Altholactone induces reactive oxygen species-mediated apoptosis in bladder cancer T24 cells through mitochondrial dysfunction, MAPK-p38 activation and Akt suppression

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Abstract. Human bladder cancer is an aggressive tumor which frequently resists chemotherapy. Therefore, the search for new therapeutic agents is of great importance. Altholactone, isolated from *Goniothalamus* sp., has been reported to inhibit the growth of various types of cancer cells. However, no prior research has been conducted to demonstrate the antiproliferative potential of altholactone on bladder cancer. In the present study, we characterized the effect of altholactone on cell growth and apoptosis in bladder cancer T24 cells. Treatment with altholactone resulted in a significant reduction in cell viability, induction of apoptosis and generation of reactive oxygen species (ROS) in T24 cells. Furthermore, our results revealed that altholactone-induced apoptosis was associated with decreased expression of Akt phosphorylation and activation of MAPK-p38. Altholactone treatment was also found to result in a significant loss of mitochondrial membrane potential, Bcl-2 downregulation and caspase-3 activation. Pretreatment of T24 cells with the antioxidant *N*-acetylcysteine (NAC) significantly inhibited activation of caspase-3 and MAPK-p38 and prevented inactivation of Akt and Bcl-2. Taken together, our data demonstrate that altholactone induces ROS-dependent apoptosis in T24 cells via a novel mechanism involving inhibition of Akt and provide the rationale for further *in vivo* and preclinical investigation of altholactone against bladder cancer.

Introduction

Bladder cancer represents the second most common neoplasm among urological malignancies and the fourth in general among all neoplastic pathologies for the male gender accounting for ~3% of all cancer-related deaths (1). At the epidemiological level, many studies have proven that bladder cancer represents more than 4% of all types of cancers in Austria (2), and in the United States, it was estimated to affect 73,510 patients in 2012 (3). Regarding the etiology of bladder cancer, at initial diagnosis, 70% of bladder tumors are non-muscle invasive and several studies have implicated chemical penetration into epithelial tissues and muscles surrounding the bladder, causing metastatic disease (3,4). Human bladder cancer is an aggressive tumor which frequently resists chemotherapy; therefore, the search for new therapeutic agents is of great importance. The study of plant-derived compounds with an effect at the molecular level has become a rational approach in the selection of new agents with antitumor activity.

Recent studies have shown that costunolide (5), wedelolactone (6), pseudolaric acid B (7) may be important chemopreventive agents for the management of bladder cancer through induction of apoptosis and cell cycle arrest. Altholactone (Fig. 1) or goniothalenol belongs to the styryllactone family and is a tetrahydrofurano-2-pyrone. It has been reported to exhibit antitumor activity, including inhibition of Bcl-2 and increased p53 expression in human cervical cancer HeLa cells (8), suppression of cell growth of human lung cancer COR-L23 cells (9), and induction of apoptosis in human HL-60 leukemia cells through oxidative stress (10) and in human colorectal cancer (CRC) cells through caspase-dependent and -independent apoptotic pathways (11).

However, no prior research has been conducted to demonstrate the chemopreventive potential of altholactone on human bladder cancer. Therefore, the present study was conducted, for the first time, to evaluate the chemopreventive/antiproliferative potential of altholactone against human bladder cancer and its mechanism of action. The current findings demonstrated that treatment with altholactone resulted in a significant decrease in cell viability, induction of apoptosis and increased generation of reactive oxygen species (ROS) in T24 cells. Furthermore, our results revealed that altholactone-induced apoptosis was associated with decreased expression of Akt phosphorylation and activation of MAPK-p38.

Materials and methods

Chemical and reagents. Altholactone was purchased from the BioBioPha Co., Ltd. of Pharmaceutical and Biological

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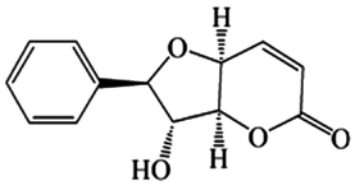

Figure 1. The chemical structure of altholactone.

