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Targeting of Wnt/β-Catenin by Anthelmintic Drug Pyrvinium Enhances Sensitivity of Ovarian Cancer Cells to Chemotherapy

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 Chongyuan Zhang
BCD 1 Zhenge Zhang
BC 1 Shuirong Zhang
F 2 Wenrong Wang
ADG 1 Ping Hu

1 Department of Obstetrics and Gynaecology, Jingzhou Central Hospital, The Second Clinical Medical College, Yangtze University, Jingzhou, Hubei, P.R. China
2 Department of Obstetrics and Gynaecology, Jingzhou Third People's Hospital, Jingzhou, Hubei, P.R. China

Corresponding Author: Ping Hu, e-mail: hupingqixing@hotmail.com
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Background: Aberrant activation of Wnt/β-catenin has been shown to promote ovarian cancer proliferation and chemoresistance. Pyrvinium, an FDA-approved anthelmintic drug, has been identified as a potent Wnt inhibitor. Pyrvinium may sensitize ovarian cancer cells to chemotherapy.

Material/Methods: The effect of pyrvinium alone and its combination with paclitaxel in ovarian cancer was investigated using an *in vitro* culture system and *in vivo* xenograft models. The mechanisms of its action were also analyzed, focusing on the Wnt/β-catenin pathway.

Results: Pyrvinium inhibited growth and induced apoptosis of paclitaxel- and cisplatin-resistant epithelial ovarian cancer cell lines A2278/PTX and SK-OV-3. Its combination with paclitaxel was synergistic in targeting ovarian cancer cells *in vitro*. In 3 independent ovarian xenograft mouse models, pyrvinium alone inhibited tumor growth. More importantly, we observed significant inhibition of tumor growth throughout the treatment when using pyrvinium and paclitaxel combined. Mechanistically, pyrvinium increased the Wnt-negative regulator axin and decreased the β-catenin levels in ovarian cancer cells. In addition, pyrvinium suppressed Wnt/β-catenin-mediated transcription, as shown by the decreased mRNA levels of MYC, cyclin D, and BCL-9. In contrast, the inhibitory effects of pyrvinium were reversed by β-catenin stabilization or overexpression, demonstrating that pyrvinium acted on ovarian cancer cells via targeting the Wnt/β-catenin signaling pathway.

Conclusions: We demonstrated that the anthelmintic drug pyrvinium targets ovarian cancer cells through suppressing Wnt/β-catenin signaling. Our work highlights the therapeutic value of inhibiting Wnt/β-catenin in ovarian cancer.

MeSH Keywords: Drug Repositioning • Ovarian Neoplasms • Pyrvinium Compounds • Wnt Signaling Pathway

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Background

Epithelial ovarian cancer is the most common type of ovarian cancer; it affects women worldwide and causes high mortality [1]. Clinical management of advanced epithelial ovarian cancer remains a challenge due to the development of chemoresistance [2]. The molecular mechanisms leading to ovarian cancer progression and chemoresistance are not well understood but might involve changes in cell adhesion (e.g., CD44 and β1 integrin), mutation and loss of *TP53* function, and activation of oncogenic pathways (e.g., KRAS and BRAF) [3]. Recent studies have shown that the Wnt/β-catenin pathway contributes to chemoresistance via promoting epithelial-to-mesenchymal transition and can serve as a prognostic maker in patients with advanced ovarian cancer [4,5]. Targeting the Wnt/β-catenin pathway might be an alternative therapeutic strategy to overcome resistance in ovarian cancer.

Pyrvinium, a FDA-approved anthelmintic drug, has been revealed to be a potent Wnt inhibitor [6]. It inhibits growth of various different tumor cell lines *in vitro* and *in vivo* [6,7–11]. Pyrvinium also enhances the efficacy of chemotherapeutic drugs in several tumors [8,10]. Apart from inhibiting the Wnt/β-catenin pathway, the mechanism of action of pyrvinium depends on cancer cell type. Other reported mechanisms of action of pyrvinium in different cancers involve mitochondrial respiration inhibition [9,12], targeting autophagy [8], and unfolded protein response [10].

In the present study, we examined the effect of pyrvinium on ovarian cancer using an *in vitro* culture cell system and an *in vivo* xenograft mouse model. We showed that pyrvinium induced apoptosis and inhibited proliferation in 2 resistant ovarian cancer cell lines regardless of their mechanisms of resistance. Pyrvinium also significantly arrested ovarian tumor growth in mice. The combination of pyrvinium and paclitaxel was synergistically active against ovarian cancer. In our analysis, we had determined and showed that the inhibitory effects of pyrvinium was due to the inhibition of the Wnt/β-catenin signaling pathways in ovarian cancer cells.

Material and Methods

Human cell lines and compounds

Human ovarian carcinoma cell lines A2780/PTX, A2780cis, and SK-OV-3 were purchased from Sigma and KeyGen Biotech Co., Ltd., respectively. Cells were cultured in Minimal Essential Media containing 10% FBS (fetal bovine serum, Hyclone, UK) and 2 mM L-glutamine (Invitrogen). Pyrvinium, lithium chloride (LiCl), and paclitaxel (Sigma) were dissolved in DMSO.

MTS proliferation assay

Cells underwent 3 days of treatment with pyrvinium. The cell proliferation activities were measured using the CellTiter 96R AQueous One Solution Cell Proliferation assay kit (Promega) according to the manufacturer's instructions.

Flow cytometry analysis

Cells underwent 3 days of treatment with pyrvinium. The cells were trypsinized and washed with PBS prior to staining with annexin V-FITC and propidium iodide (PI). Stained cells were washed with PBS and then analyzed using flow cytometry (Beckman-Coulter, USA). The annexin V-positive cells percentage was analyzed using CXP flow cytometry analysis software (Beckman-Coulter).

