

Original Article

Pimavanserin inhibits growth and invasion of bladder cancer cells

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Abstract: Pimavanserin, an antagonist of serotonin 5-hydroxytryptamine (5-HT) 2A receptors, has been used as an atypical antipsychotic. However, whether Pimavanserin affects the biological behavior of tumors remains unknown. The present study found that Pimavanserin dose-dependently reduced the viability of bladder cancer EJ cells and inhibited cell clonogenic abilities. Moreover, Pimavanserin remarkably attenuated cell migration and invasion abilities. It also induced cell cycle arrest at the G1 phase and accelerated cell apoptosis in mitochondrial pathways *in vitro*. Furthermore, Pimavanserin treatment suppressed Wnt/ β -catenin and Akt/mTOR pathways and epithelial-mesenchymal transition (EMT) process in EJ cells. Taken together, results suggest that Pimavanserin inhibits the growth and motility of bladder cancer EJ cells. The current study revealed the anti-cancer effects of Pimavanserin on bladder cancer cells, suggesting that Pimavanserin may function as a potential anti-cancer agent in bladder cancer therapy.

Keywords: Bladder cancer, Pimavanserin, Wnt/ β -catenin pathways, EMT

Introduction

Bladder cancer is the ninth most common malignancy and the thirteenth leading cause of cancer deaths, worldwide, with an approximate 429,793 new cases and 165,084 deaths in 2012 [1-3]. Incidence rates in males are higher than those in females. About three-quarters of cases are males [4]. Despite advancements in the treatment of bladder cancer in recent years (surgical resection and systemic chemotherapy), 5-year survival rates of patients with bladder cancer have not improved significantly [5, 6]. In view of the current situation, it is critical to find new anti-cancer agents that inhibit the growth and metastasis of bladder cancer. In search of novel cancer treatment options, increasing numbers of studies have focused on finding new anti-cancer drugs, including reassessment of several drugs used in other diseases, aiming to discover their potential anti-tumor effects [7].

Pimavanserin, an antagonist of serotonin 5-hydroxytryptamine (5-HT) 2A receptors, has been

used as an atypical antipsychotic for treatment of Parkinson's psychosis [8-11]. A recent study report showed that a serotonin 5-HT_{2A} receptor antagonist, Ritanserin, displayed anti-cancer activity in colorectal cancer cells. It accomplished this by reducing cell viability and inducing apoptosis [12]. However, it remains unclear whether Pimavanserin, a highly selective 5-HT_{2A} receptor antagonist, plays a role in the progression of bladder cancer.

The current study examined the effects of Pimavanserin on biological behaviors of bladder cancer cells, investigating the underlying mechanisms. Current data showed that Pimavanserin reduced proliferation, migration, and invasion levels of EJ bladder cancer cells, inducing cell cycle arrest and apoptosis. Moreover, Pimavanserin downregulated Wnt/ β -catenin and Akt pathways and the epithelial-mesenchymal transition (EMT) process in bladder cancer cells. Present data suggests that Pimavanserin may serve as a potential anti-cancer agent in bladder cancer therapy.

Materials and methods

Cell culturing

Human bladder cancer cell line EJ was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). It was maintained in DMEM medium (Hyclone, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco, USA) at 37°C with 5% CO₂.

Dose-dependent assay

EJ cells were seeded into 96-well plates and treated with different concentrations of Pimavanserin (0, 0.25, 0.5, 1, 2, 4, 8, 16, 24, 32, and 64 µM; MedChemExpress, USA) for 24 hours. After treatment, cells were cultured with CCK8 reagent (10 µl/well; Beijing Solarbio Science & Technology, China) at 37°C for 90 minutes. Absorbance values were measured at 450 nm.

