

Rottlerin-induced autophagy leads to apoptosis in bladder cancer cells

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Abstract. It has been well-established that apoptosis contributes to cancer cell death; however, the role of autophagy in cancer cell death remains unclear. The aim of the present study was to investigate the effects of rottlerin, a traditional Indian medicine, on cell growth inhibition and autophagy in EJ human bladder carcinoma cells *in vitro*. Cell viability, measured by MTT assay, was found to be suppressed in a dose- and time-dependent manner. In addition, apoptosis was significantly increased in cells treated with rottlerin, as indicated by increased annexin V-fluorescein isothiocyanate/propidium iodide staining and changes in the cell cycle distribution that indicated blockage at G1 phase. Rottlerin treatment also enhanced the activation of autophagy, with increased expression of microtubule-associated protein 1 light chain 3 (LC3)-II and the appearance of autophagosomes. The increased level of LC3-II and autophagosomes suggests that autophagy may contribute to apoptosis in these cells. In addition, no apparent alterations in the levels of pro-caspase-3, cleaved caspase-3, total poly (ADP ribose) polymerase (PARP) and cleaved-PARP were observed in cells treated with rottlerin, which indicates that caspases may not serve a key role during the process of apoptosis induced by rottlerin. Therefore, the results of the present study indicate that rottlerin promotes apoptosis and arrests the cell cycle in EJ cells, which may be caused by autophagy activation.

Introduction

Urinary bladder cancer is the ninth most common cancer worldwide (1), and occurs in a ratio of ~5:1 with respect to non-muscle-invasive vs. muscle-invasive phenotypes (2). Non-muscle-invasive tumors may be treated by combined

therapy involving transurethral resection and intravesical chemotherapy (3), whereas muscle-invasive bladder cancer can be managed with radical cystectomy and pelvic lymphadenectomy, according to the current gold standard method (4). Critically, among all tumor types, bladder cancer exhibits a significantly greater rate of recurrence following treatment, and the per-patient cost of treating this cancer is therefore considered to be the highest (5). As current therapies continue to produce unsatisfactory rates of morbidity and mortality, the development of novel treatment strategies to manage bladder cancer remains a challenge.

Rottlerin [1-(6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl)-3-phenyl-2-propen-1-one; also known as mallotoxin] is a traditional Indian medicine that is used against tapeworm, scabies and herpetic ringworm. It has been used as a protein kinase C δ (PKC- δ) inhibitor to verify the biological function of PKC- δ (6). Recent scientific research has confirmed that rottlerin has a range of molecular targets and antitumor activities, including inhibition of cell proliferation, suppression of cell growth, induction of apoptosis, anti-angiogenesis and inhibition of reactive oxygen species formation (7-10). However, the biological mechanism underlying the antitumor effect of rottlerin remains unclear.

In eukaryotic cells, autophagy is important process by which protein degradation and organelle turnover can occur, and acts as a critical adaptive response during cell stress or starvation to recycle energy and nutrients (11). The activation of autophagy is associated with various stress conditions, whereas its dysfunction is linked to numerous types of human diseases, including cancer (12). Consequently, autophagy has been widely considered to be a potential novel target for cancer therapy (13). Autophagy and apoptosis are intricately linked: Both are genetically regulated and evolutionarily conserved processes that can determine cell fate; however, while apoptosis invariably leads to cancer cell death, autophagy may occur as a survival response to growth factors or nutrient deprivation, but may also act as an important molecular mechanism for tumor cell suicide (14). Numerous studies have indicated that if cellular damage is extensive or if apoptosis is compromised, autophagy may be used to kill cells (15,16).

In the present study, the antitumor activity of rottlerin on EJ malignant bladder cancer cells was investigated and its role in the induction of autophagy was examined. Based on

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the results, we propose that rottlerin-induced autophagy may contribute to the antitumor effect via apoptosis.