Western blot (WB) analyses

Drug-treated or transfected cells were lysed by RIPA buffer (Life Technologies Inc, USA). Frozen tumor tissues were homogenized using a polytron homogenizer in ice-cold RIPA buffer for protein lysis. Total protein was measured using the bicinchoninic acid protein assay kit (Thermo Scientific, USA). Equal amounts of total proteins were resolved using denaturing with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were then processed for WB analyses. Antibodies used in WB analyses include anti-axin1, anti-axin2, anti-β-catenin, P1, and anti-β-actin (Cell Signaling Technologies, USA).

TOPflash reporter assay

Cells were electroporated with a M50 Super 8x TOPFlash plasmid [13] using the Amaxa Nucleofector kit (Lonza, Germany). At 24 h post-transfection, they were exposed to drugs for 24 h prior to β-catenin activity assessment using the luciferase assay (Promega, USA) following the manufacturer's instructions.

Transfection of β-catenin overexpression plasmid

Cells were transfected with 1.5 μg pcDNA or pcDNA-β-cat using the Amaxa Nucleofector kit (Lonza, Germany). At 24 h post-transfection, cells were exposed to pyrvinium for 3 days prior to apoptosis and proliferation analyses.

Real-time (RT) PCR

Isolation of total RNA was done using TRIzol Reagent (Ambion, USA) and first-strand cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad, CA). The PCR master mix was prepared using the SsoFast EvaGreen Supermix and amplification was done on the CFX96 RT PCR system (Bio-Rad, CA). Primer designs for the following genes are provided below.

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MYC (5'-AAT GAA AAG GCC CCC AAG GTA GTT ATC C-3'and 5'-GTC GTT TCC GCA ACA AGT CCT CTT C-3'), cyclin D (5'-CCG TCC ATG CGG AAG ATC-3' and 5'-ATG GCC AGC GGG AAG AC-3'), BCL9 (5'-AGA GAG AAG CAC AGC GCC TC-3' and 5'-CTG CAG TCT GGT ATT CTG GGA AG-3') and β-actin (5'-AAG GAT TCC TAT GTG GGC GAC G-3' and 5'-GCC TGG ATA GCA ACG TAC ATG G-3') as control. The mRNA levels of cyclin D, BCL9, and MYC were quantified using a comparative CT method with β-actin levels for normalization.

Ovarian cancer xenograft in SCID mice

All procedures were conducted in accordance with the Guide to the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee of Yangtze University. SCID mice were purchased from the Animal Resources Centre, Australia. The SCID mice were inoculated with 1 million SK-OV-9, A2780/PTX, or A2780cis cells subcutaneously in the right flank. The inoculation volume (0.1 ml) comprised a 50: 50 mixture of cells in growth media and Matrigel (BD Biosciences). When tumors were palpable, the mice were intraperitoneally treated with vehicle, pyrvinium at 0.5 mg/kg, paclitaxel at 10 mg/kg daily, or a combination of both for 3 weeks. Tumor length and width were measured every 3 days and tumor volume was estimated by applying the following equation:

$$\text{volume}=\text{length}\times\text{width}^2/2.$$

Statistical analyses

The unpaired *t* test was used in the statistical analysis for a comparison of categorical variables. A P-value less than 0.05 was deemed as statistically significant.

Results

Pyrvinium induces apoptosis and inhibits proliferation of resistant ovarian cancer cells.

To evaluate the effects of pyrvinium on ovarian cancer cells, we performed proliferation and apoptosis assays on 2 ovarian cancer cell lines: SK-OV-3 and A2780/PTX. SK-OV-3 is resistant to anti-estrogen treatment [14]. A2780/PTX is resistant to paclitaxel and displays cancer stem cell properties [15]. We found that pyrvinium significantly induced apoptosis of SK-OV-3 and A2780/PTX in a dose-dependent manner as assessed by flow cytometry for annexin V and PI staining (Figure 1A, 1B). In addition, pyrvinium potently inhibited proliferation of ovarian cancer cell lines (Figure 1C). The efficacy of pyrvinium is similar among ovarian cancer cell lines (Figure 1), suggesting that pyrvinium is active against ovarian cancer cells regardless of their various mechanisms of resistance.

Pyrvinium enhances the inhibitory effects of paclitaxel *in vitro*

We next investigated whether the combination of pyrvinium and paclitaxel is superior to a single drug in ovarian cancer cells. As shown by Figure 2A, paclitaxel slightly inhibited proliferation. However, when pyrvinium was combined with paclitaxel, the combination significantly inhibited proliferation in ovarian cancer cells. Similar synergistic effects in inducing apoptosis were also observed in cells exposed to both pyrvinium and paclitaxel (Figure 2B). These data clearly demonstrate that pyrvinium acts synergistically with chemotherapy drug paclitaxel in resistant ovarian cancer cells.

Pyrvinium suppresses Wnt/β-catenin signaling in ovarian cancer cells

Pyrvinium has been demonstrated to regulate the stability of β-catenin and axin in colon cancer cells, leading to inhibition of Wnt/β-catenin signaling [6] and inhibiting mitochondrial functions in blood cancer [12]. Our results show that pyrvinium at 100 and 500 nM, which effectively targeted growth and survival, did not affect ATP level in ovarian cancer cells, whereas pyrvinium at 1000 nM slightly decreased ATP level (Supplementary Figure 1), suggesting that pyrvinium does not act on ovarian cancer cells through mitochondrial function inhibition. As Wnt/β-catenin signaling plays an important role in the development of ovarian cancer [5,16,17], we next tested whether pyrvinium acts on ovarian cancer cells through inhibiting Wnt/β-catenin signaling.