Cell viability assay

Effects of Pimavanserin on cell viability were assayed using CCK8 assays. Briefly, cells were seeded in a 96-well plate at a density of approximately 1×10³ cells per well. They were cultured for 24 hours, then treated with Pimavanserin (20 µM) or DMSO (as negative control, NC). Following treatment for 0, 24, 48, and 72 hours, the cells were cultured with CCK8 reagent at 37°C for 1.5 hours. Absorbance values were measured at 450 nm.

Cell proliferation assay

Cells treated with either Pimavanserin (20 µM) or DMSO for 24 hours were seeded in mediums at a density of 500 cells per well and cultured for 10-14 days. Afterward, the cells were fixed with 1 mL of 4% paraformaldehyde for 30 minutes, followed by staining with crystal violet for 30 minutes. Photographs were taken and the number of colonies was counted.

Wound-healing assay

Cells treated with either Pimavanserin (20 µM) or DMSO for 24 hours were seeded in 6-well plates. Wounds were generated using pipette tips. Following scratching, the cells were cultured in serum-free medium for 24 hours. Wound closure was then analyzed using Image J software.

Cell invasion assay

Transwell chambers (EMD Millipore, USA) coated with Matrigel (BD Biosciences, USA) were used for cell invasion assays. After treatment with either Pimavanserin (20 µM) or DMSO for 24 hours, approximately 1×10⁵ EJ cells in a serum-free medium were transferred into the upper chamber. The complete medium was added into the lower chamber. Following incubation for 24 hours, invaded cells were fixed with 4% paraformaldehyde, prior to staining, with 0.1% crystal violet. The number of invaded cells was counted and captured (magnification, ×100) under a light microscope.

Cell cycle and apoptosis flow cytometry assays

Cells treated with either Pimavanserin (20 µM) or DMSO were fixed with 70% pre-cooling anhydrous ethanol at -20°C overnight. This was followed by staining with propidium iodide (PI). Cell cycle distribution was analyzed using a flow cytometer (BD FACSCanto II, BD Biosciences, USA). Assessing cell apoptosis, treated cells were collected and stained with the Annexin V-FITC-PI apoptosis detection kit (4A Biotech, China), according to manufacturer instructions. The rate of apoptosis was analyzed and calculated using a flow cytometer.

Western blotting

Following treatment with either Pimavanserin (20 µM) or DMSO for 24 hours, the cells were lysed in ice-cold RIPA Lysis Buffer (CW BIO, China). Equal amounts of protein from each sample were electrophoresed using 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Bedford, USA). The membranes were incubated with primary antibodies at 4°C overnight and treated with secondary antibodies for 1 hour. Finally, the signal was developed using an enhanced chemiluminescence detection kit (CW BIO). Antibodies against Bcl-2, Bax, active Caspase-3, Caspase-9, Cyclin D1, NUSAP1, wnt3a, β-catenin, N-cadherin, E-cadherin, Vimentin, Snail1, Snail2, and GAPDH, as well as all secondary antibodies, were obtained from the Proteintech Group (USA). Antibodies against Akt, p-Akt, mTOR, and p-mTOR were obtained from Cell Signaling Technology (USA).

Statistical analysis

Data are expressed as mean ± SD and were analyzed using GraphPad Prism 7.0 (USA). St-

