Prazosin inhibits the proliferation, migration and invasion, but promotes the apoptosis of U251 and U87 cells via the PI3K/AKT/mTOR signaling pathway

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Abstract. Prazosin, an α-adrenergic receptor antagonist, is used to treat mild to moderate hypertension. It has recently been discovered that α-adrenergic receptors may have potential antitumor properties. Therefore, in the present study, the effect of prazosin on human glioblastoma and the underlying mechanism were investigated. Human glioblastoma U251 and U87 cells were treated with different concentrations of prazosin, and a Cell Counting Kit-8 assay was performed to investigate the effects of prazosin on cell proliferation. Transwell migration and invasion assays were used to assess the effects of prazosin on cell migration and invasion. Prazosin-induced apoptosis in U251 and U87 cells was detected by flow cytometry, and the protein expression levels of anti-apoptotic proteins and proteins related to the PI3K/AKT/mTOR signaling pathway were detected by western blotting. The results suggested that following treatment with prazosin, the proliferation, migration and invasion of U251 and U81 cells were decreased. By contrast, U251 and U81 cell apoptosis, as well as the protein expression levels of Bax and active Caspase-3 were increased after prazosin treatment ($P<0.05$). Bcl-2 levels were also decreased after prazosin treatment ($P<0.05$). Additionally, the expression of phosphorylated (p)-AKT and p-mTOR, P70 and cyclin D1 were decreased in U251 and U81 cells following prazosin treatment ($P<0.05$). The present study suggested that prazosin may suppress glioblastoma progression by downregulating the activity of the PI3K/AKT/mTOR signaling pathway.

Introduction

Among the most common primary malignant tumors of the brain, human glioblastoma accounts for ~40% of intracranial tumors (1). The main clinical features of glioblastoma include high malignancy, a high relapse rate and poor prognosis (2). Local abnormal proliferation and infiltration of human glioblastoma cells in the brain is the main cause of death in patients, and at present, there are no safe and effective treatments for the disease (3). Despite significant advances in treatment strategies, including surgical resection, radiotherapy and chemotherapy, the overall 5-year survival rate for patients with glioblastoma remains poor (~10%) (4). Currently, there are only two drugs clinically used for the standardized treatment of human glioblastoma, temozolomide and carmustine; however, both drugs display serious side effects (5). Therefore, identifying novel therapeutics for human glioblastoma is essential.

As a member of the G-protein coupled receptor superfamily, adrenergic receptors play an important role in the sympathetic nervous system (6). There are three subtypes of adrenergic receptors: α1A, α1B and α1D (7). Commonly used adrenergic receptor antagonists include quinazoline-based prazosin, doxazosin and terazosin, as well as the sulfonamide derivative tamsulosin. The aforementioned drugs are not only used to treat essential hypertension (8), but are also used for the treatment of prostate cancer as they inhibit progression, induce apoptosis and reduce prostate specific antigen levels (9,10). Kyprianou *et al* (8) reported for the first time that α-adrenergic antagonists can induce apoptosis in the glandular epithelium and smooth muscle, which are present in benign prostatic hyperplasia (8). Further studies have reported that α-adrenergic antagonists can also induce apoptosis in malignant prostate cancer cells (11-13). Based on the aforementioned studies, further investigation into the effects of α1-adrenergic antagonists on other human malignancies, including mesothelioma, as well as breast and bladder cancer, has been conducted (14-16). These further studies have reported that α-adrenergic antagonists have a significant proapoptotic effect on malignant tumors. Doxazosin is an α-adrenergic receptor blocker that inhibits tumor growth and angiogenesis (17,18). Prazosin is also an α-adrenergic antagonist used to treat essential hypertension and although the effects of prazosin on human glioblastoma have been reported, the underlying mechanism remains unclear (19). In the present study, the potential antitumor effects of prazosin in glioblastoma cells

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were investigated. Referring to recently published studies investigating the mechanism underlying glioblastoma progression, U251 and U87 cell lines were used in the present study (20,21). Furthermore, the PI3K/AKT signaling pathway was investigated as a potential molecular mechanism underlying the effects of prazosin on glioblastoma cells.

Materials and methods

Cell lines and cell culture. Human malignant glioblastoma cell lines U251MG (astrocytoma) and U87 (glioblastoma of unknown origin) were purchased from Nianjing KeyGen Biotech Co., Ltd. U87 cells were authenticated by a short tandem repeat profiling method using the PowerPlex 18D system kit (cat. no. DC1802; Promega Corporation) and an ABI 3500 Genetic analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Cells were cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Beijing Solarbio Science & Technology, Co., Ltd.) at 37˚C with 5% CO_2.