Products (Kunming, China) and dissolved in 100% dimethyl sulfoxide (DMSO) (20 mM for stock solution). Cell culture medium reagents and MTT [3'-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], propidium iodide (PI), and DMSO were purchased from Sigma. Fetal bovine serum (FBS) was purchased from the Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). An Annexin V-FITC apoptosis detection and reactive species detection kits were purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Rabbit polyclonal anti-human Bcl-2, pAkt, Akt, p38 and caspase-3 antibodies were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). Mouse anti-β-actin and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ponceou and cell lysis buffer for western blots and IP were purchased from Beijing Biosynthesis Biotechnology (Beijing, China). Rhodamine 123 was purchased from Invitrogen (Eugene, OR, USA).

Cell culture. The human bladder cancer T24 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) nutrient mixture supplemented with 10% FBS and antibiotics at 37˚C in a humidified atmosphere with 5% CO_2 and 95% air. Cells were seeded in a 10-cm culture dish and allowed to grow to ~70% confluency before experimentation.

Cell proliferation assay. The cytotoxic effects of altholactone on the T24 cells were determined by MTT assay. Briefly, T24 cells were seeded at a density of $1x10^4$ cells/well in 96-well plates and were allowed to grow overnight. Cells were incubated with 100 μl of complete culture medium containing 0, 3, 6, 12.5, 25 and 100 μM of altholactone. After incubation for 24 h, the growth of cells was determined by adding 10 μl MTT (5 mg/ml in phosphate-buffered saline; PBS) to each well and incubated for 4 h. After removal of the medium, 150 μl DMSO was added to each well and shaken carefully. The absorbance was read at a wavelength of 570 nm in a plate reader (ELx800, Bio-Tek Instruments Inc.). The growth curve was plotted against mean values which were calculated using the following equation:

$$I\% = [(A570_{control} - A570_{treated})/A570_{control} \times 100].$$

Flow cytometric determination of apoptosis. The apoptosis rate of the T24 cells was examined by flow cytometry using Annexin V-FITC/PI staining. Briefly, T24 cells were cultured in 6-well plates and allowed to attach overnight. Cells were treated with 43.5 μM of altholactone for 12 and 24 h. Then cells were collected, washed and resuspended in PBS. Apoptotic cell death was measured by Annexin V-FITC and PI double staining using the Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed by flow cytometry using CellQuest software.

Flow cytometric determination of reactive oxygen species (ROS) in T24 cells. In order to determine the intracellular changes in ROS generation, T24 cells were stained with 2',7'-dichlorofluorescein-diacetate (DCFH-DA). The fluorescent dye DCFH-DA is cell membrane permeable and is converted into the cell membrane impermeable non-fluorescent compound DCFH by intracellular esterases. Oxidation of DCFH by reactive oxygen species produces highly fluorescent DCF. The fluorescence intensity of 2',7'-dichlorofluorescein (DCF) inside the cells is proportional to the amount of peroxide produced. Briefly, T24 cells were treated with 43.5 μM altholactone for 12 and 24 h. After treatment, cells were further incubated with 10 μM DCFH-DA at 37˚C for 30 min. Subsequently, cells were harvested, rinsed, re-suspended in PBS, filtered with 300 apertures and analyzed for DCF fluorescence by flow cytometry (Beckman Coulter, Epics XL).

Flow cytometric determination of mitochondrial membrane potential ($\Delta\Psi_m$). To probe for changes in $\Delta\Psi_m$, T24 cells were stained with Rhodamine 123 (1 μM) following treatment with 43.5 μM of altholactone for 12 and 24 h together with the control group. The fluorescence of Rhodamine 123 was measured by flow cytometry with excitation and emission wavelengths of 488 and 530 nm.

Western blotting. To reveal the mechanism underlying the apoptotic effect of altholactone, western blotting for apoptotic-related proteins was performed as previously described (5). Briefly, T24 cells were incubated with 43.5 μM altholactone for the indicated time periods. Cells were trypsinized, collected in a 1.5-ml centrifuge tube and washed with PBS. The cell pellets were resuspended in lysis buffer and were lysed on ice for 30 min. After centrifugation for 15 min, the supernatant fluids were collected and the protein content of the supernatant was measured by the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The protein lysates were separated by electrophoresis on 12% SDS-polyacrylamide gel and transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were soaked in blocking buffer (5% skimmed milk) for 2 h. To probe for Bax, Bcl-2, pAkt, Akt, p38, caspase-3 and β-actin; membranes were incubated overnight at 4˚C with relevant antibodies, followed by appropriate HRP-conjugated secondary antibodies and ECL detection.