Because it is a negative regulator of Wnt/β-catenin signaling, we determined axin levels in ovarian cancer cells exposed to pyrvinium [18]. We found that axin level was increased in both SK-OV-3 and A2780/PTX cells exposed to pyrvinium in a dose-dependent manner (Figure 3A). Consistently, β-catenin level was significantly decreased by pyrvinium. We next evaluated the changes in Wnt/β-catenin-mediated transcription using a luciferase-based TCF/LEF1 binding sites (TOPflash) reporter assay to ascertain the inhibitory effect of pyrvinium on β-catenin signaling. Pyrvinium inhibited TOPflash luciferase activity in ovarian cancer cells in a dose-dependent manner (Figure 3B). In addition, the levels of Wnt/β-catenin-mediated activation of target genes BCL9, cyclin D, and MYC were decreased in ovarian cancer cells exposed to pyrvinium (Figure 3C, 3D), suggesting that pyrvinium decreased Wnt/β-catenin signaling in ovarian cancer cells.

β-catenin overexpression reverses inhibitory effects of pyrviniums in ovarian cancer cells

To further confirm that β-catenin is the target of pyrvinium in ovarian cancer cells, we next rescued β-catenin levels in ovarian cells using both pharmacological (e.g., LiCl) and genetic (e.g.,

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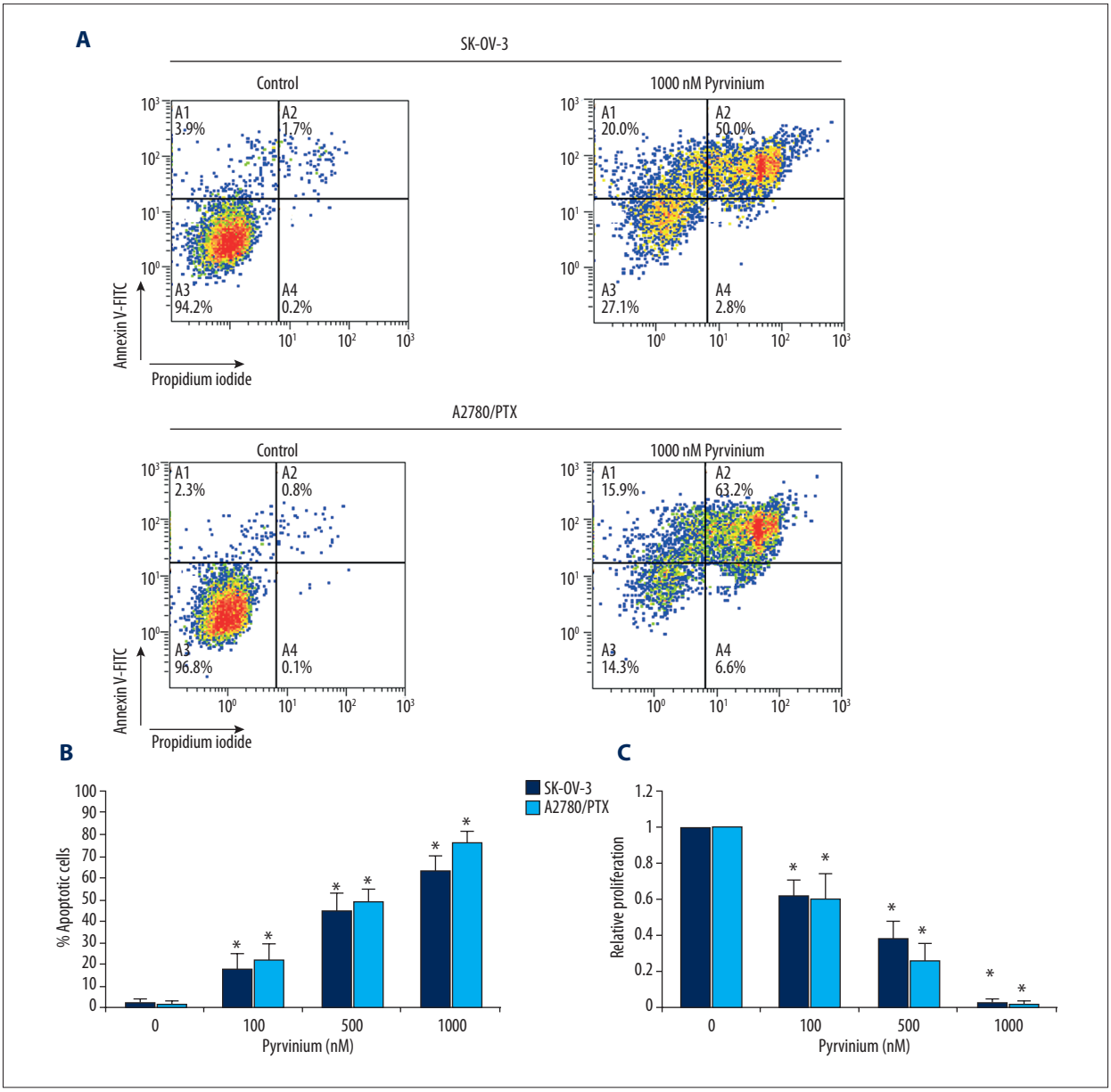

Figure 1. Pyrvinium inhibits proliferation and induces apoptosis in ovarian cancer cell lines. (**A**) Representative flow cytometry dot plots showing the percentage of annexin V and PI staining. Pyrvinium significantly induces apoptosis (**B**) and inhibits proliferation (**C**) of SK-OV-3 and A2780/PTX cells in a dose-dependent manner. Cell were treated with pyrvinium for 3 days prior to proliferation and apoptosis analysis. These data are derived from 3 independent experiments. * p<0.05, compared to control.