Drug sensitivity assays. U251 and U87 cells were seeded ($1x10^3$ cells/well) into 96-well microtiter plates containing 100 μl culture medium. Prazosin was dissolved in DMSO to 10 different concentrations: 0, 2.5, 5, 7.5, 10, 15, 20, 30, 40 and 50 μM. Different concentrations of prazosin were added to each well and incubated for 48 h at 37˚C. Subsequently, 10 μl Cell Counting Kit-8 (CCK-8) reagent (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well and the plates were incubated for 1.5 h at 37˚C. The optical density value of each well was detected at a wavelength of 450 nm using a microplate reader and a dose-response curve was plotted. The IC_{50} concentration of prazosin was calculated using GraphPad Prism software (version 7; GraphPad Software, Inc.). Each drug concentration was tested 3 times.

CCK-8 assay for cell proliferation. U251 and U87 cells were seeded ($1x10^3$ cells/well) into 96-well plates and cultured for 24 h at 37˚C with 5% CO_2. U251 and U87 cells were treated with 13.16 and 11.57 μM prazosin, respectively. Cells were incubated for 24, 48 or 72 h 37˚C. Subsequently, 10 μl CCK-8 solution was added to each well and the plates were incubated for 1.5 h at 37˚C. The absorbance was measured at a wavelength of 450 nm using a microplate reader.

Cell invasion and migration assays. The upper chamber of the Transwell plate was precoated with Matrigel® for 30 min at 70˚C. Subsequently, the Transwell membrane was hydrated with serum-free medium containing 10 g/l bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. Prior to plating, $1x10^5$ U251 or U87 cells were serum-starved for 12-24 h at 37˚C. Subsequently, cells were detached by trypsinization and resuspended in serum-free medium containing 10 g/l BSA. Cell invasion was evaluated using Transwell invasion assays. Subsequently, cells treated with prazosin for 24 h at 37˚C ($1x10^5$) were seeded into the upper chambers. Medium containing 10% FBS (500 μl) was plated in the lower chamber of the Transwell plates. Following incubation at 37˚C for 24 h, the invading cells were fixed with 4% paraformaldehyde for 10 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature. Stained cells were counted in five randomly-selected fields using a light microscope (magnification, x200).

The Transwell migration assay followed the same protocol as the Transwell invasion assay, however, the Transwell membranes were not precoated with Matrigel and $5x10^3$ cells were plated in the upper chamber of the Transwell plates.

Cell apoptosis assay. Following treatment with prazosin for 24 h, U251 and U87 cells were resuspended in binding buffer ($5x10^6$ cells/ml; CoWin Biosciences). Cells were stained with 5 μl annexin V-FITC and 10 μl propidium iodide (CoWin Biosciences) in the dark at room temperature for 5 min. Subsequently, the cell suspensions were centrifuged for 5 min at 4˚C and a speed of 447 x g, the supernatant was discarded and the pellets were resuspended in 400 μl PBS. Apoptotic cells were analyzed using a flow cytometer (BD Biosciences) and FlowJo software 7.6.1 (FlowJo, LLC).

Colony formation assay. U251 and U87 cells in the logarithmic growth phase were trypsinized, seeded ($2x10^2$ cells/dish) into 35 mm cell culture dishes and gently rotated to uniformly disperse the cells. Cells were suspended in DMEM containing 10% FBS. Subsequently, complete medium containing prazosin (IC_{50} concentration: U251, 13.16 μM; U87, 11.57 μM) was added to the experimental group and the same concentration of DMSO was added to the control group. The cells were cultured for 2-3 weeks at 37˚C. When macroscopic clones appeared in the culture dish, the medium was discarded and the cells were carefully washed three times with PBS. The cells were fixed with 4% paraformaldehyde for 20 min at room temperature and stained with crystal violet for 30 min at room temperature. Colony number was counted in 5 random fields of view using a light microscope (magnification, x40).

