Prazosin inhibits the growth and mobility of osteosarcoma cells

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Background: Osteosarcoma is a primary malignant bone tumor that frequently occurs in adolescents and children, its high aggressiveness and rapid metastasis often resulting in poor prognoses. In previous studies, Prazosin has been shown to possess anti-proliferative properties against prostate cancer and glioblastoma cells. In our study, we investigated Prazosin's underlying mechanisms and its effects on the biological behaviors of osteosarcoma cells.

Methods: Osteosarcoma cell lines MG63 and 143B were treated with different concentrations of Prazosin, and a CCK8 assay assessed its effect on cell viability. Colony formation, Transwell and flow cytometry assays were used to examine its effects on cell proliferation, cell migration, and cell invasion and apoptosis, respectively. The expression of relevant proteins was then examined using western blotting.

Results: Our data showed that Prazosin dose-dependently reduced the viability of MG63 and 143B cells and significantly inhibited their clonogenic ability. Moreover, Prazosin attenuated the cell migration and invasion abilities of MG63 and 143B cells when compared with the NC group. It also accelerated cell apoptosis in mitochondrial pathways by regulating Bcl-2/Bax axis and caspase 3. Furthermore, Prazosin treatment inactivated the Akt/mTOR pathway by down-regulating Akt and mTOR phosphorylation (p-Akt, p-mTOR) and the expression of P70 and cyclin D1.

Conclusions: Our data highlights the fact that Prazosin inhibits cell growth, inhibits the motility of osteosarcoma cells, and promotes apoptosis, suggesting that Prazosin is a potential anti-cancer agent in osteosarcoma therapy.

Keywords: Osteosarcoma; Prazosin; Akt/mTOR pathway

Introduction

Osteosarcoma is a primary malignant bone tumor that frequently occurs in adolescents and children, commonly occurring in the femur, tibia and humerus (1-3). Its incidence rate is 3–4/million worldwide, being slightly higher in males than in females (4,5). Due to the high aggressive and rapid metastasis of osteosarcoma, general treatment is severely limited to patients that exhibit metastasis, leading to a poor prognosis of less than 20%

(6,7). However, given the advancement of osteosarcoma treatment technologies in recent years (surgical resection, chemotherapy, and radiotherapy) the survival of patients with osteosarcoma had generally improved, but has more recently stagnated (8). Therefore, it is critical that we find a new anticancer agent that inhibits the metastasis and invasion of osteosarcoma.

Recent research has worked towards this end, searching for new anti-cancer drugs and re-evaluating known drugs that have been used for other diseases, to find their

potential anti-tumor effects (9). Prazosin [1-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-4-(2-furoyl) piperazine], an α1-adrenoceptor antagonist, had generally been used as a sympatholytic drug for the treatment of hypertension and posttraumatic stress disorder (PTSD) (9-11). Recently however, Lin *et al.* demonstrated that Prazosin also displays an anti-proliferative effect in prostate cancer cells; it accomplished this by inducing apoptosis via resulting in DNA damage stress (12). Similarly, Fuchs *et al.* confirm that Prazosin treatment inhibits the growth of medullary thyroid carcinoma (MTC) cells, also inducing apoptosis (9). Further studies have also revealed that Prazosin reduces cell proliferation and increases docetaxel-induced toxicity in prostate cancer cells, by modulating autophagy and apoptosis (13).

However, these studies have generally focused on the anti-proliferative effects of Prazosin without specifically studying whether Prazosin affects tumor metastasis in osteosarcoma. Therefore, in the present study, we have investigated Prazosin's underlying mechanisms and its effects on the biological behaviors of osteosarcoma cells. In brief, we have observed that Prazosin displays a significant depression of the proliferation, migration, and invasion properties of osteosarcoma cell lines MG63 and 143B, and induced apoptosis, suggesting that it may serve as a novel anti-cancer agent.

Methods

Cell culture

Osteosarcoma cell lines MG63 and 143B were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 37 ℃ with 5% CO_2.

CCK8 assay

The MG63 and 143B cells were treated with different concentrations of Prazosin (0, 2.5, 5, 7.5, 10, 15, 20, 30, 40, and 50 µM; MedChemExpress, USA), with Prazosin's effect on cell viability being analyzed by CCK8 assay. After treatment for 24 h, cells were cultured with CCK8 reagent (10 µL/well; Beijing Solarbio Science & Technology, Beijing, China) at 37 ℃ for 90 min with absorbance being measured at 450 nm.

The effect of Prazosin on MG63 and 143B cells were

further analyzed. Approximately 1×10^3 cells were seeded into each well of a 96-well plate and cultured for 24 h. Cells were then treated with either Prazosin or with dimethyl sulfoxide (DMSO) as negative control (NC). Following this treatment for 0, 24, 48, and 72 h, cells were cultured with CCK8 reagent at 37 ℃ for 90 min with absorbance being measured at 450 nm.

Clonogenic assay

About 500 of the cells treated with either Prazosin or DMSO for 24 h were seeded into mediums to be cultured at 37 ℃ with 5% CO_2 for 2 weeks. Afterwards, the colonies were fixed with 1 mL of 4% paraformaldehyde for 30 min, followed by staining with crystal violet for 30 min. Photographs were then taken and the number of colonies was counted.