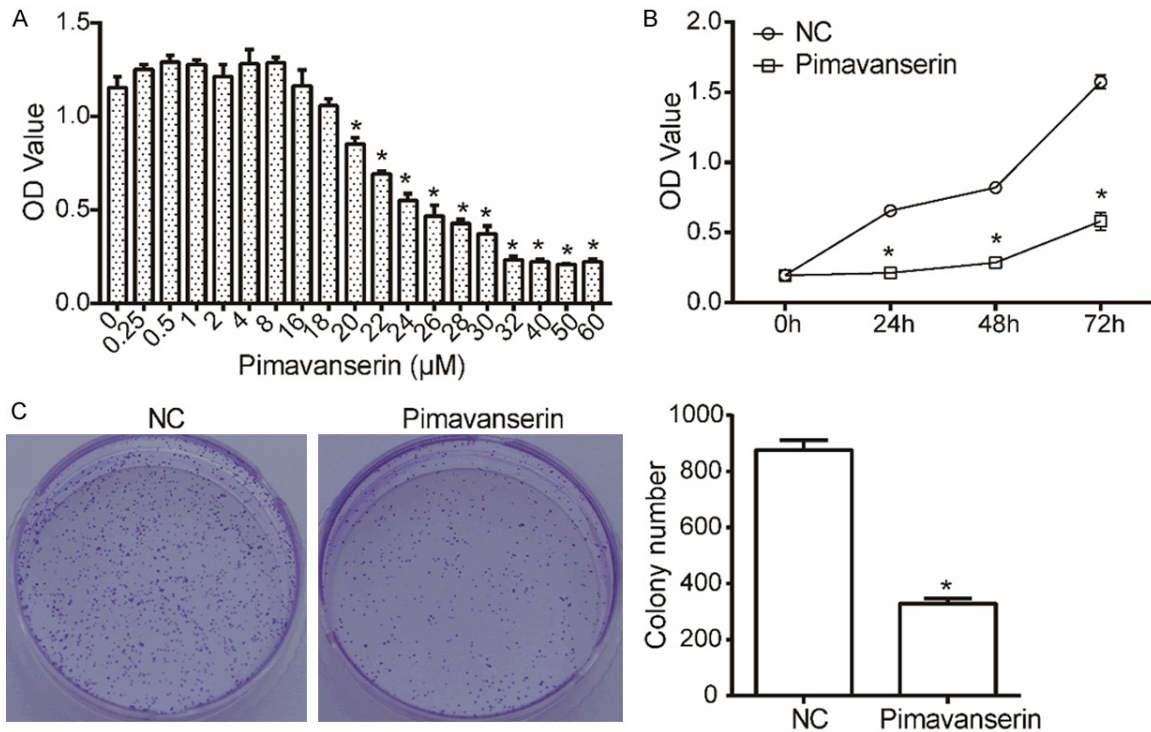


Figure 1. Pimavanserin inhibits proliferation and viability of bladder cancer cells. A. EJ cells were treated with different concentrations of Pimavanserin (0, 0.25, 0.5, 1, 2, 4, 8, 16, 18, 20, 22, 24, 26, 28, 30, 32, 40, 50, and 60 μ M). Pimavanserin inhibited viability of EJ cells in a dose-dependent manner. B. CCK8 assays showed that Pimavanserin (20 μ M) inhibited proliferation of EJ cells. C. Colony-forming assays showed that Pimavanserin (20 μ M) inhibited clonogenic ability of EJ cells. Pimavanserin: Pimavanserin treated group; NC: DMSO treated group, negative control, NC. Data are expressed as the mean $\pm$ SD. * $P < 0.05$.

udent's *t*-tests were used to compare differences between the two groups. $P < 0.05$ indicates statistical significance.

Results

Pimavanserin inhibits the viability and proliferation of bladder cancer cells

To investigate whether Pimavanserin impacts cell growth in bladder cancer, bladder cancer cell line EJ cells were treated with different concentrations of Pimavanserin (0, 0.25, 0.5, 1, 2, 4, 8, 16, 18, 20, 22, 24, 26, 28, 30, 32, 40, 50, and 60 μ M). After treatment for 24 hours, viability levels of EJ cells were examined using CCK8 assays. As shown in **Figure 1A**, Pimavanserin showed no significant effects on the viability of EJ cells at concentrations lower than 18 μ M, while it significantly decreased cell viability at greater concentrations, in a dose-dependent manner. The inhibition rate was 25.99% at a concentration of 20 μ M of Pimavanserin (**Figure 1A**). The concentration IC_{50} of