Western blot analysis. U251 and U87 cells were treated with prazosin for 48 h at 37˚C. Total protein was extracted from the cells using RIPA buffer (CoWin Biosciences). Total protein was quantified using a bicinchoninic acid assay. Protein (20 mg) was separated by 10% SDS-PAGE and transferred to PVDF membranes. Subsequently, the membranes were blocked with 5% fat-free milk for 1 h at room temperature. The membranes were incubated overnight at 4˚C with primary antibodies targeted against the following: AKT (cat. no. ab18785; 1:2,000; Abcam), phosphorylated (p)-AKT (cat. no. ab38449; 1:1,000; Abcam), mTOR (cat. no. ab2732; 1:1,000; Abcam), p-mTOR (cat. no. ab109268; 1:1,000; Abcam), Bcl-2 (cat. no. 12789-1-AP; 1:1,000; ProteinTech Group, Ltd.), Bax (cat. no. 50599-2-Ig; 1:1,000; ProteinTech Group, Ltd.), Caspase-3 (cat. no. ab32351; 1:1,000; Abcam), cyclin D1 (cat. no. ab134175; 1:1,000; Abcam), P70 (cat. no. ab184551, 1:1,000; Abcam) CDK4 (cat. no. ab108357; 1:1,000; Abcam), CDK6 (cat. no. ab124821; 1:1,000; Abcam), NUSAP1 (cat. no. ab169083; 1:500; Abcam) and GAPDH (cat. no. 10494-1-AP; 1:5,000; ProteinTech Group, Ltd.). Subsequently, the membranes were incubated with an anti-Rabbit secondary antibody (cat. no. ab6721; 1:5,000; Abcam) for 1 h at room temperature. Protein bands were visualized by ECL (CoWin Biosciences). Protein expression was quantified using Quantity One 4.6.6 (Bio-Rad Laboratories,

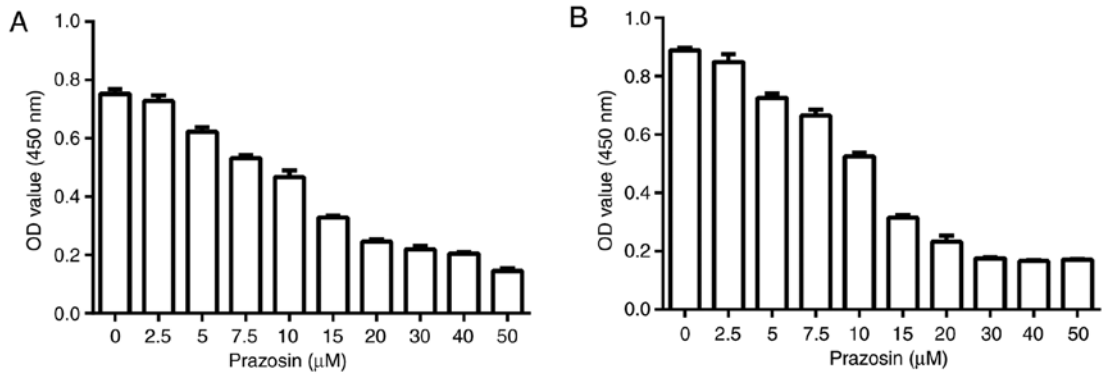

Figure 1. Dose-response curve of prazosin (0-50 μM). (A) U251 and (B) U87 cells. Each drug concentration was tested three times. OD, optical density.

Inc.) and Image J 1.41 (National Institutes of Health) software with GAPDH as the loading control.

Statistical analysis. Statistical analyses were performed using SPSS software (version 18.0; SPSS, Inc.). Data are presented as the mean ± SD. Differences were assessed using an unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

Dose-response experiment to determine the IC_{50} of prazosin. A dose-response experiment was conducted to investigate whether prazosin inhibited the proliferation of U87 and U251 cells. The IC_{50} of prazosin for glioblastoma cells was also determined using a CCK-8 assay. The IC_{50} was 13.16±0.95 and 11.57±0.79 μM prazosin for U251 and U87 cells, respectively (Fig. 1).

Prazosin inhibits U251 and U87 cell proliferation. To investigate the potential antitumor effects of prazosin, the proliferation of U251 and U87 cells treated with 13.16 and 11.57 μM prazosin, respectively, for 24, 48 or 72 h was assessed. The results suggested that prazosin significantly decreased the proliferation of U251 and U87 cells after treatment for 48 and 72 h (Fig. 2A and B).

Furthermore, the colony formation assay suggested that prazosin-treated U251 and U87 cells displayed significantly decreased colony formation compared with the NC group (58±3 vs. 149±8 and 50±2 vs. 126±6, respectively; Fig. 2C). The colony number of prazosin-treated U251 and U87 cells displayed a similar trend to colony formation (11.6 vs. 29.8 and 10 vs. 25.2%, respectively; P<0.05; Fig. 2C). Therefore, the results suggested that prazosin effectively inhibited the proliferation of U251 and U87 cells.