Statistical analysis of the data. For the statistical analysis of data, comparisons between results from the different groups were analyzed with SPSS for Windows, version 15.0. The Student's t-test was employed to determine the statistical significance of the difference between the different experimental groups and the control group. A $P<0.05$ value was defined as statistically significant. All experiments were repeated at least three times. Data are presented as mean ± standard deviation (SD).

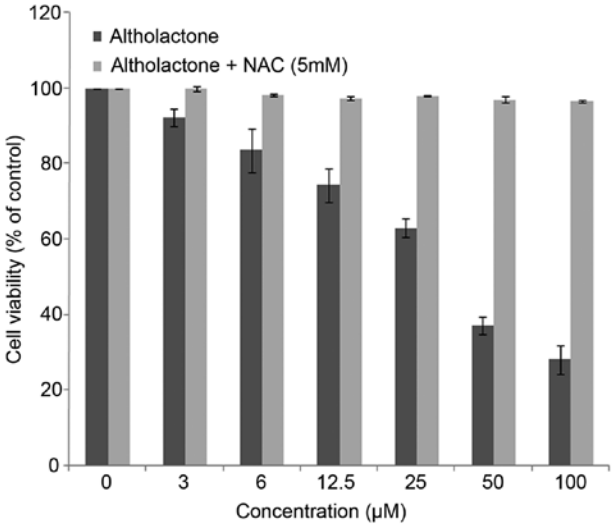

Figure 2. Altholactone inhibits cell growth and induces cell death in human bladder cancer cells. T24 cells were treated with indicated doses of altholactone in the presence or absence of NAC for 24 h, and cell viability was measured by MTT assay. Data are expressed as mean ± SD (n=3). NAC, *N*-acetylcysteine.

Results and Discussion

Altholactone induces cell growth inhibition in T24 cells. One potential source of novel anticancer agents is natural plant products. The study of plant-derived compounds exhibiting effects at the molecular level has become an important approach in the selection of new agents with antitumor activity. The present research began by the random screening of previously isolated and identified natural products from our personal repositories using human bladder cancer cell line T24, in the presence or absence of NAC, a specific ROS inhibitor. The compounds chosen were representative of particular classes of natural products that were previously reported by us (5,12-14). This screening approach led to the identification of anti-bladder cancer compounds targeting ROS metabolism. Altholactone, a plant-derived natural compound, was identified as a potent inhibitor of bladder cancer T24 cell growth during the screening process. Exposure of T24 cells to altholactone at various concentrations resulted in the reduction in the viability of cells in a dose-dependent manner when assessed by MTT assay. Notably, altholactone demonstrated a potent inhibitory effect in T24 cells, and the magnitude of inhibition increased in a dose-dependent manner. The inhibitory rate was >90% at 100 μM, and the concentration needed to achieve 50% growth inhibition (IC_{50}) was 43.5 μM. Pretreatment with NAC (5 mM), a specific ROS scavenger, abrogated altholactone-induced inhibitory effects on T24 cells indicating that altholactone exerts a cytotoxic effect on cell viability through ROS generation (Fig. 2). These results demonstrated that altholactone induced growth inhibition of T24 cells similar to the effects on other types of cancer cells including leukemia (10), colorectal (11) and cervical cancer cells (8).