β-catenin overexpression) approaches. Ovarian cells treated with LiCl (to inhibit β-catenin degradation and activate Wnt signaling) or transfected with β-catenin overexpression plasmid showed increased β-catenin levels compared to control (Figure 4A). We found that in both SK-OV-3 and A2780/PTX cells treated with lithium or overexpressing β-catenin, pyrvinium was ineffective in inhibiting growth and inducing cell death (Figures 4, 5), suggesting that β-catenin is essential for the action of pyrvinium in ovarian cancer cells.

Pyrvinium inhibits ovarian tumor growth and enhances the effect of paclitaxel *in vivo*

We further evaluated the translational potential of pyrvinium using 3 independent xenograft ovarian cancer mouse models, and investigated the *in vivo* efficacy of pyrvinium alone and its combination with paclitaxel. The xenograft ovarian cancer mouse model was derived from SK-OV-3, A2780/PTX, or A2780cis cells. A2780/PTX is paclitaxel-resistant but

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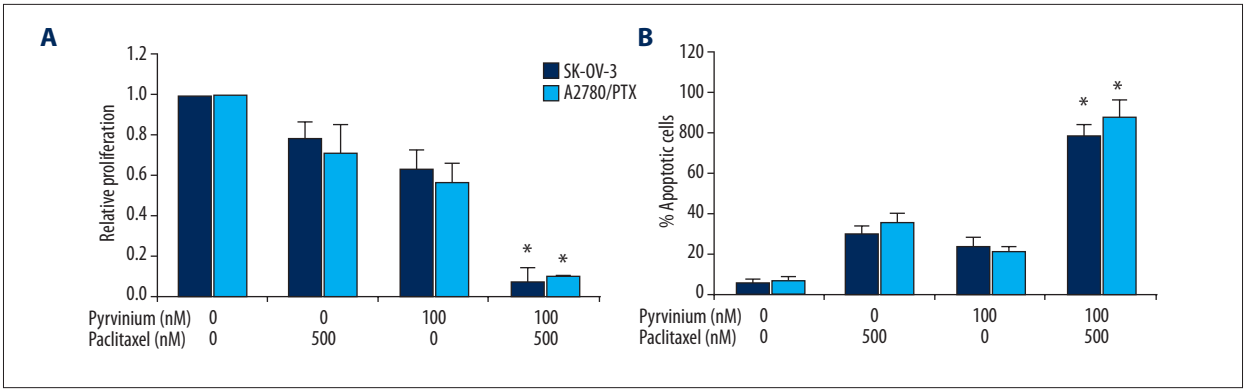

Figure 2. Pyrvinium acts synergistically with paclitaxel in ovarian cancer cell lines. The combination of pyrvinium and paclitaxel inhibits much more proliferation (**A**) and induces much more apoptosis (**B**) than a single drug alone in SK-OV-3 and A2780/PTX cells. These data are derived from 3 independent experiments. The concentrations of pyrvinium and paclitaxel used in combination studies are 0.1 μM and 0.5 μM, respectively * p<0.05, compared to single-arm treatment.

