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Original Article

Devazepide, a nonpeptide antagonist of CCK receptor, induces cell apoptosis and inhibits contraction in human prostatic stromal myofibroblasts

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Abstract: Human prostatic stromal myofibroblasts have been shown to be related to lower urinary tract symptoms (LUTS) in aging men. Devazepide, also known as a nonpeptide antagonist of CCK receptor, has been found to inhibit the proliferation in tumor cells. Here, we aim to investigate the effects of Devazepide on growth and contraction ability in human prostatic stromal WPMY-1 myofibroblasts. Human prostatic stromal WPMY-1 myofibroblasts were treated with various concentrations of Devazepide. The cell viability was determined by WST-8 assay. TUNEL assay and Annexin-V/Propidium iodide (PI) staining were performed to determine cell apoptosis. Expression levels of cleaved Caspase-3 and cleaved Caspase-9 were examined by Western blot. Contraction ability in WPMY-1 cells was evaluated by collagen gel contraction assay. We found that cell viability of WPMY-1 cells was significantly inhibited in the presence of Devazepide. Devazepide inhibited growth of WPMY-1 cells in a dose and time-dependent manner. In addition, Devazepide could significantly induce G2/M cell cycle arrest and apoptosis in WPMY-1 cells. Increased expression levels of cleaved Caspase-3 and Caspase-9 proteins were found in Devazepide-treated WPMY-1 cells. Collagen gel contraction assay further showed that Devazepide could inhibit contraction ability of WPMY-1 cells, which might be associated with growth inhibition and decreased expression levels of Col1A1 and Col1A2 in WPMY-1 cells. Taken together, Devazepide could inhibit in vitro growth and contraction ability in human prostatic stromal myofibroblasts, which might be a novel therapeutic target of LUTS in aging men.

Keywords: Devazepide, CCK receptor, myofibroblast, prostate

Introduction

Lower urinary tract symptoms (LUTS) have been shown to be an important medical challenge in aging men. The main symptoms include nocturia, intermittent or hesitant urination, weak stream, and sensation of incomplete emptying [1]. If LUTS were not treated effectively, multiple complications eventually occurred, such as urinary retention, bladder calculi, and renal impairment [2]. It has been reported that many diseases could lead to LUTS, such as benign prostatic hyperplasia (BPH) [3]. However, effective treatment of LUTS is still a medical challenge due to less understanding of etiology of LUTS.

Fibrosis is characterized by myofibroblast accumulation, collagen, and extracellular matrix (ECM) deposition [4, 5]. Multiple cell types could differentiate into myofibroblasts including fibrocytes, pericytes, and fibroblasts [6]. Recent years, multiple evidences showed that periurethral ECM deposition and fibrosis were closely associated with LUTS in aging men [7, 8]. In the study [9], it was reported that periurethral calcification (PUC) was independently associated with peak urinary flow rate (Qmax) and urinary symptoms, which provided strong evidence that periurethral fibrosis and stiffness could lead to LUTS-BPH in aging men. It was reported that prostate tissues from men with LUTS were significantly stiffer than those without LUTS [8]. Further study showed that it was associated with significantly higher collagen content and lower glandularity [5]. It was also found that the proportion of larger bundles of collagen was

significantly increased in BPH nodules, which suggested that large fibers might be involved in BPH/LUTS [10].

Currently, although anti-androgens, smooth-muscle relaxers, and surgical operation could be used to treat LUTS in aging men, they were not effective to all patients. Several findings showed that anti-fibrotic therapeutics might be effective for the treatment of LUTS [7, 11, 12]. In addition, it has been reported that myofibroblast accumulation has been found in the periurethral area of prostate. Myofibroblast accumulation was thought to be associated with ECM deposition, tissue stiffness, and urinary voiding dysfunction [8, 12]. Considering the important roles of myofibroblasts in LUTS, myofibroblasts may be a novel target for LUTS treatment in aging men.

Devazepide is known as a CCK-1 receptor antagonist, which has been shown to inhibit Ewing tumor growth and HT-29 cell proliferation [13, 14]. In this study, we investigated the effects of Devazepide on proliferation and contraction ability in human prostatic stromal WPMY-1 myofibroblasts. We found that Devazepide could induce apoptosis and inhibit in vitro contraction ability in human WPMY-1 cells.

Materials and methods

Cell culture, reagents

Human prostatic stromal myofibroblast cell line WPMY-1 was obtained from American Type Culture Collection (ATCC). WPMY-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere. The CCK-1 receptor antagonist Devazepide and CCK-2 receptor antagonist LY225910 were purchased from Tocris Biosciences (Bristol, UK). Non-selective CCK antagonist Proglumide was purchased from Sigma Aldrich (MO, USA).

WST-8 cell viability assay

Cell viability was evaluated by Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). The WPMY-1 cells were seeded at a density of 0.8×10^4 cells per well in 96-well plates. After 12 h incubation, the medium was replaced with fresh medium containing various concentrations of Devazepide, LY225910, and Proglumide. At different time points, WST-8 was used to detect cell viability. The experiments were repeated four times, and cell growth rate was computed using the following formula: Growth rate = (OD test/OD control) × 100%.