Effect of altholactone on induction of T24 cell apoptosis. Apoptosis is defined as an extremely synchronized mode of cell death. It is characterized by distinct morphological features, including chromatin condensation and nuclear fragmentation (15,16). The importance of signaling has been recognized in cell regulation during normal and disease states (17,18). Accumulated data (5,19-25) suggest that various anticancer chemopreventive agents can induce apoptosis which in turn induce death in cancerous cells. To further investigate the altholactone-induced inhibitory effect, T24 cells were treated with altholactone (43.5 μM) for different time periods, and the percentages of cells undergoing apoptosis/necrosis were determined by flow cytometric analysis following staining with Annexin V-FITC and PI. The ratio of apoptosis in T24 cells was significantly higher than that in the control cells. We observed a significant increase in both early and late apoptosis in the 24-h treatment group whereas the 12-h treatment group showed a significant increase only in early apoptosis when compared with the control group. Our results showed that altholactone caused time-dependent apoptosis. The results of

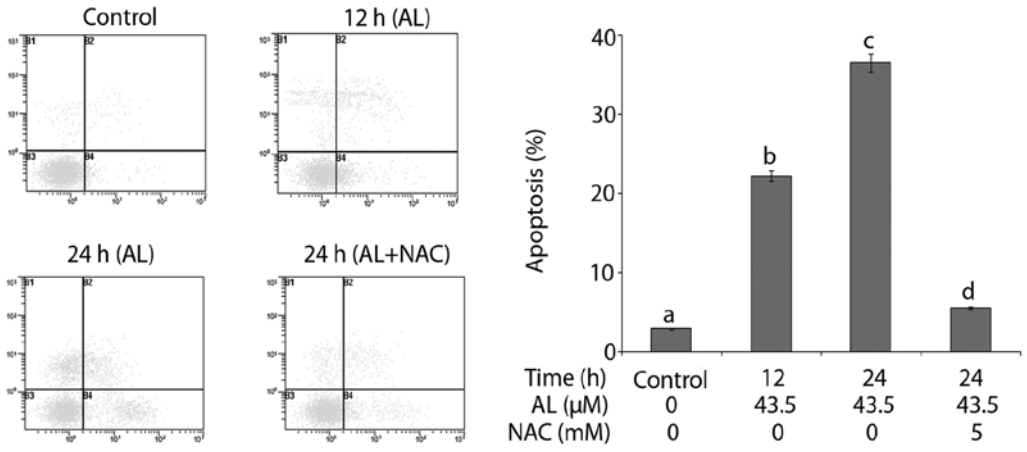

Figure 3. Apoptosis is induced by altholactone in T24 cells. Human bladder cancer T24 cells were treated with 43.5 μM of altholactone (AL) for 12 and 24 h in the presence or absence of NAC. The cells were then stained with FITC-conjugated Annexin V and PI for flow cytometric analysis. The flow cytometric profile indicates Annexin V-FITC staining on the x-axis and PI on the y-axis. Data are expressed as mean ± SD (n=3). Columns (histogram) not sharing the same superscripted letter differ significantly (P<0.05). NAC, *N*-acetylcysteine.

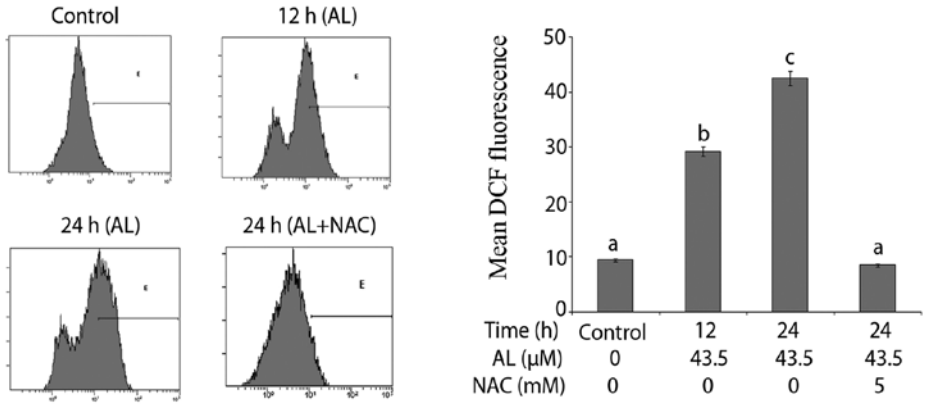

Figure 4. Flow cytometric analysis of ROS generation. T24 cells were treated with 43.5 μM of altholactone (AL) for 12 and 24 h in the presence or absence of 5 mM NAC for 24 h. Data are expressed as mean ± SD (n=3). Columns (histograms) not sharing the same superscripted letter differ significantly (P<0.05). NAC, *N*-acetylcysteine; DCF, 2',7'-dichlorofluorescein.