Materials and methods

Cell culture. EJ human bladder carcinoma cells were purchased from ATCC (Manassas, VA, USA) and maintained in Invitrogen RPMI-1640 medium (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore, Billerica, MA, USA), 1% non-essential amino acids and 1% penicillin/streptomycin in a humidified incubator at 37°C supplied with 5% CO₂.

Reagents. Rottlerin (Sigma-Aldrich; Merck Millipore) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 20 mM and stored at -20°C. Human microtubule-associated protein 1 light chain 3 (LC3; cat. no. ABC432; working dilution, 1:1,000) antibody was purchased from Sigma-Aldrich (Merck Millipore). Human caspase-3 (cat. no. ab13585; dilution, 1:1,000) and human poly (ADP-ribose) polymerase (PARP; cat. no. ab75607; dilution, 1:400) antibodies were purchased from Abcam (Cambridge, MA, USA). The human anti-β-actin antibody (cat. no. sc-130065; dilution, 1:1,000) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated donkey anti-rabbit (cat. no. NA934; dilution, 1:1,000) and anti-mouse (cat. no. N1034; dilution, 1:10,000) IgG secondary antibodies were obtained from GE Healthcare Life Sciences (Uppsala, Sweden).

MTT cell viability assay. Cell viability was measured by MTT assay. EJ cells (5,000/well) were seeded into 96-well plates and cultured in a CO₂ incubator overnight. Rottlerin at various concentrations was then added. Following incubation periods of 24, 48 and 72 h, 10 μl of MTT solution (Sigma-Aldrich; Merck Millipore), dissolved in autoclaved phosphate-buffered saline (PBS) at 5 mg/ml, was added. After 4 h, 150 μl of DMSO was added to dissolve the formazan crystals, and the absorbance (optical density; OD) of each well was measured using an automatic microplate reader (Thermo Fisher Scientific, Inc.) at 490 nm. Each experiment was repeated three times. Cell viability results were calculated as a percentage, as follows: Cell viability % = $(OD_{\text{treatment}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100$.

Transmission electron microscopy (TEM). TEM was used to determine the appearance of autophagosomes in treated and control EJ cells. Cells treated with rottlerin (2 μM) or without for 48 h were harvested by trypsinization, washed with ice-cold PBS and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, then post-fixed in 1% osmium tetroxide buffer. Following dehydration in a graded series of ethanol, the cells were embedded in Spurr resin. Ultra-thin sections (60 nm) were cut on an ultramicrotome and were subsequently observed under a JEM-1230 transmission electron microscope.

Western blot analysis. Cells were treated with rottlerin (2 μM) and washed with ice-cold PBS at 48 h post-treatment, and cell lysate was isolated using Protein Extraction Reagent (Beyotime Institute of Biotechnology, Nantong, China). Equal amounts of cell lysate protein (30 μg) were separated by gel electrophoresis

on a 6-12% gradient SDS-PAGE gel and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk in PBS for 1 h at room temperature prior to the application of the specific primary antibodies for incubation overnight at 4°C. Subsequently the membranes were washed three times with PBS containing Tween-20, and incubated with secondary antibodies at room temperature for 1 h. The blots were detected with an ECL kit (Beyotime Institute of Biotechnology) using a Storm 840 PhosphorImager system (Molecular Dynamics, Inc., Sunnyvale, CA, USA). The images were further analyzed using Image Quant TL 8.1 software (GE Healthcare Life Sciences).

Flow cytometric analysis. Apoptosis was analyzed by flow cytometry using propidium iodide (PI) and an annexin V-fluorescein isothiocyanate FITC staining kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells were detached by trypsinization at 37°C for 5 min. Detached cells were rinsed twice with RPMI-1640 medium and cold PBS, then separated by centrifugation at 168 x g for 5 min at room temperature. Cells were resuspended in 1X binding buffer at a concentration of 1x10⁶ cells/ml. Cell/buffer solution (100 μl; 1x10⁵ cells) was transferred to a round-bottom tube and incubated with 5 μl of PI and annexin V-FITC for 15 min at room temperature in darkness. Finally, 400 μl of 1X binding buffer was added to each sample tube and evaluated by flow cytometry (FACSCalibur; BD Biosciences) according to the manufacturer's instructions.

