were notably increased by involucrasin A. These results suggested that the Akt/MDM2/p53 signaling pathway may have a key role in the anticancer function of involucrasin A. The Akt protein kinase has important roles in several interconnected molecular signaling axes related to proliferation, angiogenesis, apoptosis and cell metabolism. Thus, Akt represents a therapeutic target, especially in colon cancer and triple-negative breast cancer (TNBC), where the Akt signaling axis is largely hyper-activated (38). For example, a clinical trial showed that the Akt inhibitor ipatasertib plus paclitaxel could improve progression-free survival of patients with locally advanced/metastatic TNBC (39). Additionally, the constitutive activation of the Akt pathway generally induces chemotherapy resistance (17). p53 inhibits cancer development through transcriptionally activating related genes, and the functional link between Akt and p53 has been reported (40,41). MDM2, a key downstream protein of Akt, can be phosphorylated by p-Akt. p-MDM2 can translocate to the nucleus and modulate p53 levels by targeting p53 for protein degradation (42). Therefore, the Akt/MDM2/p53 signaling pathway serves a key role in regulation of the cell cycle, proliferation and apoptosis. Novel agents that can inhibit this pathway are an emerging strategy to treat cancer.

In the present study, the results suggested that involucrasin A could significantly decrease the phosphorylation levels of Akt and MDM2, and upregulate p53 protein expression in HCT-116 cells. To confirm the involvement of the Akt/MDM2/p53 pathway in the antitumorigenic functions of involucrasin A, overexpressed CA-Akt and p53 KO in HCT-116 cells were assessed. The results showed that CA-Akt and p53 KO attenuated involucrasin A-induced anti-proliferative and pro-apoptotic effects. Bax has been verified to be involved in p53-mediated apoptosis (19). Upon apoptotic stimuli, the protein expression levels of Bax are upregulated directly by p53, thereby stimulating programmed cell death (20).

Notably, the present study found that the expression levels of Bax were upregulated by involucrasin A. However, p53 KO attenuated involucrasin A-induced Bax expression. These results suggested that involucrasin A induced cancer cell apoptosis by p53-modulated Bax signaling. To confirm this hypothesis, Bax knockdown in HCT-116 cells was performed. The results showed that Bax knockdown attenuated involucrasin A-induced pro-apoptotic effects and proliferation inhibition. During apoptosis, Bax induces the release of cytochrome *c* from the mitochondria, and subsequently stimulates the caspase-9 pathway (30,31). This process is a classical signaling pathway, more attention was therefore focused on the protein levels of cleaved-caspase 9 rather than cytochrome *c* in the present study. Notably, Bax knockdown significantly attenuated the induction of cleaved-caspase 9 following treatment with involucrasin A.

In summary, the present study indicated that involucrasin A may exert a potent anticancer effect on colon cancer cells through inducing apoptosis and cell cycle arrest. Mechanistically, involucrasin A exhibited anticancer functions by modulating the Akt/MDM2/p53 pathway. For the present study, involucrasin A was isolated from *S. involucrata* (Wall.) Wight & Arn by our team. Unfortunately, it was too difficult to extract enough involucrasin A for use in animal studies. Based on these results, involucrasin A is a promising natural compound for treating colon cancer, and worthy of further exploration in preclinical and clinical trials.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CW, ZY and WM conceived and designed the experiments. JD, YS, ZiW, QL, FZ, WL, ZhW and JC performed the experiments and analyzed the results. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy. CW and WM wrote the manuscript. CW and WM confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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