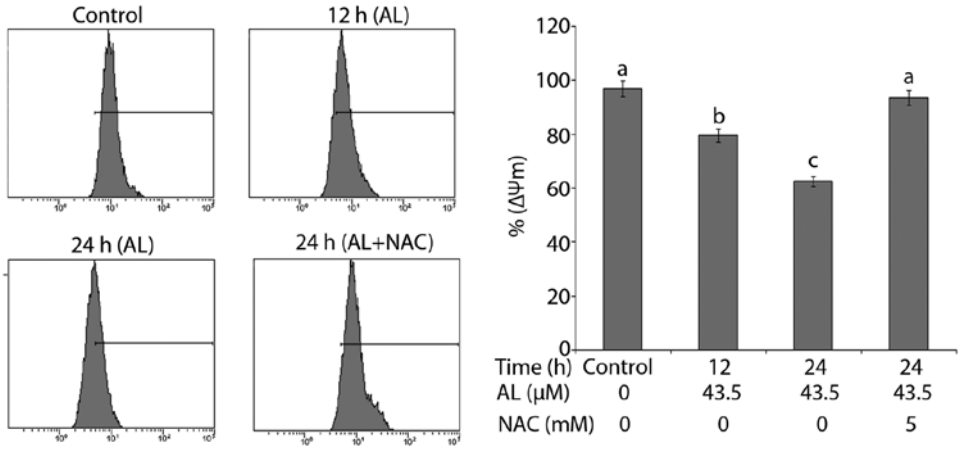

Figure 5. Effects of altholactone on mitochondrial transmembrane potential ($\Delta\Psi_m$) of T24 cells were determined by flow cytometry. The values indicate the percentages of Rhodamine 123 fluorescence in the T24 cells treated without or with 43.5 μM of altholactone (AL) for 12 and 24 h in the presence or absence of NAC. Data are expressed as mean ± SD (n=3). Columns not sharing the same superscripted letter differ significantly (P<0.05). NAC, *N*-acetylcysteine.

the flow cytometric analysis showed that the rates of apoptosis were 22.98±2.26 and 36.57±2.96% at 12 and 24 h, respectively when compared to an apoptosis rate of 2.85±0.89% in the control cells (Fig. 3).

Several reports have revealed that ROS can trigger apoptotic cell death in various types of cancer cells following treatment with anticancer drugs (26,27). To determine whether ROS play an important role in altholactone-induced apoptosis in T24 cells, we pretreated cells with the ROS scavenger NAC (5 mM) for 2 h, and then we treated the cells with altholactone (43.5 μM) for an additional 24 h. Pretreatment with NAC markedly abrogated the apoptotic effects of altholactone indicating that induction of apoptosis was in an ROS-dependent manner (Fig. 3). Therefore, these results suggest that intracellular ROS play an essential role in altholactone-induced apoptotic cell death in T24 cells, which was consistent with our observed cell survival data and compatible with previously reported research (5).

Altholactone promotes ROS generation in bladder cancer T24 cells. ROS, active, transitory and oxygenic compounds are known mediators of intracellular signaling cascades, including H_2O_2, O_2 and hydroxyl radicals are metabolites of biochemical processes in the body. Regarding genesis, ROS are the result of disordered mitochondrial function and metabolite augmentation, and there may exist strategies by which to regulate ROS selectively in cancer cells (28). In the present study, we hypothesized that altholactone arouses ROS levels, which may be involved in altholactone-induced apoptosis. Therefore, the intracellular ROS level was measured using the ROS-detecting fluorescence dye 2,7-dichlorofluorescein diacetate (DCF-DA) since the DCF assay is highly sensitive, linear, and precise for measuring oxidative stress in irradiated cells (29). The level of ROS was significantly increased in a time-dependent manner following treatment of the cells with altholactone (43.5 μM) for 12 and 24 h. Our results indicated that ROS generation was effective diminished by pretreatment with the ROS scavenger when cells were treated with altholactone.