Immunofluorescence assay

Human WPMY-1 cells were grown on glass coverslips and fixed in 4% paraformaldehyde for 15 min. After washing with PBS, WPMY-1 cells were permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Then, 5% goat serum was used to block nonspecific binding. Cells were incubated with rabbit polyclonal KI-67 antibody at a dilution of 1:200 overnight at 4°C, and subsequently were exposed to goat anti-rabbit IgG Rhodamine (TRITC) (1:500 dilution) for one hour at 37°C. DAPI was used to stain nuclei of cells.

RNA extraction and quantitative real-time RT-PCR

Total RNA in WPMY-1 cells was extracted using the RNAiso Plus reagent (TaKaRa) according to the manufacturer's instruction. Complementary DNA (cDNA) was obtained from RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser kit (TaKaRa). Expression levels of KI-67, COL1A1, COL1A2, and GAPDH mRNA were examined by quantitative real-time RT-PCR (qRT-PCR). The qRT-PCR was performed using the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa). The primer sequences for qRT-PCR were shown as follows: for KI-67, 5'-CCCAAGACACCTTTGGAGAA-3' (forward) and 5'-ATCCTCACCTCCTGGTACTT-3' (reverse); for COL-1A1, 5'-CCCTGGAAAGAATGGAGATG-3' (forward) and 5'-CCACTGAAACCTCTGTGTCC-3' (reverse); for COL1A2, 5'-CAGAACATCACCTACCACTGCAA-3' (forward) and 5'-TTCAACATCGTTGGAACCCTG-3' (reverse); for GAPDH, 5'-TGCACCACCAACTGCTTAGC-3' (forward) and reverse primer 5'-GGCATGGACTGTGGTCATGAG-3' (reverse).

Analysis of cell cycle

Human WPMY-1 cells were seeded in 60-mm dishes and treated with Devazepide. Cells were subsequently collected and analyzed for DNA content at different time points. To analyze cell cycle, WPMY-1 cells were fixed with 75%

cold ethanol. Then, cells were treated with RNase and propidium iodide for 10 min at room temperature, and further analyzed by flow cytometry.

Western blot

Human WPMY-1 cells were treated with Devazepide. Then, WPMY-1 cells were treated with RIPA lysis buffer on ice for 15 min. Subsequently, proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF). Non-fat milk (5%) in TBS-T (Tris-buffered saline containing 0.1% Tween-20) was prepared to block nonspecific binding. The membranes were immunoblotted with the primary antibodies overnight at 4°C, and subsequently incubated with secondary antibody goat anti-rabbit IgG (H+L) HRP (1:5000 dilution, Bioworld Technology, Minneapolis, USA) for one hour at room temperature. The primary antibodies in this study were shown as follows: Polyclonal rabbit anti-GAPDH (1:5000 dilution, Bioworld Technology, Minneapolis, USA), p-Chk1 (Ser280) (1:1000 dilution, Bioworld Technology, Minneapolis, USA), p-Chk2 (Thr68) (1:500 dilution, Bioworld Technology, Minneapolis, USA), Cyclin B1 (1:1000, Bioworld Technology, Minneapolis, USA), p21 (1:1000, Bioworld Technology, Minneapolis, USA), Caspase-3 (1:1000 dilution, Abcam, Cambridge, UK), Caspase-9 (1:1000 dilution, Abcam, Cambridge, UK), cleaved Caspase-3 (1:1000 dilution, Abcam, Cambridge, UK), and cleaved Caspase-9 (1:1000 dilution, Abcam, Cambridge, UK).

TUNEL assay

TUNEL assay was conducted with the one-step in situ TUNEL fluorescent kit (Beyotime, China). Briefly, WPMY-1 cells were treated with Devazepide and were fixed with 4% paraformaldehyde for 15 min at room temperature. Subsequently, cells were permeabilized with 0.2% Triton X-100/PBS for 15 min, followed with Cyanine 3 (Cy3)-labelled TUNEL reaction mixture for one hour at 37°C. The Cy3-labeled cells were photographed using fluorescent microscope.

Flow cytometric analysis of cell apoptosis

Apoptosis was analyzed using Annexin V-FITC/Propidium iodide Apoptosis Detection kit according to the manufacturer's instruction (Dojindo Molecular Technologies, Japan). Briefly, after Devazepide treatment, cells were harvested and suspended in 200 μL binding buffer containing 10 μL Annexin V-FITC and 5 μL PI. Cells were incubated at room temperature for 15 min, and then analyzed using flow cytometry.

Collagen gel contraction assay

Collagen gel contraction assay was performed to evaluate contraction ability of myofibroblasts using modifications of a previously described method [12]. Briefly, collagen gel was prepared on ice by mixing 3 ml of collagen solution (Stem Cell Technologies, Vancouver, BC) with 5 ml DMEM. The mixture was adjusted to pH 7.4. Then, 600 μl of the mixture was added to each well of 24-well plate and allowed to solidify at 37°C. WPMY-1 cells were seeded on solidified collagen gels, and allowed to attach for 24 h. Solidified collagen gel was detached from the wall of 24-well plate and cells were treated with Devazepide at 37°C in a 5% CO_2 incubator. The gels were photographed at different time points. Gel surface areas were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., MD, USA).