Prazosin inhibits U251 and U87 cell invasion and migration. The effect of prazosin on the migration and invasion of U251 and U87 cells was detected using Transwell assays. The migratory ability of U251 and U87 cells was significantly decreased following prazosin treatment compared with the negative control cells (282±14 vs. 67±6 and 202±11 vs. 42±5, respectively; P<0.05; Fig. 3). The invasive ability of U251 and U87 cells decreased significantly following prazosin treatment compared with the negative control cells (153±9 vs. 34±3 and 163±8 vs. 31±3; P<0.05; Fig. 3A). The results indicated that prazosin inhibited the migration and invasion of U251 and U87 cells.

Prazosin suppresses the PI3K/AKT/mTOR signaling pathway in U251 and U87 cells. The PI3K/AKT/mTOR signaling pathway plays a key role in the regulation of cell proliferation (22). To investigate the effects of prazosin on the PI3K/AKT/mTOR signaling pathway in glioblastoma, U251 and U87 cells were treated with prazosin and protein expression levels were determined by western blotting. Following prazosin treatment, the expression of p-AKT and p-mTOR was significantly reduced in U251 and U87 cells compared with the negative control cells (P<0.05; Fig. 4A). Furthermore, the expression levels of P70 and cyclin D1, which are downstream target genes of the PI3K/AKT/mTOR signaling pathway (23), were decreased in prazosin-treated cells compared with negative control cells (P<0.05; Fig. 4B). The results suggested that prazosin treatment decreased the protein expression of components of the PI3K/AKT/mTOR signaling pathway in U251 and U87 cells.

Based on the result that cyclin D1 expression was reduced by prazosin treatment, the expression levels of cell cycle related genes, cyclin dependent kinase (CDK)4/6 and nucleolar and spindle associated protein 1 (NUSAP1), were measured to investigate whether prazosin affected the cell cycle. The expression levels of CDK4/6 and NUSAP1 were significantly decreased in the prazosin treatment groups compared with the NC groups (P<0.05; Fig. 4B). The results suggested that prazosin may inhibit the proliferation of U251 and U87 cells by blocking the cell cycle.

Prazosin induces apoptosis in U251 and U87 cells. The effect of prazosin on U251 and U87 cell apoptosis was detected by flow cytometry. The percentage of apoptotic U251 and U87 cells was significantly increased in the prazosin treatment groups compared with the NC groups (9.28±0.89 vs. 1.96±0.23 and 12.46±1.07 vs. 4.20±0.56%, respectively; Fig. 5A). Western blot analysis indicated that the expression of the antiapoptotic protein Bcl2 was decreased, and the expression of the proapoptotic proteins Bax and active Caspase-3 was increased in the prazosin treatment groups compared with the NC