Cell cycle analysis. Cells were trypsinized, washed twice with cold PBS, fixed in ice-cold 70% ethanol and stored at 4°C. Prior to analysis, cells were washed again with PBS, suspended in 50 g/ml PI and 0.25 g/ml RNase A, and further incubated for 30 min in the dark. Cytometric analyses were performed using flow cytometer and CellQuest v1.0.2 software (BD Biosciences). For each determination, ~10,000 cells were counted.

Clonogenic (colony formation) assay. The clonogenic assay was performed to evaluate *in vitro* cell survival following treatment with rottlerin. Cells were seeded in 6-well plates (2x10⁵/well) and treated with rottlerin (2 μM) at 24 h after seeding. Untreated cells were used as a negative control for this analysis. After a further 24 h incubation, cells were trypsinized and washed once with PBS, then counted and seeded in 6-well plates (200 cells/well). Each experiment was repeated three times. All experimental samples were incubated in an environment of 5% CO₂ at 37°C for 14 days to form appropriately large clones consisting of ≥50 cells. Cells were subsequently stained with crystal violet staining solution (Beyotime Institute of Biotechnology). The visible colonies (≥50 cells) were counted and typical images were captured using a Nikon camera.

Statistical analysis. All statistical analyses were performed with SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Results are presented as the mean ± standard error of the mean. Different test conditions were compared using a one-way analysis of variance or Student's *t*-test, with P<0.05 considered to indicate a statistically significant difference.

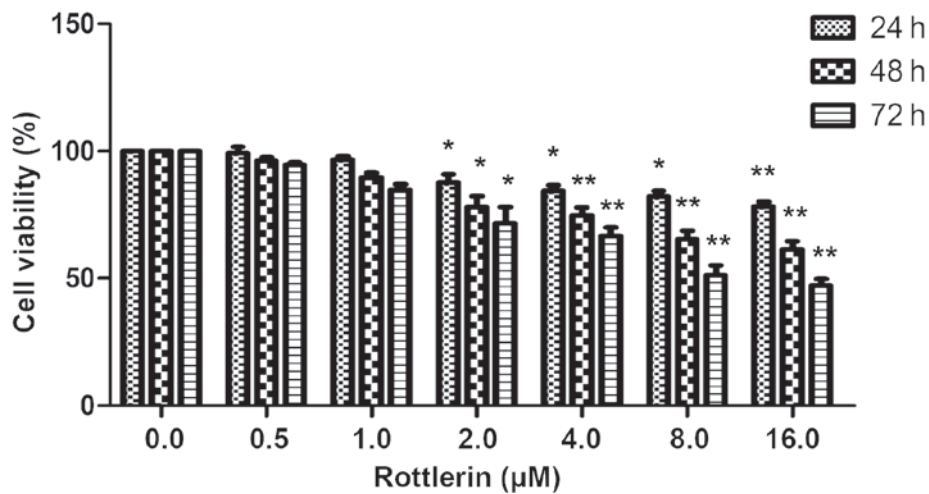


Figure 1. Dose-response and time-response studies for EJ cells treated with rottlerin. EJ cells were exposed to rottlerin at concentrations of 0, 0.5, 1, 2, 4, 8 or 16 μmol/l for 24, 48 or 72 h. Cell viability was evaluated by MTT assay. The plot depicts the percentage of cell viability of the rottlerin-treated cells compared with those of the untreated control cells (the viability of control cells was set as 100%). Results are presented as the mean ± standard error of the mean from three independent experiments. *P<0.05 compared with the control group; **P<0.01 compared with the control group.