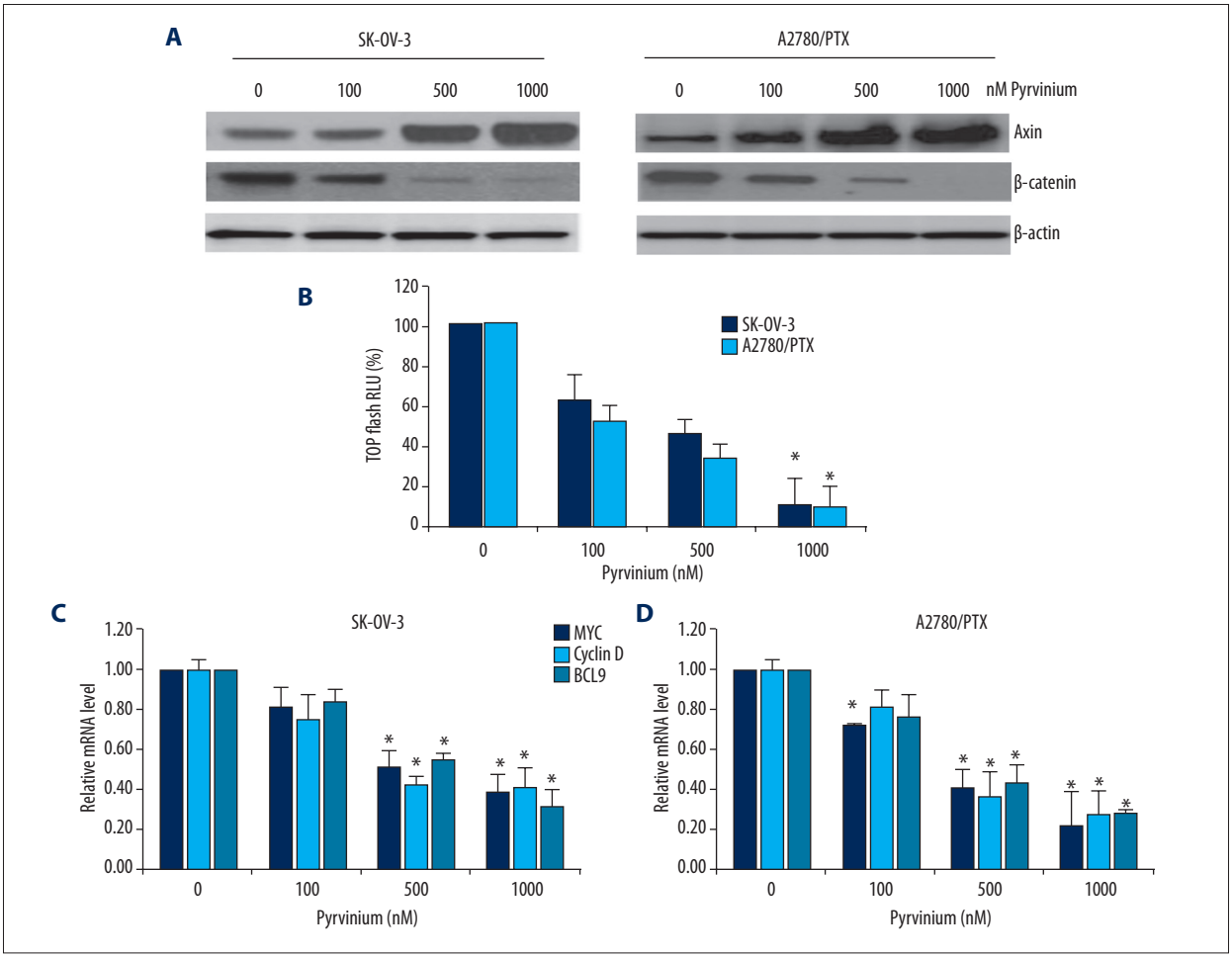

Figure 3. Pyrvinium inhibits Wnt/β-catenin signaling in ovarian cancer cells. (**A**) Pyrvinium increases axin and decreases β-catenin levels in ovarian cancer cell lines. SK-OV-3 and A2780/PTX cells were treated with pyrvinium for 24 h. Cell lysates were immunoblotted for axin, β-catenin, and actin (loading control). (**B**) Pyrvinium inhibits TOPflash activation in ovarian cells. Cells transfected with TOPflash plasmid were treated as indicated. Results shown are relative to control. RLU, relative light units. Pyrvinium dose-dependently decreases mRNA levels of MYC, cyclin D, and BCL9 in SK-OV-3 (**C**) and A2780/PTX (**D**) cells. * p<0.05, compared to control.

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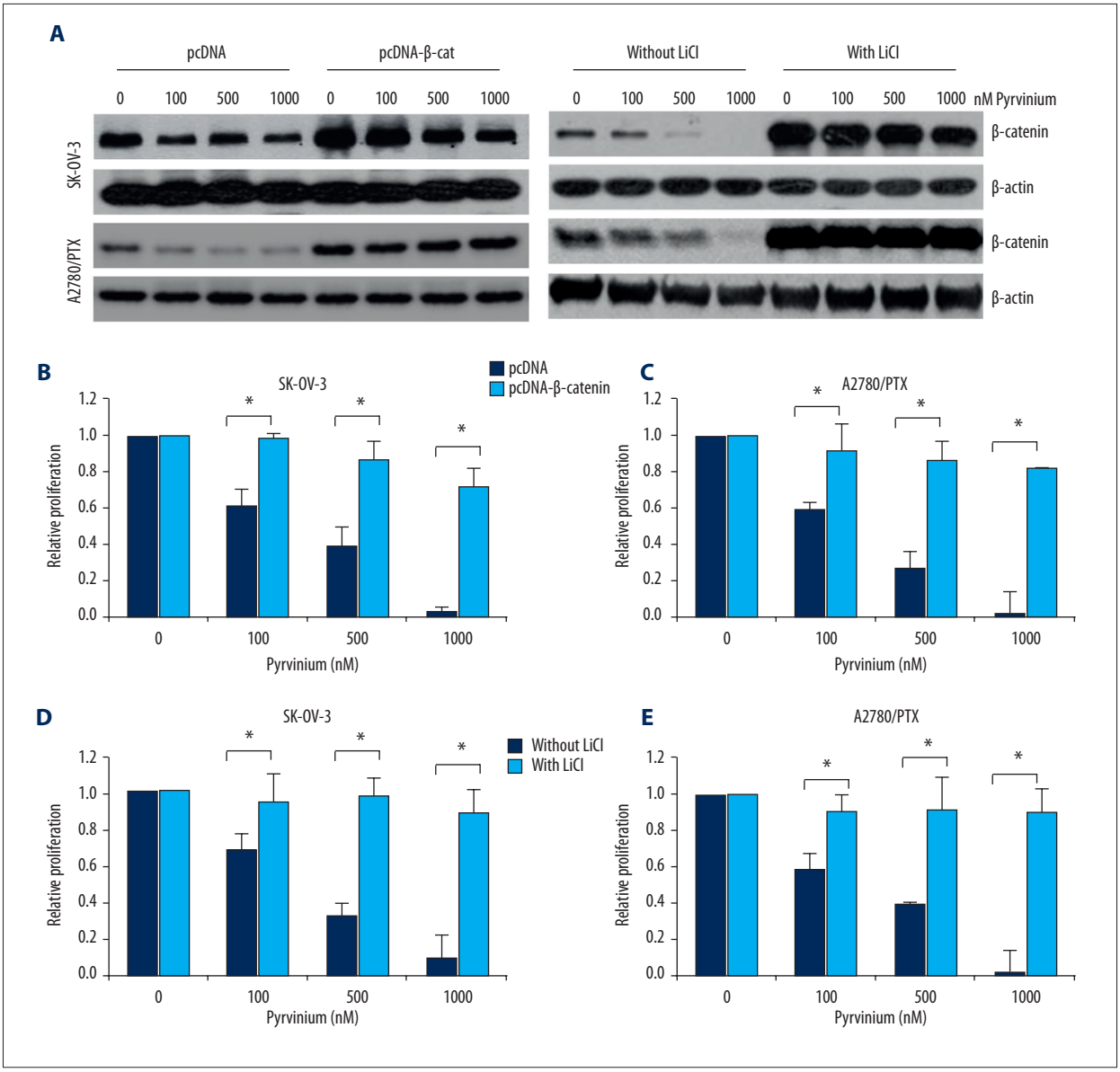

Figure 4. The inhibitory effects of pyrvinium are abolished by β-catenin overexpression in ovarian cancer cells. (**A**) β-catenin levels are increased in ovarian cancer cells exposed to 1 mM LiCl or transfected with β-catenin overexpression plasmid in the presence or absence of pyrvinium. The inhibitory effects of pyrvinium on proliferation (**B, C**) or survival (**D, E**) were abolished by β-catenin overexpression. Cells were electroporated with 2 μg pcDNA or pcDNA-β-cat and cultured for 24 h prior to WB, MTS, apoptosis assays. * p<0.05, compared to wild-type cells.