Statistical analysis

All data were calculated based on at least three independent experiments and expressed as means ± standard derivations (SD). Statistical differences were analyzed by Student's t test for intergroup comparison using SPSS 19.0 software (SPSS Inc., USA). A P value < 0.05 was statistically considered significant.

Results

Devazepide inhibited proliferation in human prostatic stromal WPMY-1 myofibroblasts

Devazepide is a benzodiazepine drug, which could selectively block CCK-1 receptor site for the hormone cholecystokinin (CCK). The non-selective CCK receptor antagonist Proglumide and selective CCK-2 receptor antagonist LY2-25910 have also been developed for research on anxiety and pain. It was shown that Devazepide could inhibit the growth and induce apoptosis in Ewing tumor cells [13]. We also

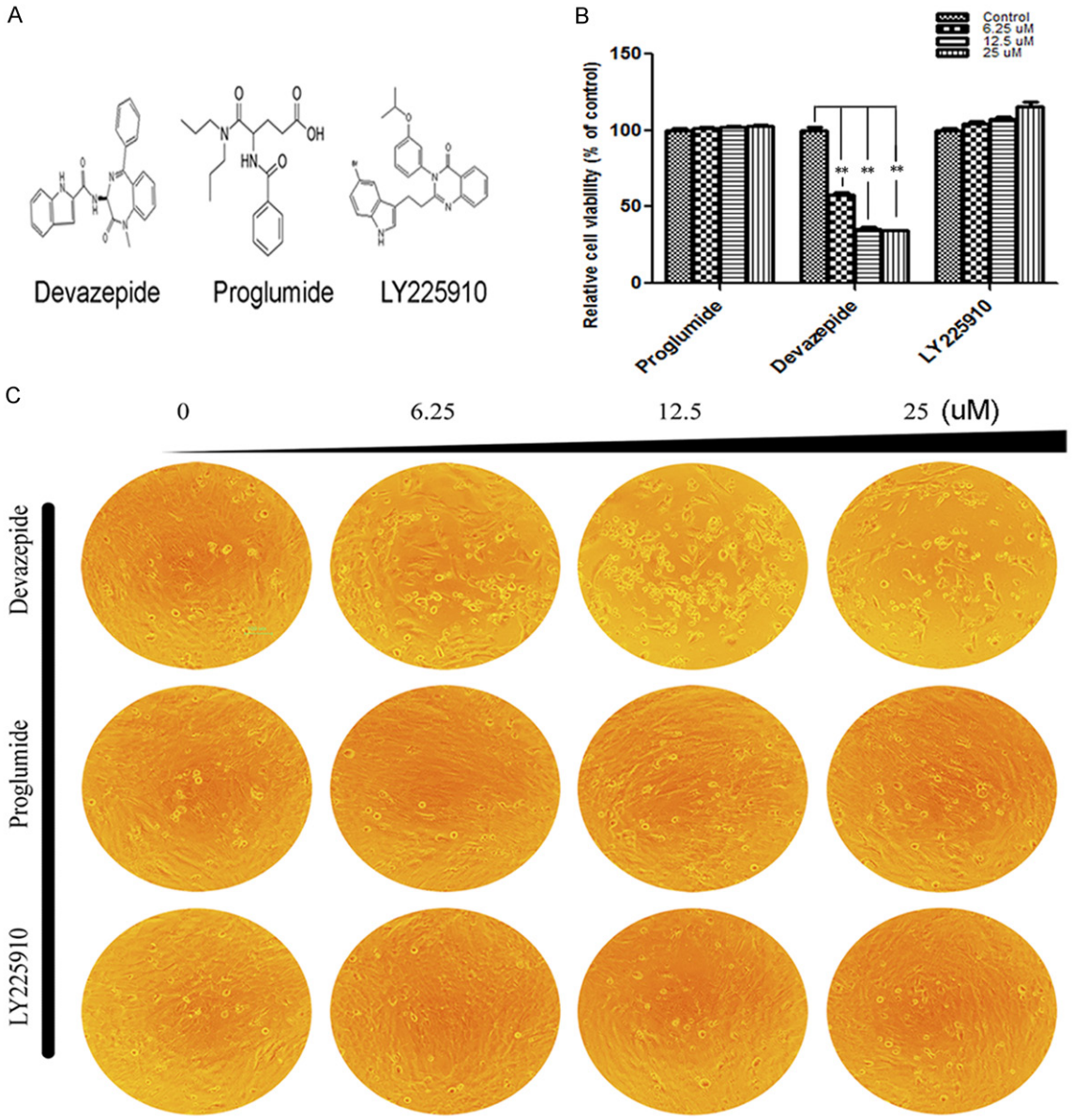

Figure 1. Proliferation inhibition of WPMY-1 cell was affected by treatment of selective CCK-1 receptor antagonist Devazepide. A. Chemical structures of the selective CCK-1 receptor antagonist Devazepide, selective CCK-2 receptor antagonist LY225910, and non-selective CCK receptor antagonist Proglumide. B. WPMY-1 cells were treated with indicated concentrations of Devazepide, LY225910, and Proglumide. Cell viability was evaluated by WST-8 assay. The data were shown as means ± SD. Asterisks indicate statistically significant difference (**P < 0.01). C. Devazepide treatment exhibited growth-inhibitory effects in WPMY-1 cells. The morphological characteristics were observed in WPMY-1 cells in the presence of indicated concentrations of Devazepide, LY225910, and Proglumide under light microscope.