Pimavanserin was 23.6 μ M in EJ cells. Moreover, 20 μ M of Pimavanserin was used in the remaining experiments. DMSO was used as the negative control (NC). The viability of EJ cells was significantly blocked by 20 μ M of Pimavanserin, even after 72 hours of treatment ($P < 0.05$, **Figure 1B**). The inhibition rate of Pimavanserin was 63.05% after 72 hours of treatment (**Figure 1B**). In addition, colony-forming assays were used to further confirm the effects of Pimavanserin on growth of EJ cells. Consistent with the above assays, current data revealed a significant decrease in the number of colonies in the Pimavanserin-treated group, compared with the NC group ($P < 0.05$, **Figure 1C**). Data suggests that Pimavanserin inhibited the viability and proliferation of bladder cancer cells.

Pimavanserin decreases migration and invasion abilities of bladder cancer cells

To further assess the effects of Pimavanserin on progression of bladder cancer, a wound-

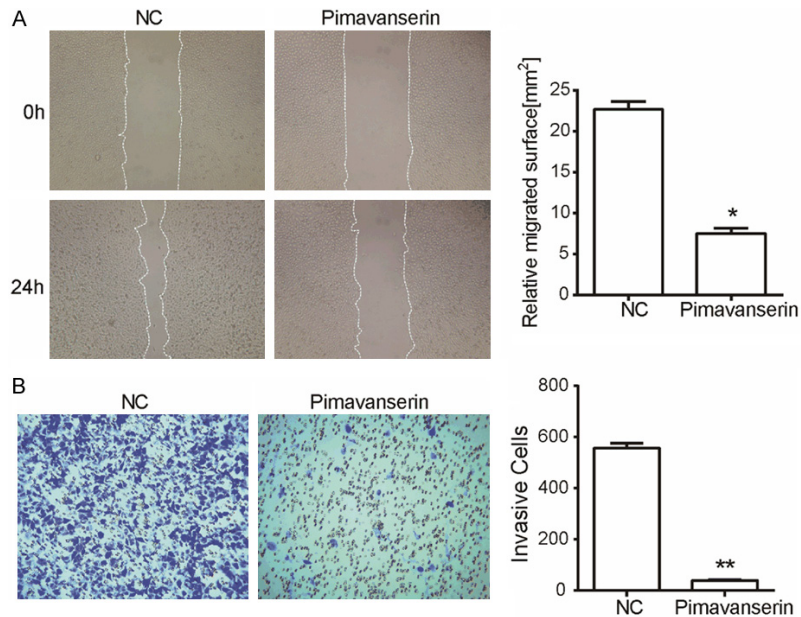


Figure 2. Pimavanserin inhibits migration and invasion of bladder cancer cells. A. Wound-healing assays revealed inhibition of migration of EJ cells by Pimavanserin (20 μ M) treatment. B. Transwell assays showing inhibition of invasion of EJ cells by Pimavanserin (20 μ M) treatment. Pimavanserin: Pimavanserin treated group; NC: DMSO treated group, negative control, NC. Data are expressed as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

healing assay was performed to detect cell migration ability of EJ cells. As shown in **Figure 2A**, the wound-healing assay revealed that Pimavanserin significantly decreased the wound closure ability of EJ cells, compared with the NC group ($P < 0.05$). Furthermore, using a Matrigel-coated Transwell chamber assay, compared with NC cells, the Pimavanserin-treated cells displayed a significant depression in the ability to invade through the Matrigel into the empty space (**Figure 2B**, $P < 0.01$). Collectively, current data suggests that Pimavanserin (20 μ M) showed significant suppression of the cell motility of bladder cancer cells *in vitro*.