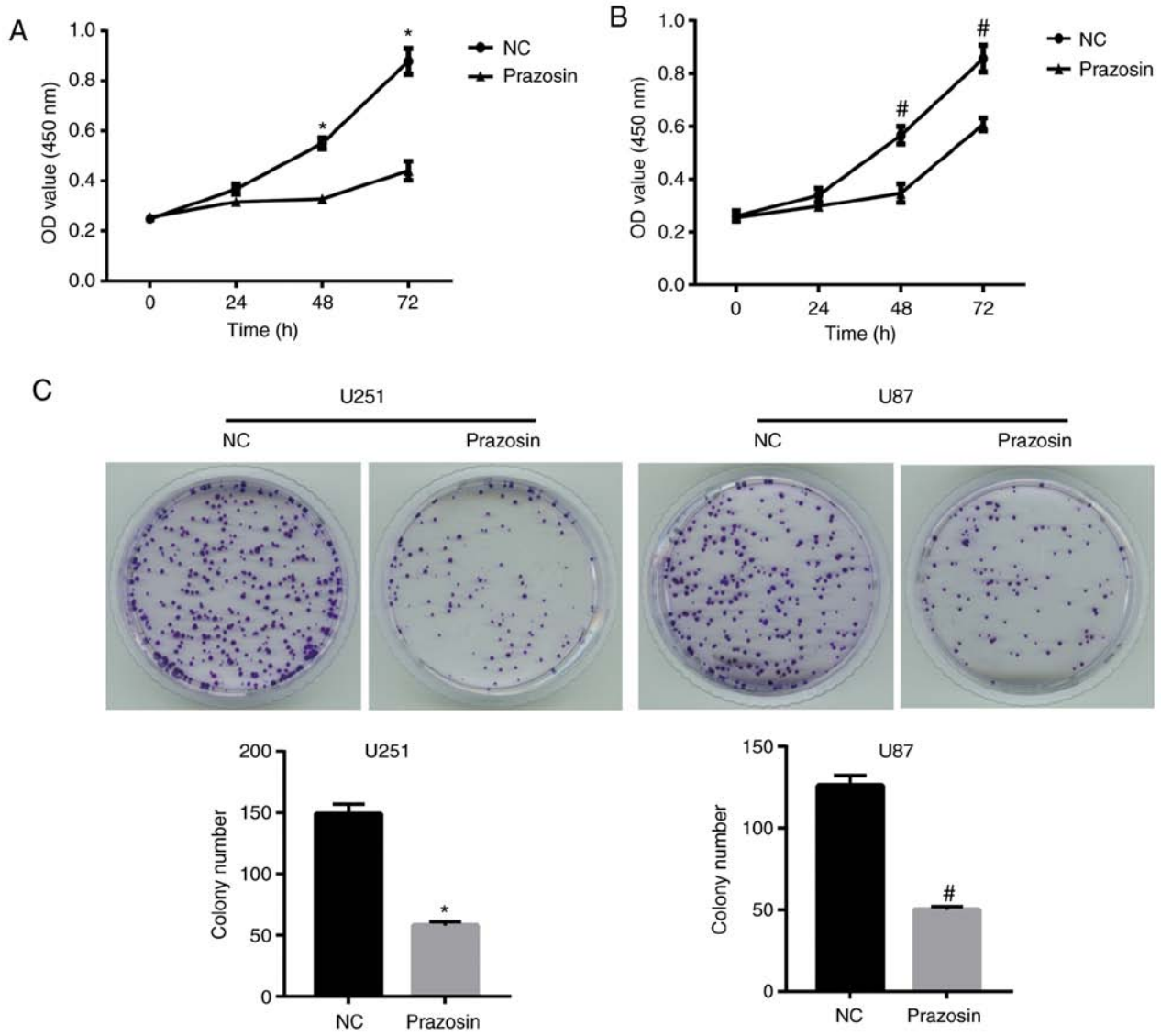

Figure 2. Effect of prazosin on the proliferation of U251 and U87 cells. (A) U251 and (B) U87 cell proliferation, as determined by a Cell Counting Kit-8 assay. (C) Prazosin reduced the colony forming ability of U251 and U87 cells. *P<0.05 vs. respective U251 NC. #P<0.05 vs. respective U87 NC. NC, negative control; OD, optical density.

groups (Fig. 5B). The results suggested that prazosin induced apoptosis in U251 and U87 cells, indicating that the antitumor activity of prazosin may be related to apoptosis induction.

Discussion

To the best of our knowledge, the present study suggested for the first time that prazosin inhibits the proliferation, migration and invasion of U251 and U81 cells via the PI3K/AKT/mTOR signaling pathway.

Glioblastoma is one of the most common adult malignant brain tumors worldwide, with a median survival time of 13-15 months (24). At present, there are no safe and effective treatments for the disease, and the standard treatment involves a combination of surgery, radiotherapy and chemotherapy (25). However, the standard therapeutic strategies display a number of problems and limitations: i) Glioblastoma invades the adjacent brain parenchyma, which makes it difficult to completely remove the tumor (26); ii) orthotropic tumors grow malignantly, which causes surrounding tumors to grow and leads to brain tissue edema, which is one of the major causes of the high mortality rate observed in patients with glioblastoma (27); iii) the maximum dose of radiation does not completely eradicate tumor cells (27); iv) movement, language and cognitive deficits can occur following surgery, resulting in poor patient quality of life and increased mortality (28,29); and v) the anatomy of the human brain, for example, the blood-brain barrier, limits the efficacy of cancer therapeutics (30). Therefore, there is an urgent requirement for the identification of novel therapeutic targets for glioblastoma.