investigated the effects of CCK-1 receptor antagonist Devazepide on human prostatic stromal WPMY-1 myofibroblasts. WPMY-1 cells were treated with indicated concentrations of Devazepide and cell viability was evaluated by WST-8 assay. After treatment for 72 h, it was found that Devazepide could inhibit the proliferation of WPMY-1 cells by about 41.7% compared with the control group. In addition, we found that Devazepide could inhibit the growth of WPMY-1 cells in a time and dose-dependent manner (**Figure 1**). Furthermore, for non-selective CCK receptor antagonist Proglumide and selective CCK-2 receptor antagonist LY225910, no inhibitory effects were found on the proliferation of WPMY-1 cells (**Figure 1**).

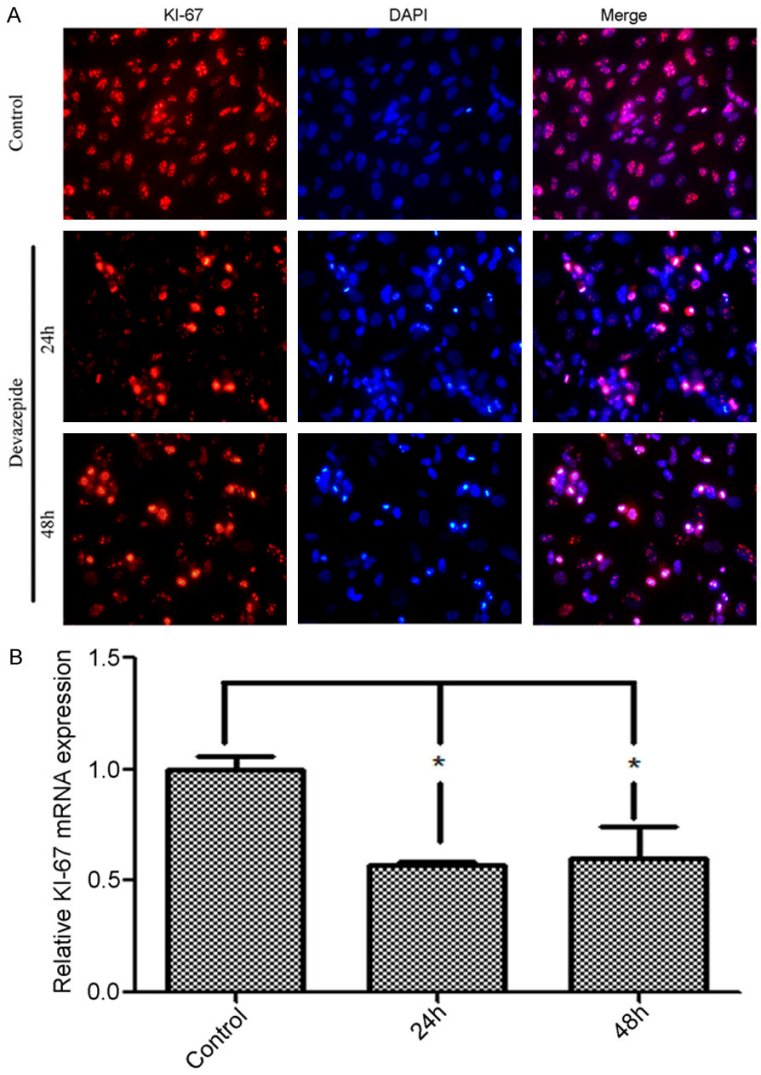

Figure 2. Selective CCK-1 receptor antagonist Devazepide downregulated KI-67 expression in WPMY-1 cells. A. Representative images of low expression of KI-67 in Devazepide-treated WPMY-1 cells by immunofluorescence staining. Cells were stained using 6-diamidino-2-phenylindole (DAPI) to visualize nuclei. B. KI-67 mRNA expression was decreased in Devazepide-treated WPMY-1 cells by quantitative real-time RT-PCR. Data are presented as means ± SD. Asterisks indicate statistically significant difference (*P < 0.05).

We also analyzed the changes in cell morphology in the presence of Devazepide. After Devazepide treatment, WPMY-1 cells became shrunken and floating. Hoechst 33258 staining was also used to detect nuclear change under Devazepide treatment (data not shown). We found that Devazepide-treated WPMY-1 cells showed morphological changes including nuclear chromatin condensation, which were consistent with cell apoptosis. In Proglumide- and LY225910-treated groups, no apoptotic morphological changes were found (**Figure 1**).

Devazepide downregulated KI-67 expression

KI-67 is a nuclear protein which is closely associated with cell proliferation. Several studies showed that KI-67 was also related to ribosomal RNA transcription [15, 16]. It was well known that KI-67 was a valuable cellular marker for proliferation, which was associated with cell proliferation [17]. We found that Devazepide could inhibit the growth of WPMY-1 cells. This led us to investigate the effect of Devazepide on KI-67 expression. Using immunofluorescence assay, we found that KI-67 was mainly localized in nuclei of WPMY-1 cells. Furthermore, weak staining of KI-67 protein was found in Devazepide-treated WPMY-1 cells (**Figure 2**). In addition, we found that Devazepide also inhibited KI-67 mRNA expression in WPMY-1 cells (**Figure 2**).