Pimavanserin induces cell cycle arrest at the G1 phase in EJ cells

As illustrated in **Figure 3A**, compared with the NC group, a significant increase in the proportion of cells in the G1 phase and a significant decrease in the proportion of cells in the S phase was observed in Pimavanserin-treated cells ($P < 0.05$). It was further demonstrated that expression of Cyclin D1, a key regulator promoting the cell cycle from G1 phase to S phase, was significantly downregulated by Pimavanserin treatment ($P < 0.05$, **Figure 3C**). In

addition, another essential protein involved in mitosis, nucleolar and spindle-associated protein 1 (NUSAP1), was inhibited by Pimavanserin treatment in EJ cells ($P < 0.05$, **Figure 3C**). Results indicate that Pimavanserin blocked the bladder cancer cell cycle at G1 phase by downregulating expression levels of Cyclin D1 and NUSAP1.

Pimavanserin promotes cell apoptosis of EJ cells

Since Pimavanserin has been found to inhibit cell proliferation, the current study examined cell apoptosis using flow cytometry. The aim was to evaluate the effects of Pimavanserin on survival of bladder cancer cells. As indicated in **Figure 3B**, Annexin V/PI staining

showed that the percentage of apoptotic cells was significantly upregulated in Pimavanserin-treated cells, compared with the NC group ($P < 0.05$). Moreover, expression levels of Bcl-2, Bax, active Caspase-3, and Caspase-9 were detected using Western blotting, investigating relevant mechanisms underlying the Pimavanserin-induced apoptosis. Expression of Bcl-2, a pivotal anti-apoptotic protein, was significantly blocked in Pimavanserin-treated cells ($P < 0.05$), while expression of pro-apoptosis proteins Bax, active Caspase-3, and Caspase-9 were upregulated by Pimavanserin, compared with NC cells ($P < 0.05$, **Figure 3C**). Results suggest that Pimavanserin induces mitochondrial apoptosis pathways in bladder cancer cells.

Pimavanserin downregulates Wnt/ β -catenin and Akt/mTOR pathways and epithelial-mesenchymal transition (EMT) in EJ cells

To further investigate mechanisms underlying the inhibitory effects of Pimavanserin on cell proliferation, viability, migration, and invasion of EJ cells, the current study assessed alterations in Wnt/ β -catenin and Akt/mTOR pathways. These have been shown to play pivotal roles in regulating tumor growth and metastasis.