Previous studies have reported that the activation of α1-adrenergic receptors increases the proliferation of nerve cells, vascular smooth muscle cells and vascular endothelial cells during development (31-35). Furthermore, it has been reported that α1-adrenergic receptors play a crucial role in embryonic brain development (36). In the present study, U251 and U87 cells were treated with prazosin to investigate the

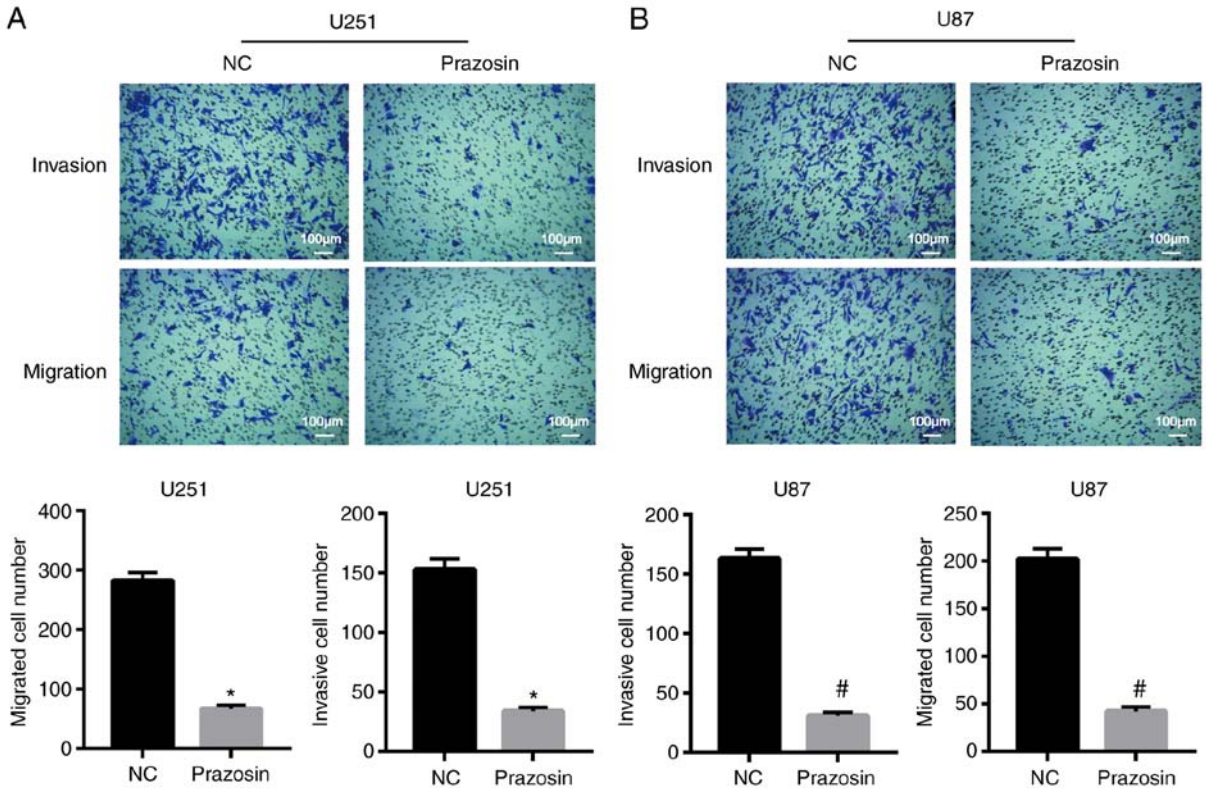

Figure 3. Transwell assays were performed to investigate the effect of prazosin on U251 and U87 cell invasion and migration. (A) U251 and (B) U87 cells. *P<0.05 vs. U251 NC. #P<0.05 vs. U87 NC. NC, negative control.