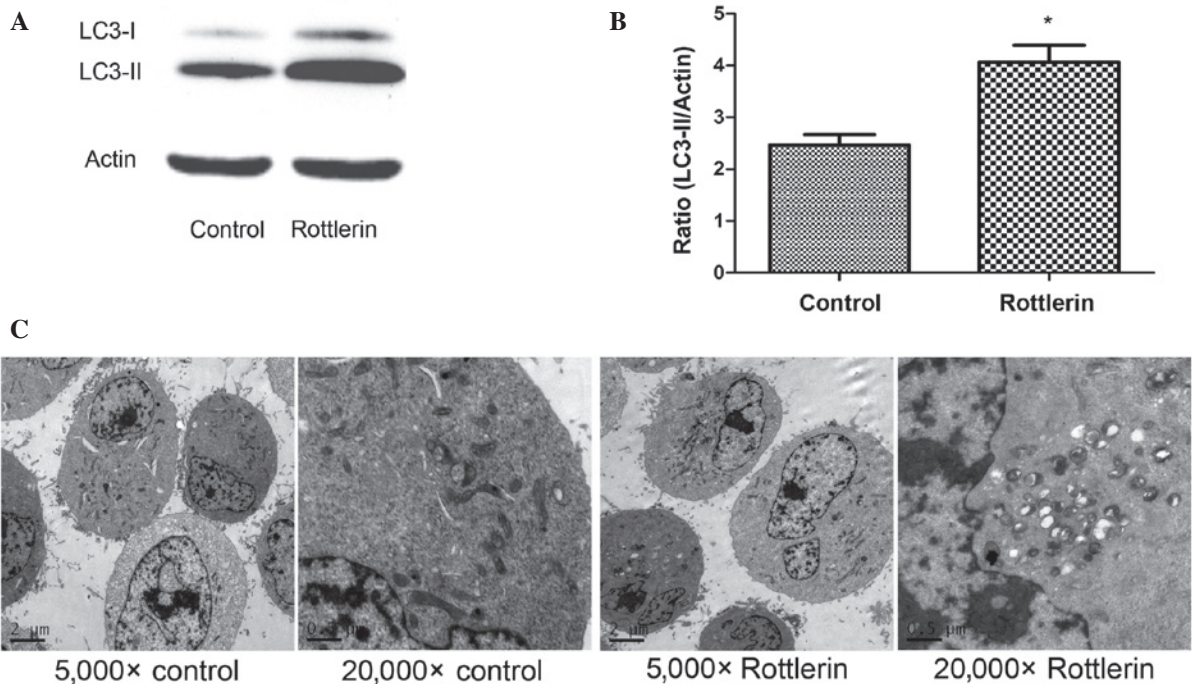


Figure 2. Activation of autophagy by rottlerin in EJ cells. (A) The expression level of LC3-II increased following treatment with rottlerin for 48 h. Equal loading and transfer were verified by probing the membranes with an anti-β-actin antibody. (B) Quantification of the protein expression levels revealed significant changes in cells treated with rottlerin for 48 h. The results are presented as the mean ± standard error of the mean from three independent experiments. *P<0.05 compared with the control group. (C) TEM studies revealed a greater amount of autophagosomes in rottlerin-treated EJ cells, whereas no autophagosomes were observed in the untreated control group. Magnification, x5,000 and x20,000. LC3, microtubule-associated light chain 3.

Results

Rottlerin inhibits the growth of human malignant bladder cancer EJ cells. Using an MTT assay, the effects of rottlerin on EJ bladder cancer cells were determined. The cells were treated with 0-16 μM rottlerin for 24, 48 or 72 h prior to the assessment of cell viability. As shown in Fig. 1, rottlerin had a growth inhibitory effect on EJ cells in a dose-dependent and time-dependent manner, indicating that rottlerin has antitumor effects in this cell type; however, concentrations

of 0, 0.5 and 1 μM rottlerin had non-significant effects on the cells, and 2 μM was therefore selected for further experiments.