Devazepide induced G2/M cell cycle arrest in human WPMY-1 cells

Inhibition of cell proliferation in human WPMY-1 cells further led us to explore whether Devazepide could arrest WPMY-1 cells in a particular cell cycle phase. To determine this speculation, we treated human WPMY-1 cells with indicated concentrations of Devazepide. Then, cell cycle was analyzed using flow cytometry at different time points. Representative cell cycle profiles and histograms of Devazepide-untreated and -treated cells were shown in **Figure 3**. It was found that Devazepide significantly decreased the percentage of WPMY-1 cells in S phase and increased the percentage of WPMY-1 cells in G2/M phase. After Devazepide treatment, the

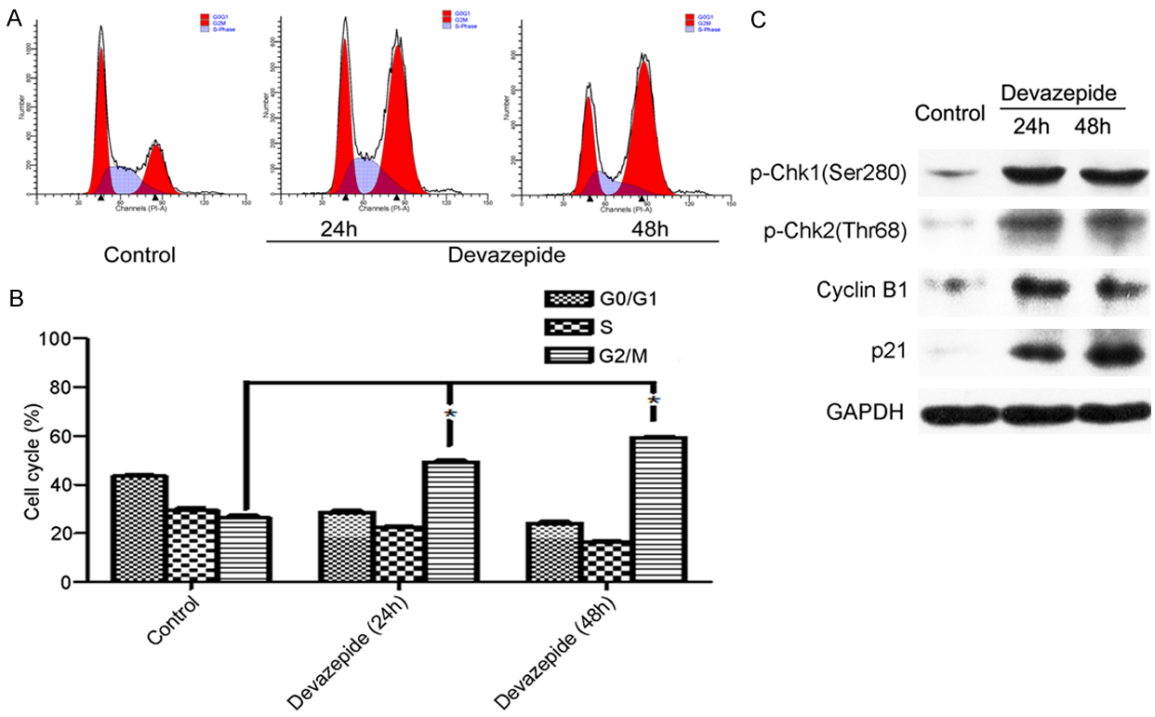

Figure 3. Devazepide induced G2/M cell cycle arrest in human WPMY-1 cells. A. WPMY-1 cells were treated with or without indicated concentration of Devazepide. The cell cycle analysis was performed in human WPMY-1 cells by flow cytometry. B. The percentage of WPMY-1 cells in the G2/M phase significantly increased in the presence of Devazepide. Consistently, the percentage of WPMY-1 cells in the S phase decreased. C. Human WPMY-1 cells were treated with or without indicated concentrations of Devazepide, and then proteins were extracted to detect the expression levels of p-Chk1, p-Chk2, Cyclin B1, and p21 by Western blot. GAPDH was used as an internal control. Data were representative of three individual experiments and presented as means ± SD. Asterisks indicate statistically significant difference (*P < 0.05).

percentage of WPMY-1 cells in G0/G1 phase significantly decreased to 25.45±2.77% and 21.88±2.49%, respectively. Consistently, the percentage of Devazepide-treated WPMY-1 cells in G2/M phase significantly increased to 45.90±4.26% and 50.56±7.78%, respectively.