As shown in Fig. 4, the ratio of DCF-positive cells following treatment with 43.5 μM of altholactone for 12 and 24 h was significantly higher (27.73±2.32 and 38.37±1.65 vs. 9.45±0.62 in control group; P<0.05). The findings confirmed

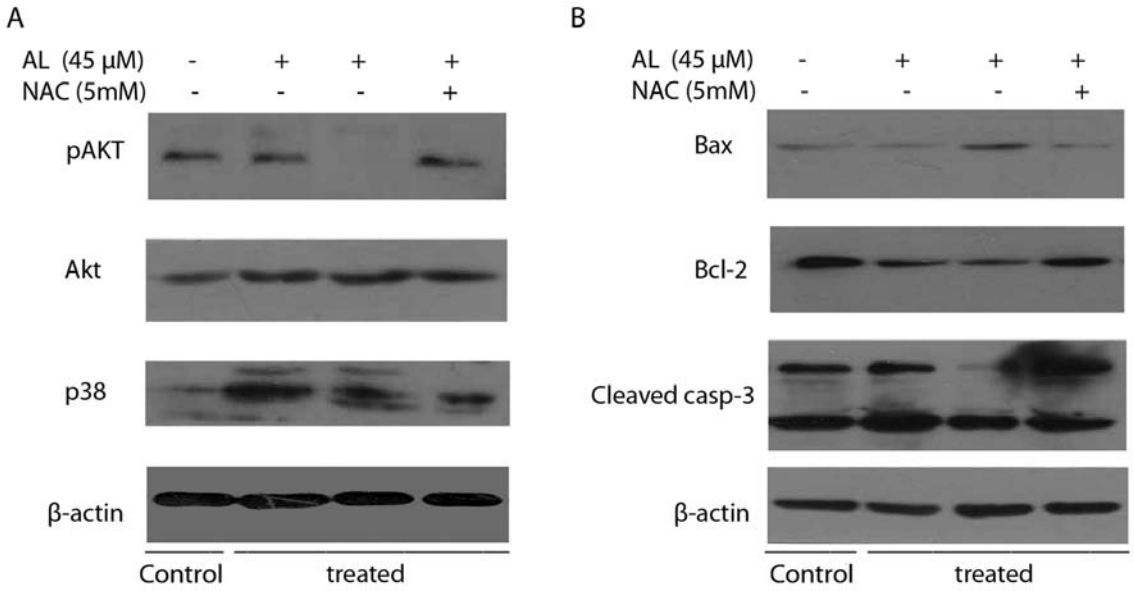

Figure 6. Effect of altholactone (AL) on the expression of major apoptosis regulatory proteins. T24 cells were exposed to 43.5 μM of altholactone for specified time intervals. Equal amounts of lysate protein were subjected to gel electrophoresis. Expression levels of (A) pAkt, Akt, p38 and (B) Bax, Bcl-2 and caspase-3 were assessed by western blot assay. β-actin was used as loading control. Data are representative of three independent experiments with similar results.

that altholactone promotes the generation of ROS in T24 cells. Chemotherapeutic agents causing enhancement in oxidative stress are likely to be toxic to cancer cells as they are found to be involved in biological processes such as cell cycle arrest, DNA repair and apoptosis (5).

Effect of altholactone on mitochondrial membrane potential ($\Delta\Psi_m$). Mitochondria are important organelles of the apoptosis execution machinery, and contain pro-apoptotic proteins (e.g., cytochrome *c*) (15). It is increasingly becoming apparent that the mitochondria play critical roles in the regulation of various apoptotic processes leading to cell death (30), including drug-induced apoptosis (31). Disruption of mitochondrial integrity is an important component of the apoptosis execution machinery. The effects of altholactone on the mitochondrial membrane potential of T24 cells were determined by flow cytometry using Rhodamine 123 staining. The rates of depletion of the mitochondrial membrane potential were 79.57±2.03 and 64.61±1.44% in the cells treated with 43.5 μM of altholactone for 12 and 24 h, respectively, as compared to 97.11±0.67% in the control group. To further confirm the involvement of ROS in disruption of the mitochondrial membrane potential, cells were treated with 5 mM NAC. Pretreatment with NAC completely prevented the dissipation of mitochondrial membrane potential, indicating that this was ROS-dependent (Fig. 5).