Rottlerin activates autophagy in EJ cells. To evaluate the activation of autophagy by rottlerin, the expression levels of the autophagy-related protein LC3 were assessed by western blotting analysis and autophagosome formation was observed by TEM. During autophagosome formation, the microtubule-associated LC3-I is converted to the membrane-bound

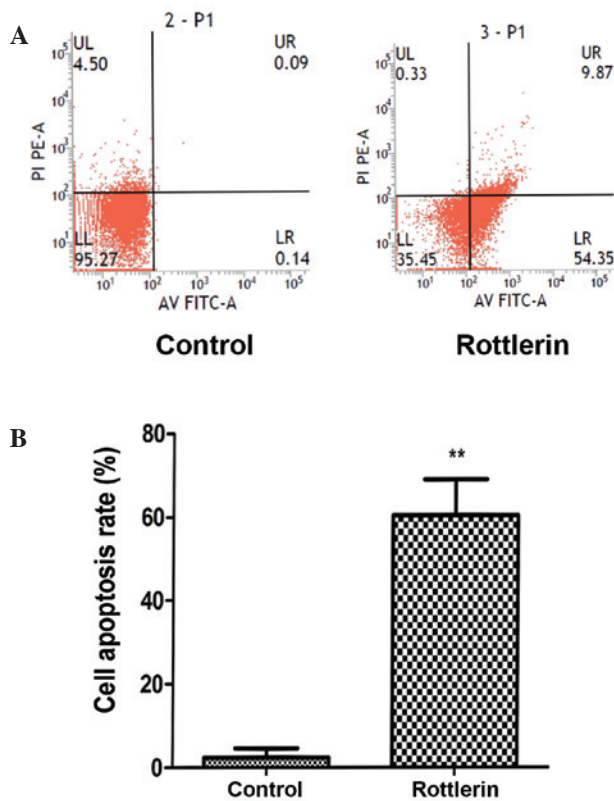


Figure 3. Rottlerin can induce apoptosis. (A) EJ cells were treated with 2 μ M rottlerin for 48 h and apoptotic cell death was evaluated by flow cytometric analysis with annexin V-FITC and PI staining. Relative proportions of early and late apoptotic cells are indicated in lower and upper right quadrants, respectively, in each treatment group. (B) Quantification of the apoptosis expression levels revealed a significant difference in apoptosis rate in cells treated with rottlerin. Results are presented as the mean \pm standard error of the mean from three independent experiments. ** P <0.01 compared with the control group. FITC, fluorescein isothiocyanate; PI, propidium iodide.

form LC3-II (17). In EJ cells, the expression level of LC3 increased following treatment with rottlerin (Fig. 2A) as compared with the control cells, and quantification of the protein expression levels demonstrated that this difference was significant ($P=0.014$) (Fig. 2B). These results indicate that autophagy is activated by rottlerin. To further confirm this finding, TEM studies were performed, revealing a greater amount of autophagosomes in rottlerin-treated EJ cells, whereas no autophagosomes were observed in the untreated control group (Fig. 2C). Collectively these data suggest that autophagy is induced by rottlerin in EJ cells.

Rottlerin promotes apoptosis and cell cycle arrest in EJ cells. To assess apoptosis, a PI and annexin V-FITC assay was performed. The results revealed that a marked increase in apoptosis occurred following a 48-h treatment (Fig. 3A). Quantification of the apoptosis rates demonstrated a significant increase in apoptosis in cells treated with rottlerin (P <0.01) (Fig. 3B). Cell cycle distribution was also analyzed by flow cytometry (Fig. 4A and B). EJ cells were predominantly blocked in G1 phase, indicating that the cell cycle distribution of EJ cells was affected by rottlerin. These results indicate that, after 48 h treatment, the percentage of cells arrested in G1 phase was increased from 54.4 ± 2.5 to $69.7 \pm 1.1\%$ ($P=0.005$).