Devazepide upregulated phosphorylation of Chk1 and Chk2 in human WPMY-1 cells

It has been reported that phosphorylation of Chk1 and Chk2 may be involved in G2/M cell cycle arrest in human cervical carcinoma HeLa cells when treated with 8-ADEQ [18]. Because Devazepide could induce G2/M cell cycle arrest in human WPMY-1 cells, we examined the effects of Devazepide on phosphorylation levels of Chk1 and Chk2. We found that Devazepide significantly upregulated phosphorylation levels of Chk1 and Chk2 at Ser280 and Thr68 sites (**Figure 3**), respectively. It suggests that Cantharidin increased cell population in G2/M phase and Cyclin B1 expression in hepatocellular carcinoma stem cells [19]. In this study, increased expression level of Cyclin B1 was detected in WPMY-1 cells in the presence of Devazepide (**Figure 3**). In addition, Devazepide could also upregulate p21 expression in WPMY-1 cells (**Figure 3**).

Devazepide induced cell apoptosis in human WPMY-1 cells via intrinsic apoptosis pathway

To investigate the effects of Devazepide on apoptosis, we treated human WPMY-1 cells with indicated concentrations of Devazepide. Using TUNEL assay, apoptosis in WPMY-1 cells was detected (**Figure 4**). Furthermore, Annexin V-FITC apoptosis detection kit was used to evaluate apoptosis in WPMY-1 cells in the presence of Devazepide. It was found that Devazepide could significantly induce apoptosis in Devazepide-treated WPMY-1 cells (**Figure 4**). Upregulation of cleaved Caspase-9 has been shown to be associated with activation of intrinsic

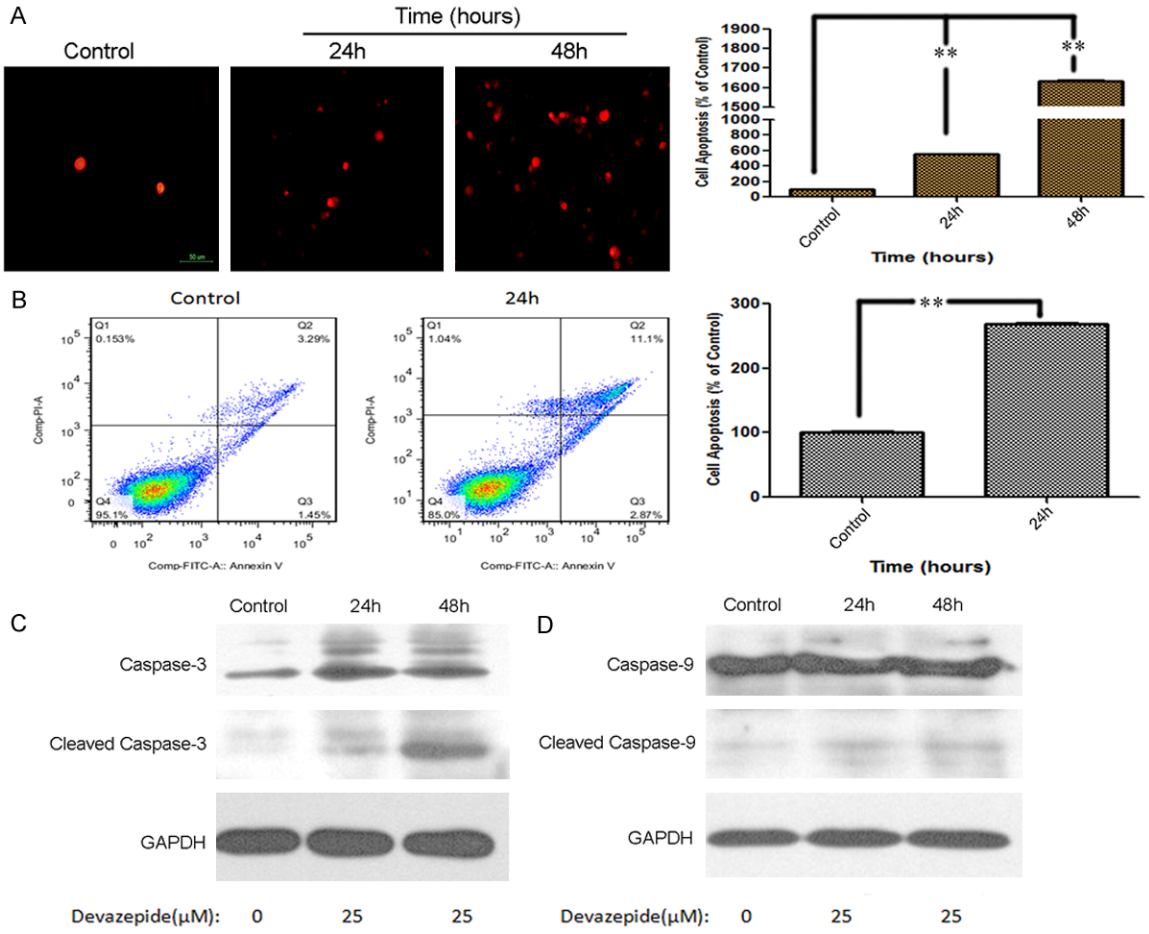

Figure 4. Devazepide activated intrinsic apoptosis pathway and induced apoptosis in human WPMY-1 cells. A. TUNEL assay showed positive staining of Devazepide-induced apoptotic WPMY-1 cells. Representative images were shown. Scale bar indicates 50 μm. B. Human WPMY-1 cells were treated with Devazepide. Then, WPMY-1 cells were stained with Annexin V-FITC/Propidium iodide and analyzed by flow cytometry. C. Western blot showed that Devazepide upregulated expression level of cleaved Caspase-3 in WPMY-1 cells. D. Increased expression level of cleaved Caspase-9 was found in Devazepide-treated WPMY-1 cells by Western blot. GAPDH was used as internal control. Data were presented as mean ± SD. Asterisks indicate statistically significant difference (**P < 0.01).

apoptosis signaling. In this study, we found that Devazepide could significantly upregulate cleaved Caspase-9 and cleaved Caspase-3 by Western blot (**Figure 4**).