Altholactone inhibits Akt and enhances p38 MAPK activation. The phosphatidylinositol 3-kinase-Akt signaling pathway is implicated to be one of the most important pathways for cell survival and inhibition of apoptosis (32). It has been demonstrated that Akt can regulate a number of cellular processes such as cell proliferation and apoptosis (33). Inhibition of phosphorylated-Akt (pAkt) may induce cancer cell apoptosis (34). Inactivation of pAkt is a key mechanism of the apoptosis induced by various anticancer drugs (35,36). For this reason, we sought to determine whether altholactone induces pro-apoptotic action via inactivation of Akt phosphorylation activity in T24 cells. T24 cells were treated with altholactone for different time periods, and proteins were extracted and immunoblotted with antibodies against activated-Akt and total Akt. As shown in Fig. 6A, constitutive activation of Akt was inactivated following treatment with altholactone in T24 cells; however, there was no effect on the expression of total Akt. Next, to determine whether regulation of the MAPK-p38 signaling pathway is necessary for altholactone-induced apoptosis, we investigated the expression levels of MAPK-p38 in T24 cells following treatment with altholactone for 24 h. The results demonstrated that phosphorylation of p38 was increased in a time-dependent manner in response to altholactone (Fig. 6A).

To better understand the role of ROS release in the inactivation of Akt and the activation of MAPK-p38, we pretreated T24 cells with NAC (5 mM) for 2 h followed by treatment with altholactone (43.5 μM) for various time intervals. Proteins were prepared and immunoblotted with antibodies against p-AKT and MAPK-p38. As shown in Fig. 6A, altholactone treatment inactivated Akt and increased MAPK-p38 phosphorylation but pretreatment with NAC prevented altholactone-induced inactivation of Akt and activation of MAPK-p38. These data clearly demonstrate that altholactone treatment caused a release of ROS that, in turn, caused inactivation of Akt and upregulation of MAPK-p38. The exact mechanism by which ROS release leads to inactivation of Akt remains unknown.

Altholactone-induced apoptosis mediated by Bcl-2 family modulation and caspase-3 activation. Accumulated data (37,38) indicate that the mitochondrial death pathway is controlled by members of the Bcl-2 family, which plays a central regulatory role in deciding the fate of cells via

the interaction between pro- and anti-apoptotic members, required for the complete release of cytochrome *c*. To further characterize this cell-specific apoptotic effect of altholactone in bladder cancer cells, we analyzed the levels of Bcl-2 family proteins following treatment with altholactone (43.5 μM) in a time-dependent manner. We observed that the expression of anti-apoptotic Bcl-2 was downregulated, whereas the level of pro-apoptotic Bax expression was markedly upregulated (Fig. 6B).

Caspases play important roles in apoptosis via triggering death receptors and mitochondrial pathways to emit various pro-apoptotic signals to accomplish programmed cell death (39,40). To examine whether regulation of caspase-3 is necessary for altholactone-induced apoptosis, we investigated the expression level of caspase-3 following treatment with altholactone (43.5 μM) for different time intervals. As shown in Fig. 6B, altholactone treatment was found to result in a significant increase in cleaved caspase-3, which is indicative of induction of apoptosis.

In conclusion, our data provide evidence that altholactone inhibits the growth of human bladder cancer T24 cells by inducing apoptosis. The present study is the first to describe the role of ROS in the induction of apoptosis in bladder cancer cells. In addition, in the present study, we also found that altholactone downregulated the expression of phosphorylated Akt and upregulated MAPK-p38 in T24 cells, which, to our knowledge, is the first observation that altholactone downregulates the expression of the anti-apoptotic protein pAkt in T24 cells. Moreover, the pathway that we described herein is novel and has not been previously elucidated. Furthermore, altholactone induced apoptosis in T24 cells by modulating mitochondrial Bcl-2 family proteins and caspase-3. These findings support a prominent insight into how altholactone exerts its cytotoxic effect against bladder cancer T24 cells. Thus, altholactone may become a potential lead compound for future development of anti-bladder cancer therapy. Further investigation is needed to validate the contribution of altholactone to tumor therapy *in vivo*.

Acknowledgements

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