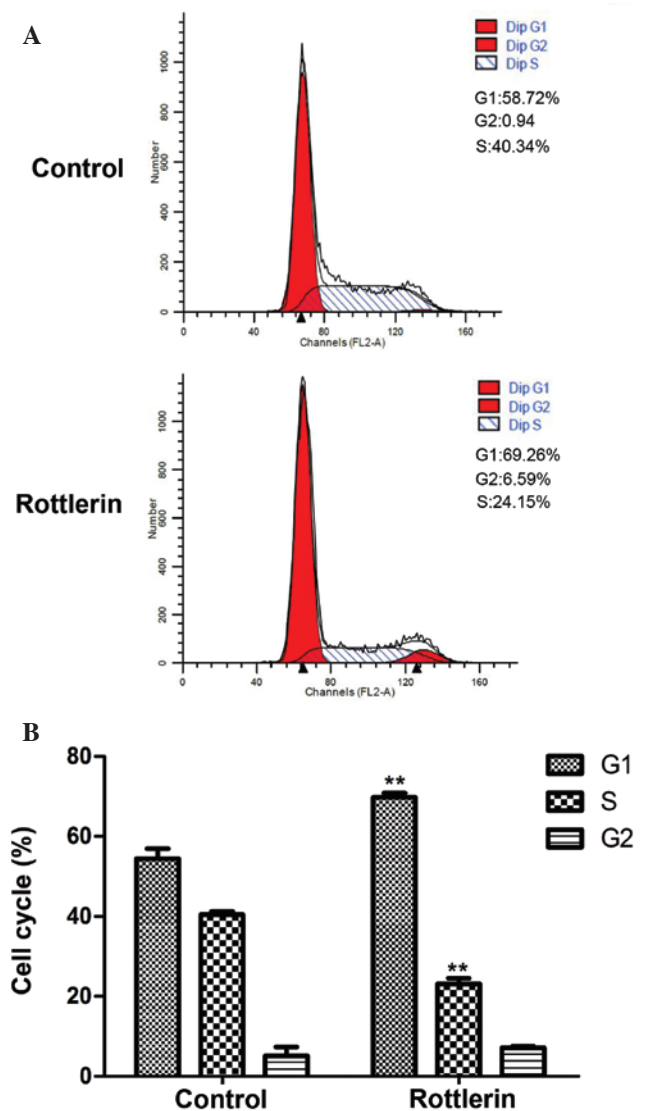


Figure 4. Effects of rottlerin on cell cycle distribution. (A) The cell cycle distribution showed an increase in G1 phase and reduction in S phase following treatment with rottlerin for 48 h, as assessed by flow cytometric analysis. (B) The change in cell cycle distribution indicated that cells arrested in G1 phase following treatment. Results are presented as the mean \pm standard error of the mean from three independent experiments. ** P <0.01 compared with the control group.

Rottlerin induces apoptotic cell death through a caspase-independent pathway. Rottlerin induces apoptosis in certain cancer cell lines (18), and the activation of a caspase cascade is important in apoptotic induction and execution (19). To confirm the involvement of caspases in rottlerin-induced apoptosis, the expression levels of pro-caspase-3 and PARP were measured by western blot. The results indicated that pro-caspase-3 and PARP levels were not increased in rottlerin-treated cells (Fig. 5A-C), and the cleaved forms of caspase-3 and PARP were also not detected (Fig. 5A). These findings indicate that caspases are not involved in rottlerin-induced apoptosis in EJ cells.