Devazepide inhibited in vitro contraction ability of human WPMY-1 cells

It suggests that myofibroblasts could form contractile stress fibers on stiff collagen gels [20]. The contraction ability of WPMY-1 cells may be associated with LUTS in aging men. Therefore, collagen gel contraction assay was performed to evaluate the effect of Devazepide on in vitro contraction ability of WPMY-1 cells. We found that Devazepide could significantly inhibit the contraction of WPMY-1 cells at different time points (**Figure 5**). Devazepide also induced cell death in WPMY-1 cells (**Figure 5**), which might influence gel contraction. In addition, we found that Devazepide could downregulate the expression levels of COL1A1, and COL1A2 (**Figure 5**).

Discussion

Devazepide, as a benzodiazepine drug, could block CCK-mediated activation of CCK-1 receptor. It suggests that CCK could affect the contraction of gall bladder and relaxation of Sphincter of Oddi (Glisson's sphincter), which is involved in the delivery of bile into the small intestine. Here, we reported a novel finding that Devazepide could induce apoptosis in human

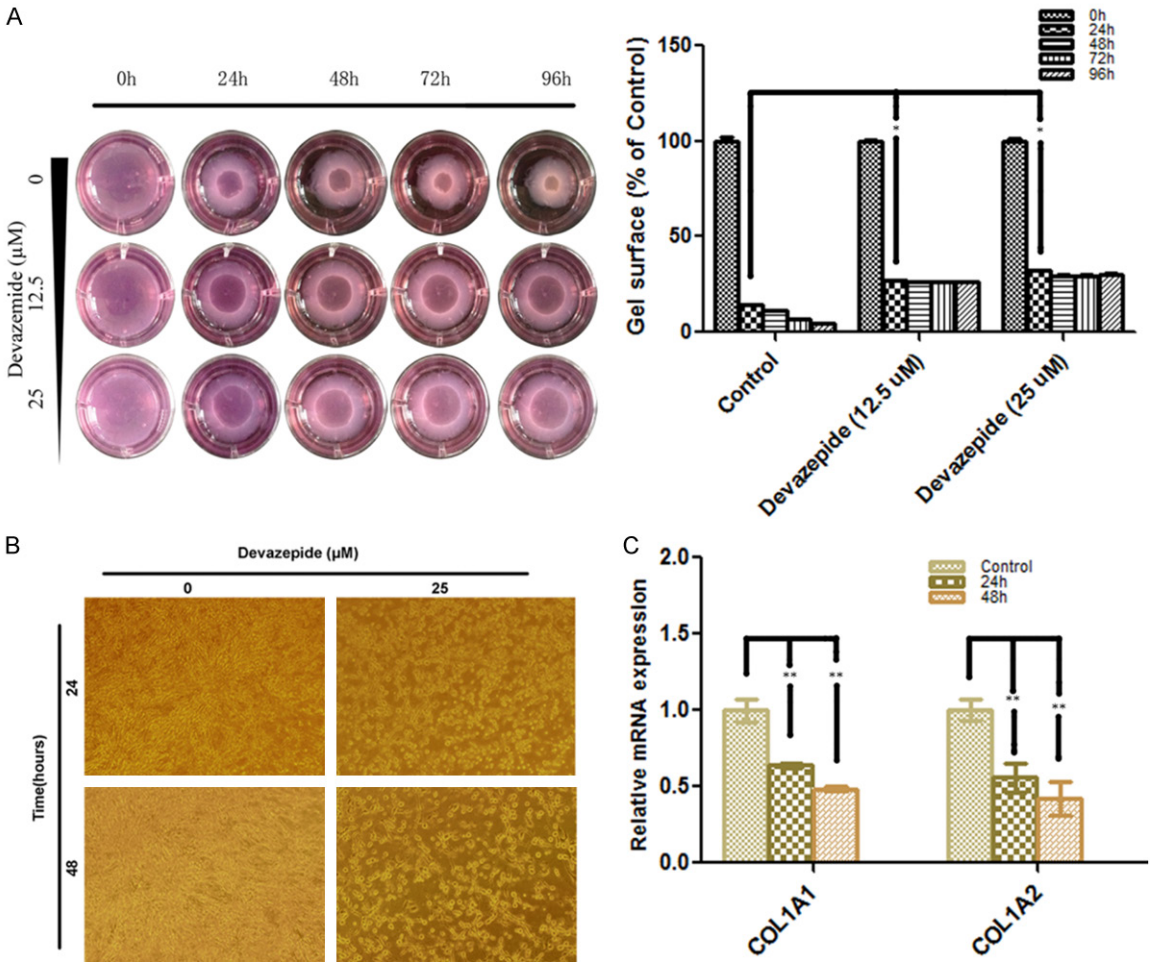

Figure 5. Devazepide inhibited in vitro contraction ability and decreased COL1A1 and COL1A2 expression in human WPMY-1 cells. A. Human WPMY-1 cells were seeded on solidify collagen gels. The gels were detached when cells were attached. Then, WPMY-1 cells were treated with indicated concentrations of Devazepide at different time points. Gel contraction was photographed and measured as a reduction of gel surface. B. The morphologic changes of human WPMY-1 cells were photographed when treated with indicated concentrations of Devazepide. C. Decreased mRNA expression levels of COL1A1 and COL1A2 were detected in Devazepide-treated human WPMY-1 cells by qRT-PCR. Quantification of COL1A1 and COL1A2 mRNA expression was normalized by GAPDH. Data were presented as mean ± SD. Asterisks indicated statistically significant difference (*P < 0.05, **P < 0.01).

WPMY-1 cells. In other studies, the effects of Devazepide on cell growth have been reported. It suggests that Devazepide could induce apoptosis in HT-29 cells [14]. It was also found that Devazepide could inhibit the growth of Ewing tumor cells [13]. Our studies firstly showed that Devazepide could induce death of human WPMY-1 cells.

KI-67 has been extensively studied in past several years and was reported to be closely associated with cell proliferation. Furthermore, it was found that KI-67 could regulate ribosomal RNA transcription and influence cell proliferation. KI-67 has been known as a marker for cell proliferation in scientific research of glioma [21], breast cancer [22], and prostate cancer [23]. In our study, it was observed that KI-67 expression was significantly decreased in Devazepide-treated WPMY-1 cells, which was a valuable marker for cell proliferation in the presence of Devazepide.

Cell cycle is series of events occurring in cell growth, which could cause division and replication of DNA to produce daughter cells. In mammals, cell cycle consists of three periods: interphase, the mitotic (M) phase, and cytokinesis. The G2/M checkpoint, as an important cell cycle checkpoint, ensures that cells don't initiate mitosis before repairing of damaged DNA. In the study [13], it was reported that