A2780cis is cisplatin-resistant. Both of them have been developed by chronic exposure of A2780 cell line to paclitaxel or cisplatin [15,19]. When a palpable (~200 mm^3) tumor developed, the mice were given intraperitoneal pyrvinium, oral paclitaxel, or a combination of both for 3 weeks. We did not observe any significant toxicity in any drug-treated mice (data not shown). We found that pyrvinium alone inhibited SK-OV-3 or A2780/PTX and A2780cis tumor growth (Figure 6A, 6B, and Supplementary Figure 2A). It was noted that the combination of pyrvinium and paclitaxel resulted in much more inhibition of tumor growth than a single drug alone (Figure 6A, 6B and Supplementary Figure 2A). Importantly, β-catenin levels were decreased in tumors derived from pyrvinium-treated mice (Figure 6C and Supplementary Figure 2B), demonstrating that pyrvinium inhibited Wnt/β-catenin *in vivo*. These results are consistent with our *in vitro* data, and confirm the inhibitory effects of pyrvinium in ovarian cancer.

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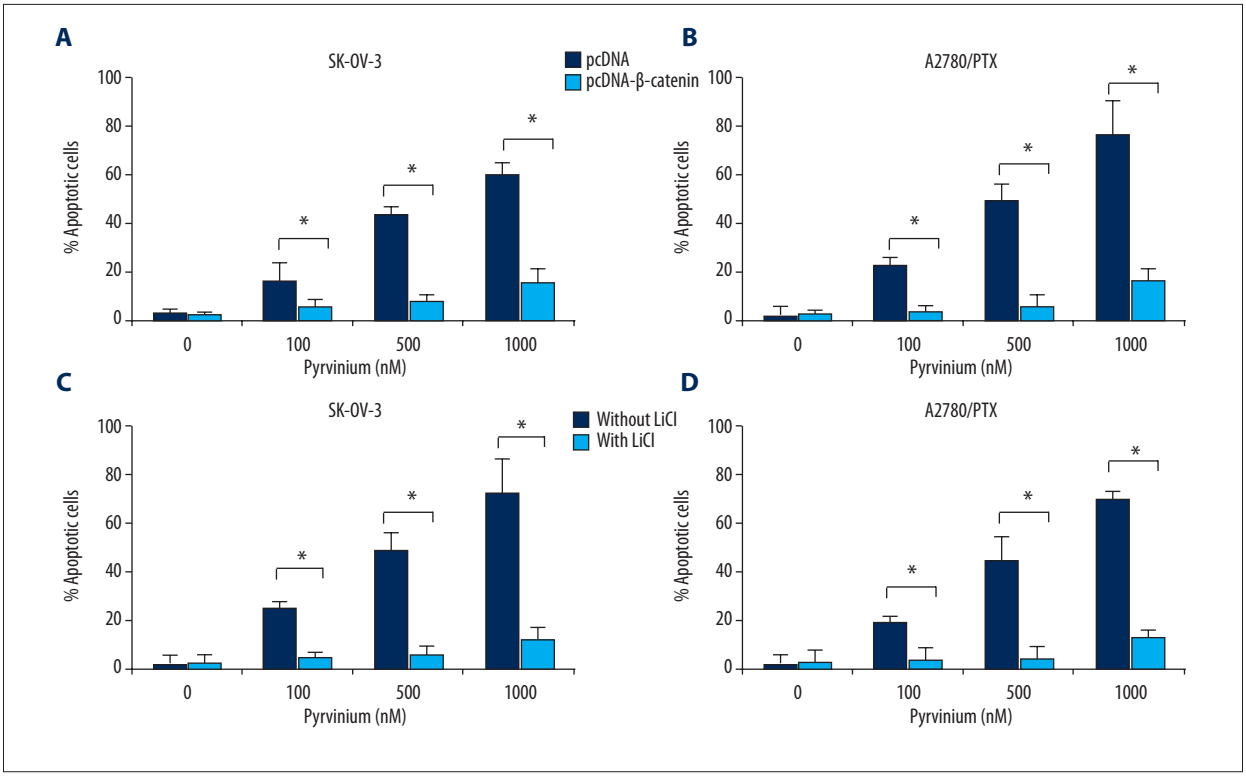

Figure 5. The inhibitory effects of pyrvinium are abolished by LiCl in ovarian cancer cells. Pyrvinium is significantly less effective in inhibiting proliferation of SK-OV-3 (**A**) and A2778/PTX (**B**) cells. Pyrvinium is significantly less effective in inducing apoptosis of SK-OV-3 (**C**) and A27780/PTX (**D**) cells. One mM LiCl and pyrvinium at different concentrations were added in ovarian cancer cells for 3 days prior to proliferation and apoptosis analysis. Results shown are relative to control. * p<0.05, compared to wild-type cells.