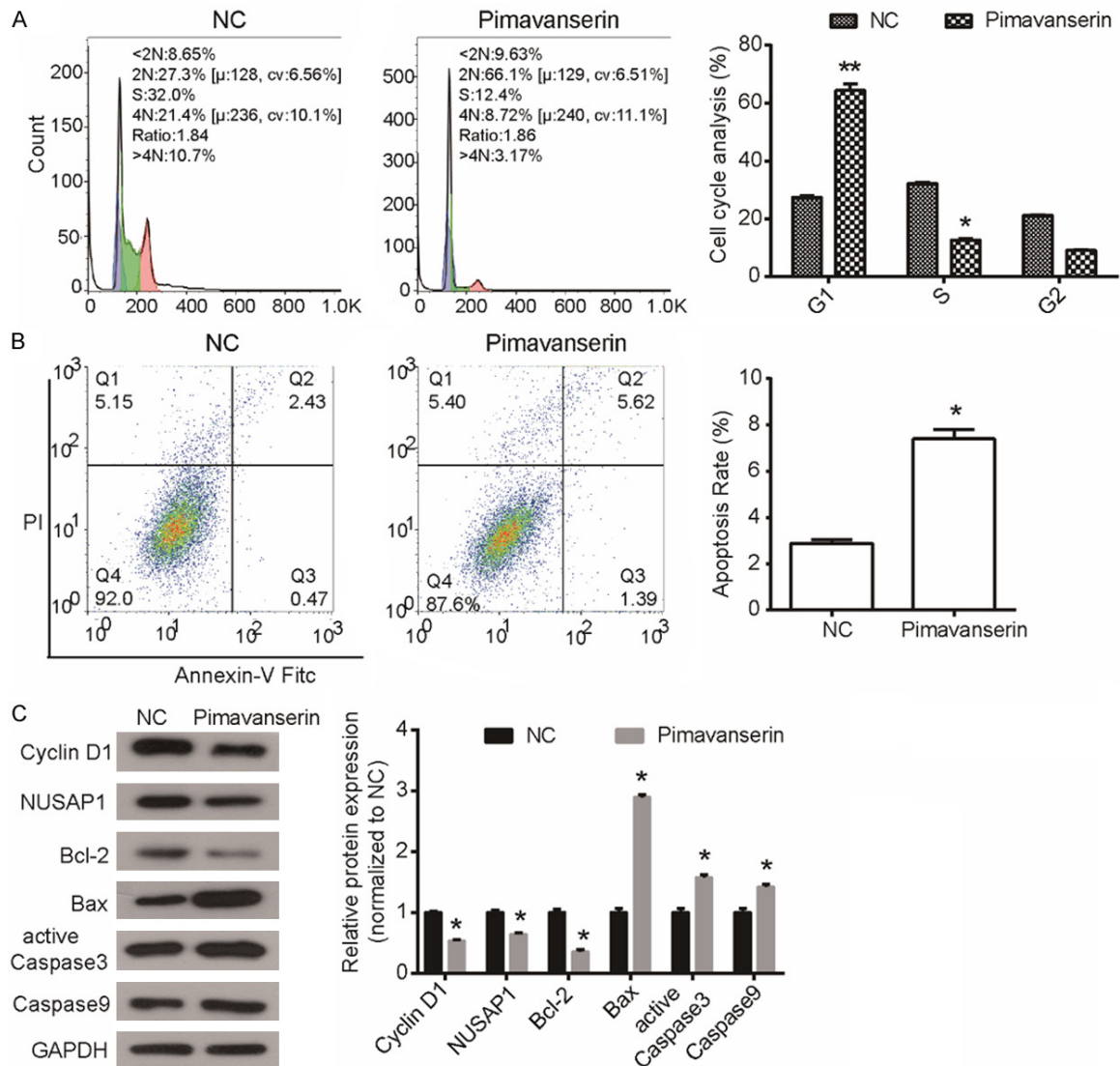


Figure 3. Pimavanserin induces cell cycle arrest and apoptosis in EJ cells. **A.** Effects of Pimavanserin (20 μ M) on the EJ cell cycle. Cell cycle distribution was assessed using PI staining, followed by flow cytometry. **B.** Pimavanserin (20 μ M) promoted apoptosis of EJ cells determined by flow cytometry. **C.** Western blot analysis showed that Pimavanserin decreased expression of cell cycle-related proteins (Cyclin D1 and NUSAP1) and anti-apoptotic protein (Bcl-2), while increasing expression of pro-apoptotic proteins (Bax, active Caspase-3, and Caspase-9). Pimavanserin: Pimavanserin treated group; NC: DMSO treated group, negative control, NC. Data are expressed as the mean $\pm$ SD. * P <0.05.

sis. Western blotting results revealed that expression of wnt3a and β -catenin, key components of Wnt/ β -catenin pathways, was significantly suppressed by Pimavanserin, compared with the NC group (P <0.05, **Figure 4A**). Moreover, expression of N-cadherin, Vimentin, Snail1, and Snail2, important acquired markers of the EMT process, were significantly decreased in Pimavanserin-treated cells. Levels of epithelial marker E-cadherin were increased accordingly (P <0.05, **Figure 4A**), indicating that the EMT process was suppressed in Pimavanserin-treated EJ cells. In addition, as presented in

Figure 4B, Pimavanserin decreased phosphorylation levels of Akt (p-Akt) and mTOR (p-mTOR) in EJ cells (P <0.05), leading to the inactivation of the Akt/mTOR pathways in bladder cancer cells. Current data suggests that Wnt/ β -catenin and Akt/mTOR pathways may be involved in anti-cancer activities of Pimavanserin.