Devazepide could arrest Ewing tumor cells in G2/M cell cycle phase. In our study, we found that WPMY-1 cells in G2/M cell cycle phase were significantly increased when treated with Devazepide. This suggested Devazepide could induce G2/M cell cycle arrest not only in tumor cells, but also in normal human prostate myofibroblasts. Therefore, Devazepide might induce cell death via affecting G2/M cell cycle checkpoint.

The underlying molecular mechanisms regulating G2/M cell cycle in cells are still not well understood. It reports that Chk1, as a Serine/threonine-specific protein kinase in humans, regulates the G2/M phase transition [24]. And Chk1 plays an important role in the initiation of cell cycle checkpoints, and cell cycle arrest. Chk2 is another important checkpoint kinase, which could phosphorylate the cell-cycle transcription factor E2F1 and promyelocytic leukemia protein [25]. The phosphorylation of Chk1 and Chk2 has been shown to influence G2/M phase transition. Cyclin B1 is also a vital molecule regulating G2/M phase transition, and found to be upregulated in cells of G2/M cell cycle arrest. In this study, we found that phosphorylation levels of Chk1 and Chk2 were significantly increased in human WPMY-1 cells in the presence of Devazepide. Meanwhile, increased expression of Cyclin B1 was also detected in WPMY-1 cells when treated with Devazepide. These findings suggested that Devazepide could induce G2/M cell cycle arrest in WPMY-1 cells, which was involved in activation of Chk1, and Chk2.

It has been found that apoptosis is a process of programmed cell death. Apoptosis could be activated through intrinsic and extrinsic pathways. In intrinsic apoptotic pathway, Caspase-9 is activated and the activated Caspase-9 could further cleave and activate other executioner Caspases, such as Caspase-3. In this study, we found that Devazepide could activate Caspase-9 and Caspase-3 in WPMY-1 cells. These findings suggested that Devazepide induced apoptosis in human WPMY-1 cells via activating intrinsic apoptotic pathway.

Type I collagen is found to be the most abundant collagen of human body, which is present in scar tissue and involved in fibrosis [26]. Myofibroblasts could express type I collagen and play a vital role in fibrosis [27]. It suggests that COL1A1 gene encodes a component of type I collagen, also known as pro-alpha1(I) chain, which combines with another a pro-alpha2(I) chain (encoded by COL1A2 gene) to produce a molecule of type I procollagen. Hence, both COL1A1 and COL1A2 genes could affect the production of type I collagen. In the study, we found that Devazepide could inhibit COL1A1 and COL1A2 expression in human WPMY-1 myofibroblasts. In addition, we also found that Devazepide inhibited in vitro contraction ability of human WPMY-1 cells. We speculated that Devazepide could inhibit myofibroblasts-mediated fibrosis via suppressing the production of type I collagen.

On the whole, we found that Devazepide could induce cell death and inhibit contraction ability of human prostate WPMY-1 myofibroblasts. Considering the important roles of myofibroblasts in fibrosis, Devazepide may be a novel target for LUTS treatment in aging men.

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Disclosure of conflict of interest

None.

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