Discussion

The Wnt/β-catenin pathway has been shown to play essential roles in ovarian cancer initiation, progression, and chemoresistance [5,17,20]. Targeting the Wnt/β-catenin pathway by Wnt inhibitors or other chemical compounds is effective in inhibiting ovarian cancer cells [21,22]. Here, we report that pyrvinium, an FDA-approved anti-parasitic drug that has been identified as a novel Wnt inhibitor [6,23], is an attractive candidate for ovarian cancer treatment. We showed that pyrvinium potently targeted ovarian cancer cells *in vitro* and *in vivo*, and acted synergistically with the chemotherapy drug paclitaxel. Pyrvinium has a potential advantage over other Wnt inhibitors due to its known pharmacokinetics and toxicity, which facilitates the rapid translation of our data into clinic practice.

Our study analyzed 2 different ovarian cancer cell lines that differ in patterns of oncogenic pathway activation and mechanisms of resistance. SK-OV-3 is resistant to hormone therapy (e.g., anti-estrogen) [14] and A2780/PTX is resistant to chemotherapy (e.g., paclitaxel) [15]. Pyrvinium significantly induced apoptosis and inhibited proliferation of both SK-OV-3 and A2780/PTX (Figure 1), suggesting that pyrvinium targets ovarian cancer regardless of the mechanisms of resistance and patterns of driving molecular pathways. The IC_{50} of pyrvinium was 0.3–0.5 μM (Figure 1), which shows the potent anti-proliferative and pro-apoptotic effects of pyrvinium. The potent efficacy of pyrvinium in ovarian cancer was further demonstrated by the xenograft mouse model, in which pyrvinium at 0.5 mg/kg arrested *in vivo* tumor growth (Figure 6A). Our data add to the recent studies that support anti-cancer activities of pyrvinium [8,9,11,24,25]. Our work also adds ovarian cancer to the growing list of pyrvinium-targeted cancer types [11].

Importantly, the pyrvinium and paclitaxel combination is synergistic in inhibiting cultured ovarian cancer cells as well as ovarian cancer xenograft (Figures 2, 6 and Supplementary Figure 1). Our study results agree with and support previous work showing that combination therapy of pyrvinium with doxorubicin is significantly more effective than monotherapy in prostate cancer [10]. The synergy shown by the combination of pyrvinium with chemotherapy drugs suggests that pyrvinium is a potential candidate for cancer treatment.

A significant finding of this study is that the molecular mechanisms of action of pyrvinium on ovarian cancer are through

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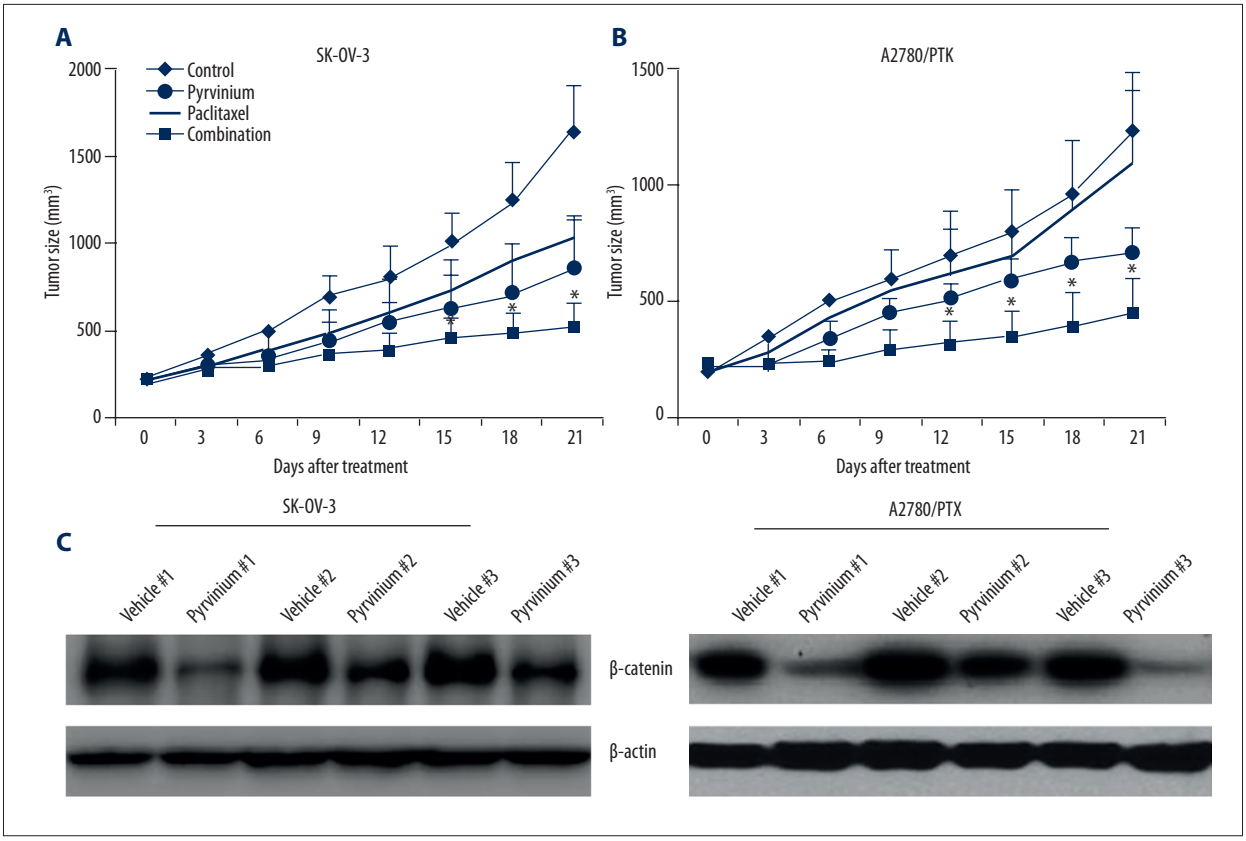