Rottlerin reduces the clonogenic capacity of EJ cells. As shown in Fig. 6, EJ cells exposed to rottlerin 48 h treatment

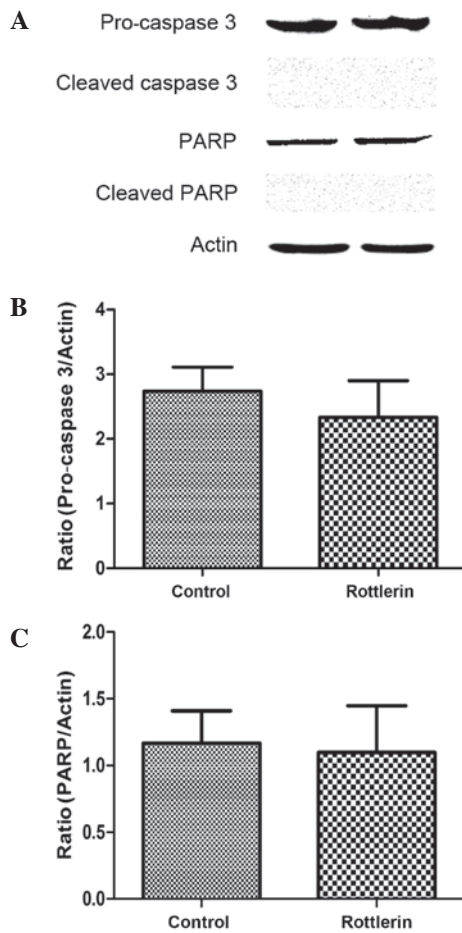


Figure 5. Expression of apoptosis-associated proteins. (A) Following treatment of EJ cells with rottlerin for 48 h, western blot analysis was performed to assess the levels of the apoptotic markers pro-caspase-3, cleaved caspase-3, total PARP and cleaved PARP. (B) Quantification of the protein expression levels revealed no apparent changes in cells treated with rottlerin for 48 h. Results are presented as the mean \pm standard error of the mean from three independent experiments. PARP, poly (ADP-ribose) polymerase.

exhibited a prominent reduction in the clonogenic capacity compared with untreated cells ($P=0.003$).

Discussion

The present study provides evidence that induction of autophagy potentiates the rottlerin-induced apoptotic cell death in human bladder cancer cells *in vitro*.

Rottlerin is a natural polyphenolic ketone that may be isolated from the pericarps of *Mallotus philippinensis* (20). Herbs and their derived products have been the mainstay of traditional medicines all over the world (21). The phytochemicals presenting in these plants and their food products are generally non-toxic and have the capacity to prevent chronic diseases (22). Generally the plant products encompass high concentrations of flavonoids and phenolic content (23). Flavonoids serve a vital role in protection against human diseases, including lipid peroxidation involved in atherogenesis, thrombosis, carcinogenesis, hepatotoxicity and a variety of other disease conditions (24,25). Rottlerin as a plant extract has numerous different functions.

The present results demonstrate that rottlerin has a growth inhibitory effect on EJ cells in a dose-dependent and