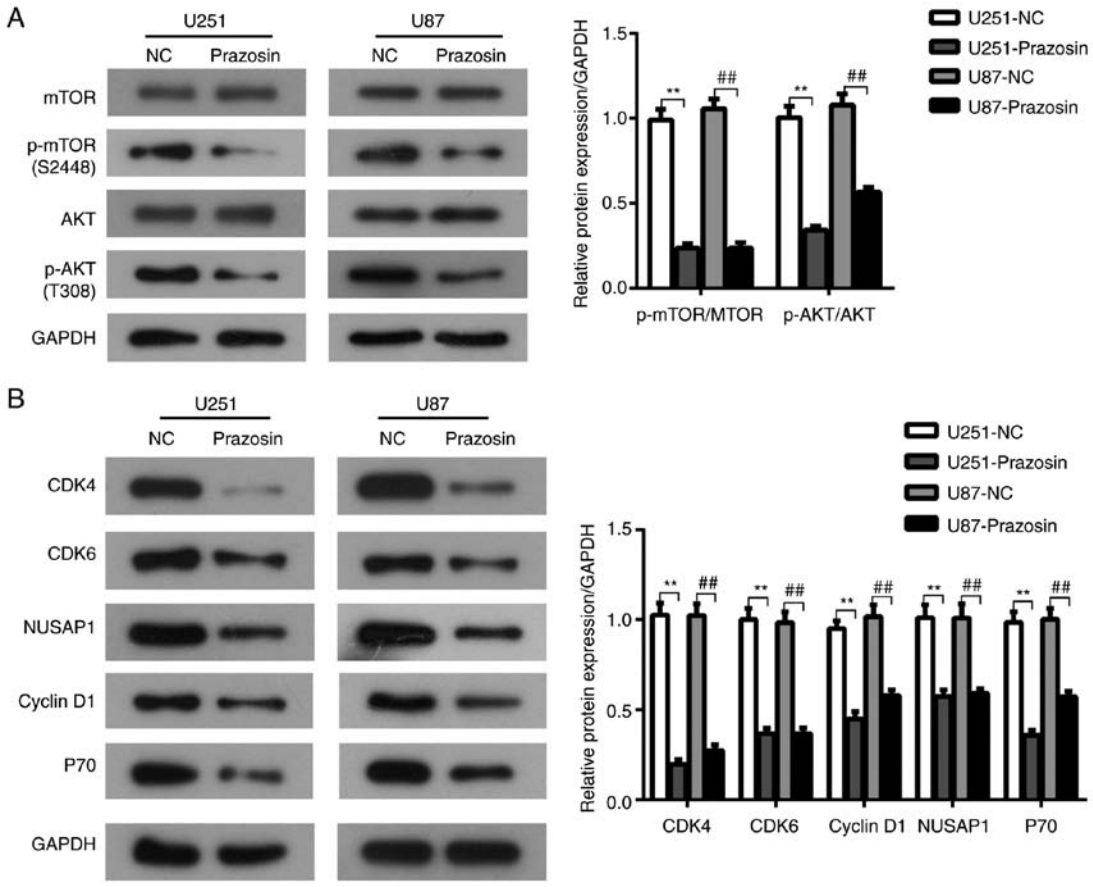

Figure 4. Effect of prazosin on the PI3K/AKT/mTOR signaling pathway and the cell cycle. Expression levels of proteins associated with (A) the PI3K/AKT/mTOR signaling pathway and (B) the cell cycle were determined by western blotting and quantified. **P<0.01 vs. U251 NC. ##P<0.01 vs. U87 NC. NC, negative control; CDK, cyclin dependent kinase; NUSAP1, nucleolar and spindle associated protein 1; p, phosphorylated.