Figure 6. Pyrvinium enhances the inhibitory effects of paclitaxel *in vivo*. Pyrvinium and paclitaxel inhibits ovarian tumor growth derived from SK-OV-3 (**A**) or A2780/PTX (**B**) cells. The combination of pyrvinium and paclitaxel synergistically arrests growth of ovarian cancer xenograft. Mice were treated with equal volume of vehicles, intraperitoneal pyrvinium at 0.5 mg/kg, and oral paclitaxel at 10 mg/kg or combination of both. (**C**) Representative Western blotting photos of cellular β-catenin in tumors are shown. * p<0.05, compared to single-arm treatment.

inhibition of the Wnt/β-catenin pathway, which is in agreement with previous reports [6,23]. We showed that pyrvinium decreased β-catenin levels and inhibited Wnt/β-catenin-mediated transcription via increasing axin in ovarian cancer cells (Figure 3). β-catenin stabilization or overexpression reversed the inhibitory effects of pyrvinium (Figures 4, 5), confirming Wnt/β-catenin as the target for pyrvinium in ovarian cancer cells. Our data support the essential roles of Wnt/β-catenin in ovarian cancer and are in line with previous work showing that targeting Wnt/β-catenin is an alternative therapeutic strategy in ovarian cancer treatment. Interestingly, recent studies revealed that 2 other anthelminthic/antibiotic drugs, niclosamide and tigecycline, also target Wnt/β-catenin in cancer cells [26,27].

Conclusions

We are the first to report that pyrvinium is effective against ovarian cancer cells *in vitro* and *in vivo*. Our findings demonstrate that inhibition of the Wnt/β-catenin pathway is the mechanism of action of pyrvinium. Importantly, our data demonstrate that the combination of pyrvinium and paclitaxel was synergistic in inhibiting ovarian cancer. Our work emphasizes the potential therapeutic utility of targeting Wnt/β-catenin in human ovarian cancer treatment and suggests that pyrvinium can be added to the list of options for treatment of ovarian cancer.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgement

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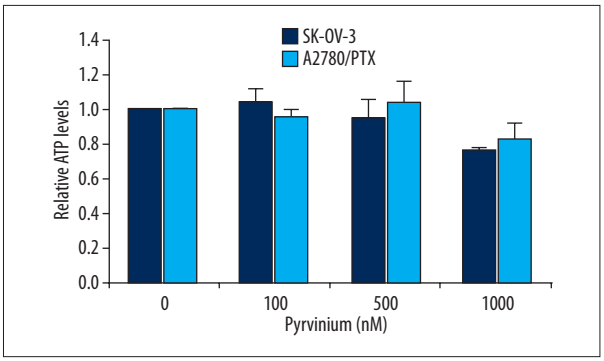
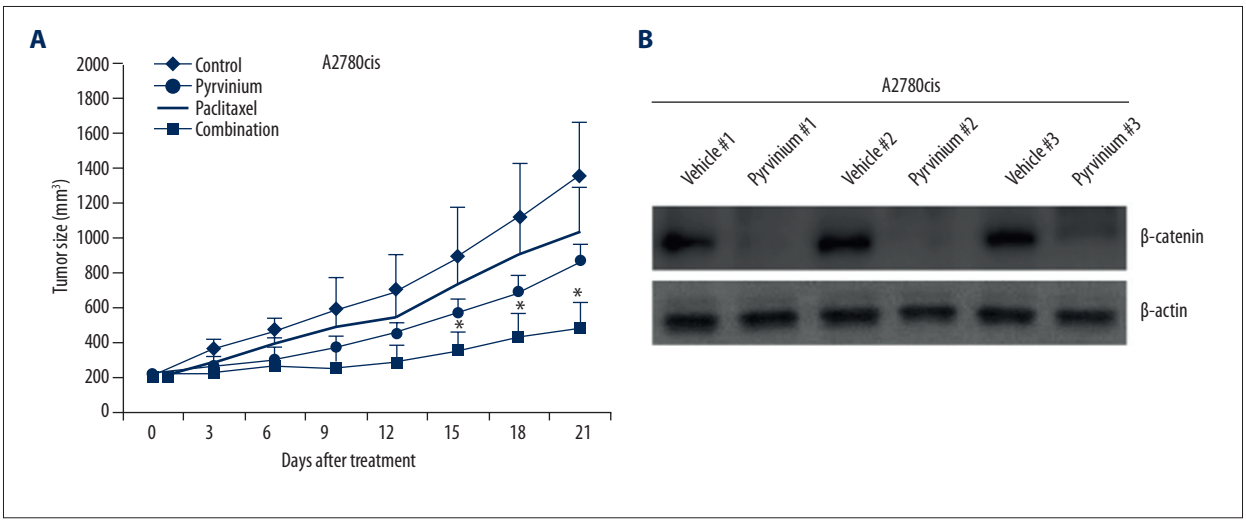
Supplementary Figures



Supplementary Figure 1. The effect of pyrvinium on ATP production in ovarian cancer cells. ATP levels were measured by CellTiter-Glo Luminiescent Cell Viability Assay (Promega, WI, US) according to the manufacturer's instructions.



Supplementary Figure 2. Pyrvinium enhances the inhibitory effects of paclitaxel *in vivo*. (**A**) Pyrvinium and paclitaxel inhibits ovarian tumor growth derived from A2780cis cells. Combination of pyrvinium and paclitaxel synergistically arrests growth of ovarian cancer xenograft. Mice were treated with equal volume of vehicles, intraperitoneal pyrvinium at 0.5 mg/kg and oral paclitaxel at 10 mg/kg or combination of both. (**B**) Representative western blotting photos of cellular β-catenin in tumors are shown. * p<0.05, compared to single arm treatment.

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