Discussion

Increasing evidence has demonstrated that there is neuroendocrine differentiation in bladder cancer [13]. A monoamine neurotransmit-

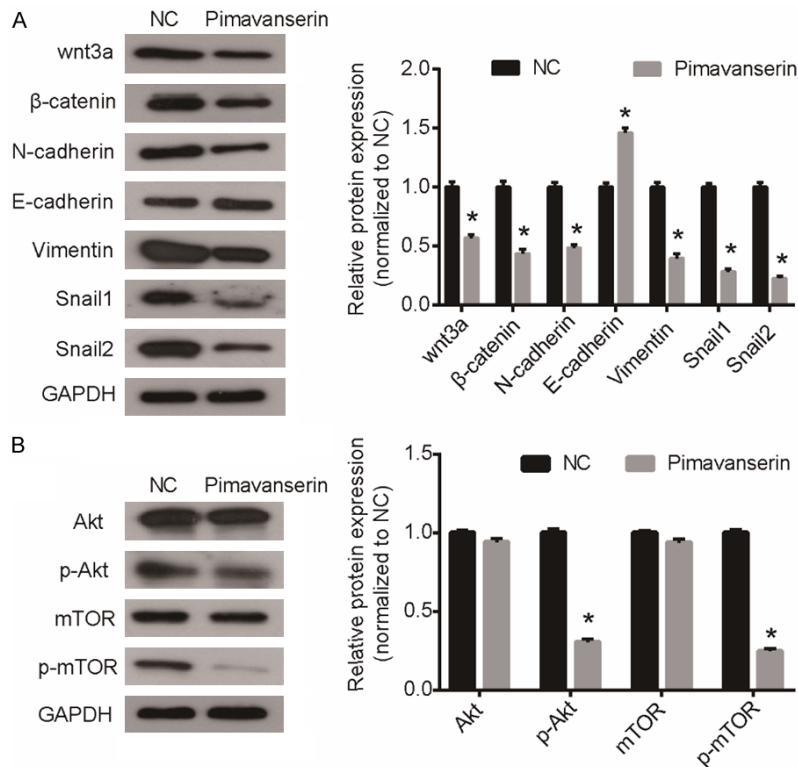


Figure 4. Pimavanserin inhibits Wnt/β-catenin and Akt/mTOR pathways in EJ cells. A. Western blot analysis showed that Pimavanserin inhibited Wnt/β-catenin pathways and the EMT process. B. Western blot analysis showed that Pimavanserin inhibited Akt/mTOR pathways. Pimavanserin: Pimavanserin treated group; NC: DMSO treated group, negative control, NC. Data are expressed as the mean ± SD. *P<0.05.

ter, 5-HT spreads across multiple tissues of the body. It has been shown to act as a growth factor in several types of tumor cells. The serotonin 5-HT₂ receptor (5-HT₂R) family has been identified to contain three subtypes, including 5-HT_{2A}R, 5-HT_{2B}R, and 5-HT_{2C}R, of which 5-HT_{2A}R has been found to be widely distributed in peripheral tissues, functioning as a mitogenic receptor in different cell types [12, 14, 15]. Siddiqui E J et al. report that 5-HT_{2A}R protein is expressed in bladder cancer tissues and HT1376 cells [16]. A recent study revealed that a 5-HT_{2A}R antagonist, ritanserin, displayed depression in cell viability levels of colorectal cancer [12]. The current study demonstrated, clearly, that the highly selective 5-HT_{2A}R antagonist, Pimavanserin, decreased viability levels of bladder cancer EJ cells, in a dose-dependent manner. It also inhibited cell proliferation, migration, and invasion abilities, displaying anti-cancer effects on the growth and invasion of bladder cancer cells.

Normal cell cycle progression is important for tumor cell proliferation and growth. Therefore, this study further investigated potential mechanisms of Pimavanserin in inhibiting the proliferation and viability of EJ cells. It was observed that Pimavanserin induced cell cycle arrest at the G1 phase in EJ cells by down-regulation of expression of cell cycle regulator protein Cyclin D1, indicating a new role of Pimavanserin in blocking tumor cell cycle.