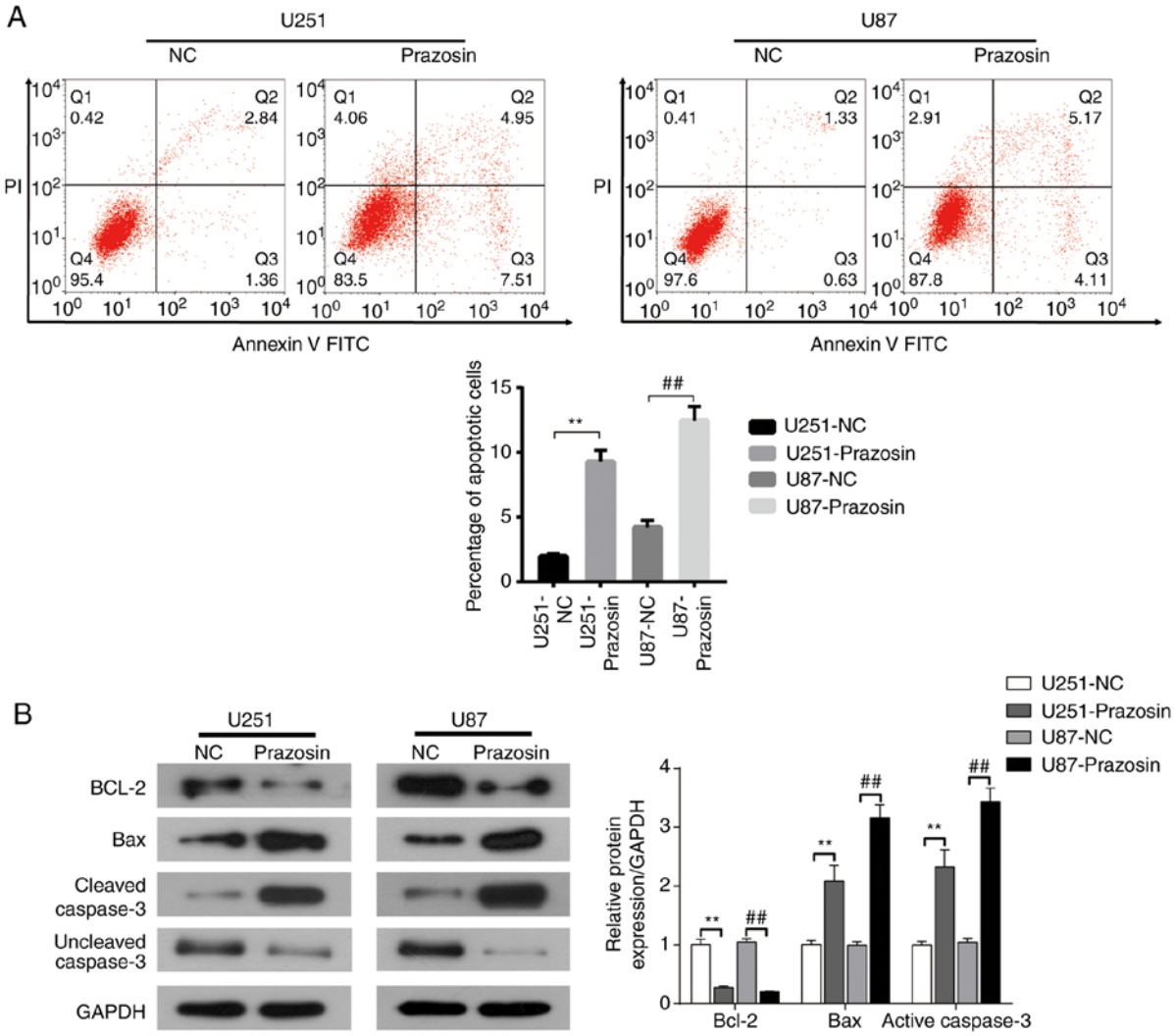

Figure 5. Effect of prazosin on U251 and U87 cell apoptosis. (A) Apoptotic cells were detected by flow cytometry. (B) Prazosin altered the expression of apoptosis related genes in U251 and U87 cells. **P<0.01 vs. U251 NC. $^{\#\#}$P<0.01 vs. U87 NC. NC, negative control; PI, propidium iodide.

effect of the drug on human glioblastoma *in vitro*. Prazosin inhibited the proliferation, migration and invasion of U251 and U87 cells, as indicated by CCK-8, colony formation and Transwell assays. However, a limitation of the present study is that the Transwell assay detects the 3D migration of cells, whereas the wound healing assay, which detects the horizontal migration of cells, was not performed in the present study.

The molecular mechanism underlying the anticancer activity of prazosin on human glioblastoma cells was also investigated. The PI3K/AKT/mTOR signaling pathway plays a crucial role in tumor cell proliferation, migration, invasion and apoptosis (22). Numerous studies have reported that the PI3K/AKT/mTOR signaling pathway is abnormally activated during human glioblastoma (23,37). mTOR is a downstream molecule in the PI3K/AKT signaling pathway that plays a key role in the activation of P70 and cyclin D1 (38), which are associated with apoptosis (39,40). The results of the present study indicated that the expression levels of p-AKT, p-mTOR, P70 and cyclin D1 were significantly reduced in the prazosin-treated group compared with the control group, suggesting that prazosin inhibited the PI3K/AKT/mTOR signaling pathway in U251 and U87 cells.

Both doxazosin and terazosin are clinically effective α1-adrenergic receptor antagonists that trigger tumor apoptosis (41). A number of previous studies have reported that quinazoline-derived α1-adrenergic receptor antagonists display anti-prostate cancer effects. In addition, doxazosin induces apoptosis and inhibits angiogenesis (11,42-45). The results of the present study suggested that prazosin increased the expression of proapoptotic proteins Bax and Caspase-3, and decreased the expression of the anti-apoptotic protein Bcl-2. The results were consistent with the effect of prazosin on apoptosis, as determined by flow cytometry, which indicated that the percentage of apoptotic cells was increased in the prazosin-treated group compared with the control group.

In conclusion, the present study suggested that prazosin inhibited the proliferation, migration and invasion, and promoted the apoptosis of U251 and U87 cells by inhibiting the PI3K/AKT/mTOR signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JZ and JF designed the experiments. JZ performed the experiments; JZ and JF analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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