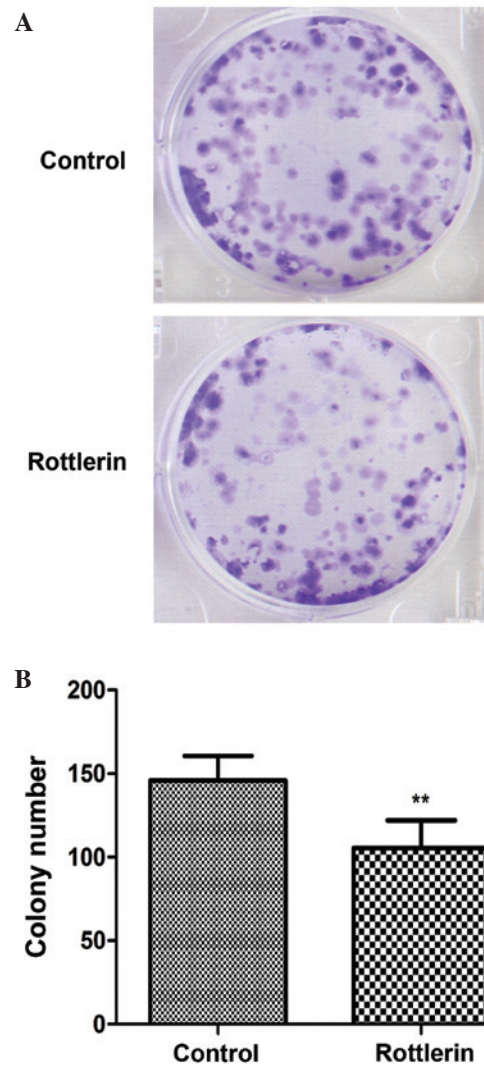


Figure 6. Colony formation assay. (A) Representative images of the colony formation assay show that, following treatment with rottlerin, cells formed fewer colonies compared with control cells. (B) The number of clones formed by the EJ cells were derived from the colony formation assay. Results are presented as the mean \pm standard error of the mean from three independent experiments. ** $P<0.01$ compared with the control group.

time-dependent manner, and also reduces the clonogenic capacity of these cells; thus, *in vitro* cell survival capacity is reduced following treatment with rottlerin. Recently, increasing evidence has demonstrated that autophagy and apoptosis often occur in the same cells, initiated in response to the same stimuli (26). In this circumstance, autophagy either promotes or inhibits apoptosis. The majority of studies have indicated that autophagy inhibition sensitizes tumor cells to a wide spectrum of cancer therapies, while others have shown that treatment-induced tumor cell death requires intact autophagic machinery (27-32). For instance, atorvastatin is considered by some to cause autophagy to promote apoptosis in human prostate cancer cells (33), whilst others have a contrasting views, and consider that inhibition of autophagy potentiates atorvastatin-induced apoptotic cell death (34). Although there is known crosstalk between autophagy and apoptosis, since they share certain signaling pathways and proteins, the mechanism where this bridging occurs has not been fully defined (35). In the present study autophagy was

induced by rottlerin, as indicated by a number of observations. Firstly, the morphological appearances of autophagy were identified, including increased amounts of autophagosomes on TEM, which is the gold standard. In addition, quantification of LC3-II protein expression levels revealed apparent changes in treated cells. Finally, biochemical changes that are characteristic of apoptotic cell death were observed, including an increased sub-G1 population.

As expected, treatments with rottlerin induced autophagy and also produced a higher rate of apoptotic cell death compared with controls in human bladder cancer cells *in vitro*. Apoptosis is a kind of programmed cell death that is important in maintaining adult tissue homeostasis and supporting the embryonic tissue remodeling (36). There are three main apoptotic pathways: i) The mitochondrial (or intrinsic) pathway, mediated by the Bcl-2 superfamily members which interact with the mitochondrial membrane; ii) the death receptor (extrinsic) pathway, governed by specific death receptors that bind specific ligands, including tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (which binds to the DR4 and DR5 death receptors), and FasL (a ligand that binds to the Fas receptor); and iii) the endoplasmic reticulum stress pathway, which is predominantly regulated by inositol-requiring enzyme 1 and CHOP-mediated proapoptotic signaling (37-40).

Caspases, a family of cysteine-dependent aspartate-directed proteases, play a key role during the process of apoptosis. Most of the apoptotic signaling pathways converge on the activation of intracellular caspases and generate a complex biochemical cascade that propagates death signaling (41). Rottlerin-induced apoptotic cell death is considered to be mediated through a decrease of mitochondrial membrane potential and translocation of apoptosis-inducing factor (AIF) into the nucleus; results suggest that rottlerin-induced apoptosis is mediated through mitochondrial membrane depolarization and AIF translocation into the nucleus, via a caspase-independent pathway (42). However, other researchers have suggested that rottlerin-induced apoptosis is mediated through the caspase pathway (43). The results of the present study reveal that pro-caspase-3, cleaved caspase-3, total PARP and cleaved-PARP protein expression levels were not apparently altered in cells treated with rottlerin. From our results, it may be concluded that rottlerin induces apoptosis through a caspase-independent pathway. Further investigation should focus on the regulation of apoptosis mechanisms to increase the antitumor effect of rottlerin treatment.

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