Apoptosis is another strict regulatory mechanism controlling cell growth. Dysregulated apoptosis and resistance to cell death are common hallmarks of tumor cells. One of the important features of Pimavanserin, according to the current study, was the induction of apoptosis in EJ cells (Figure 3). Apoptosis is a self-killing process that involves numerous proteolytic

events, mainly mediated by the family of cysteine proteases, especially the caspase family. This family is the pivotal executioner in triggering apoptosis [17]. Caspase-3 is well known to be a key rate-limiting enzyme, determining the degree of apoptosis. Caspase-3 could be activated by Caspase-9, which is an essential initiation factor in intrinsic apoptosis pathways [18-20]. Upregulation of active Caspase-3 and Caspase-9 was observed in Pimavanserin-treated cells, indicating that over-activated caspase cascades were involved in the induction of cell apoptosis caused by Pimavanserin in bladder cancer. The Bcl-2 protein located in the mitochondrial membrane. It also plays an essential role in initiation of apoptosis by triggering mitochondrial pathways [21, 22]. Additionally, it has been confirmed that Bcl-2 and Bax are frequently abnormal regulated in bladder cancer [23]. In this study, a significant decrease in expression of Bcl-2 was observed in EJ cells exposed to Pimavanserin, while Bax expression

was increased. Taken together, results suggest that Pimavanserin promotes apoptosis by triggering mitochondrial pathways.

Wnt/ β -catenin pathways are involved in the regulation of various biological cellular processes, including gene expression, cell growth, and metastasis [24]. Wnt/ β -catenin pathways also play a crucial role in driving the EMT process that damages epithelial integrity and promotes tumor metastasis and invasion [25, 26]. The present study found that Wnt/ β -catenin pathways were significantly blocked by Pimavanserin through decreasing expression of Wnt3a and β -catenin (**Figure 4A**). Moreover, Pimavanserin upregulated expression of E-cadherin and downregulated expression of N-cadherin, Vimentin, Snail1, and Snail2. The EMT process is a key activity driving tumor metastasis and invasion. Decreased expression of E-cadherin and increased expression of N-cadherin and Vimentin in epithelial cells indicates that tumors are susceptible to metastatic phenotypes [27, 28]. E-cadherin, the most classical epithelial cell marker, is expressed in epithelial cells and downregulated during EMT. N-cadherin is a mesenchymal cell marker expressed in mesenchymal cells, fibroblasts, tumor cells, and nervous tissues, with increased expression during EMT. Vimentin is a cytoskeletal marker. Expression of Vimentin is positively related to tumor invasion and metastasis. Snail, a family of transcription factors, is the most widely known E-cadherin suppressor [29]. As described above, current results raise an interesting hypothesis, suggesting that Pimavanserin inhibits the growth, migration, and invasion of bladder cancer cells by blocking Wnt/ β -catenin pathways and suppressing the EMT process. In addition, current results showed that Akt/mTOR pathways, another known factor involved in cell growth and metabolism, were significantly inhibited by Pimavanserin in EJ cells (**Figure 4B**). This suggests that Akt/mTOR pathways may be also involved in the mechanisms underlying the anti-growth activity of Pimavanserin in bladder cancer.

In summary, for the first time, the current study revealed that Pimavanserin inhibits the growth and motility of human bladder cancer EJ cells. Moreover, it promotes apoptosis and induces cell cycle arrest. Current data also demonstrates that Pimavanserin could inhibit Wnt/ β -catenin and Akt/mTOR pathways, suppressing

the EMT process. Therefore, Pimavanserin, the highly selective 5-HT_{2A} receptor antagonist, may serve as a potential anticancer agent in bladder cancer therapy.

Disclosure of conflict of interest

None.

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