Transwell assay

Transwell chambers were performed to assess the migration and invasion abilities of MG63 and 143B cells. Before assaying, the Transwell chambers were coated with Matrigel. Following treatment with Prazosin or DMSO for 24 h, the cells in serum-free culture medium were transferred to the upper chamber, with complete medium being added to the lower chamber. After incubation for 24 h, the invaded cells were fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet for 20 min. Photograph was then taken and the number of invaded cells was counted under the microscope. The migration assay was similarly conducted except for the absence of Matrigel.

Flow cytometry assay

In order to assess apoptosis of the cells, the treated MG63 and 143B cells were cultured in serum-free medium for 24 h in order to induce starvation. They were then stained with the Annexin V-FITC-PI apoptosis detection kit (4A Biotech, China) as according to the instructions. The apoptosis rate was analyzed using a flow cytometer (BD FACSCanto II, BD Biosciences, USA), and calculated using BD FACSDiva software.

Western blot assay

Cells were lysed using ice-cold RIPA Lysis Buffer (CWBIO, China) after 24 h of Prazosin or DMSO treatment to extract proteins. Twenty µg of protein from each sample
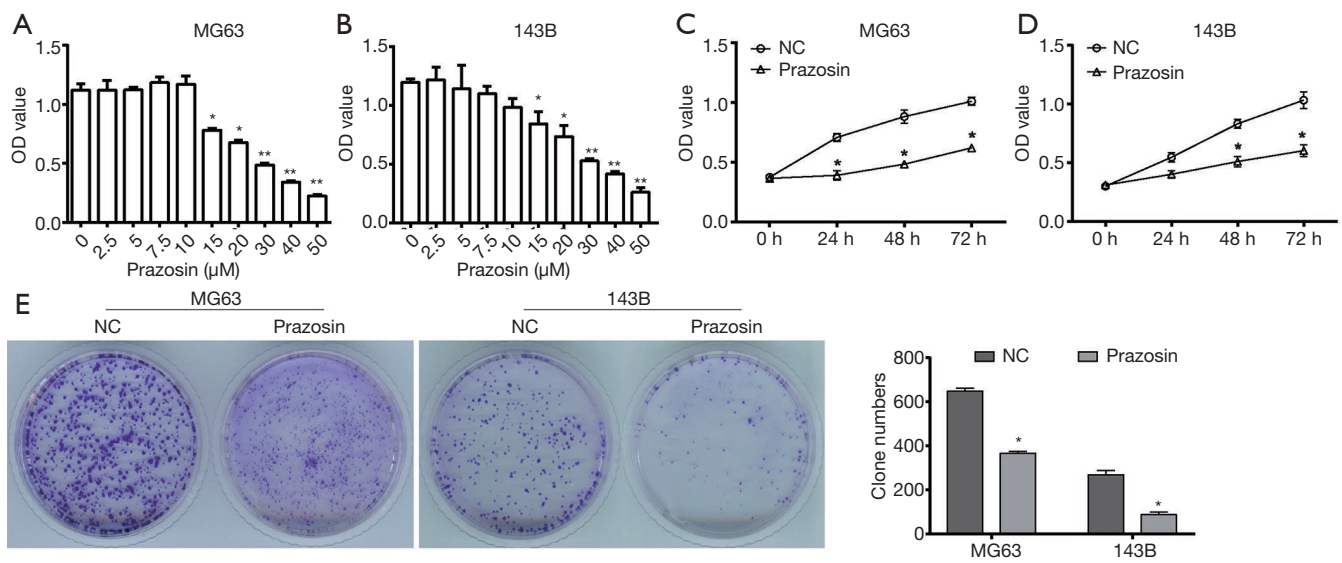
Figure 1 Prazosin inhibits the growth of MG63 and 143B cells. (A,B) Prazosin inhibits the viability of MG63 (A) and 143B (B) cells in a dose-dependent manner after 24 h treatment; (C) Prazosin (20 µM) inhibits the proliferation of MG63 cells after 24, 48 and 72 h treatment by CCK8 assay; (D) Prazosin (25 µM) inhibits the proliferation of 143B cells; (E) colony-forming assay showing the inhibitory effects of Prazosin (20 or 25 µM) on clonogenic abilities of MG63 and 143B cells. Prazosin, Prazosin treated group; NC, DMSO treated group. Data is expressed as the mean ± SD. *, P<0.05, **, P<0.01 *vs.* NC group. SD, standard deviation; DMSO, dimethyl sulfoxide; NC, negative control; OD, optical density.

was electrophoresed on 10% 24 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, USA). The membrane was blocked with 5% non-fat milk for 1 h prior to incubation with primary antibodies at 4 ℃ overnight. Following incubation with secondary antibody for 1 h, the signal was then developed using an enhanced chemiluminescence detection kit. Bcl-2, Bax, active caspase 3, P70, cyclin D1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Proteintech Group (USA), and Akt, p-Akt, mTOR and p-mTOR antibodies were obtained from Cell Signaling Technology (USA). The horseradish peroxidase-conjugated secondary antibodies were obtained from Proteintech Group.

Statistical analysis

All experiments were repeated in triplicate with the values being presented as mean ± standard deviation (SD). SPSS 18.0 statistical software was utilized to carry out statistical analysis. Student's *t*-test or one-way analysis of variance (ANOVA) was performed for statistical analysis between groups, and P<0.05 was considered statistically significant.

Results

Prazosin inhibits the growth of MG63 and 143B cells

In order to investigate whether Prazosin effects the cell growth of osteosarcoma, MG63 and 143B cells were treated with different concentrations of Prazosin (0, 2.5, 5, 7.5, 10, 15, 20, 30, 40, and 50 µM). We found that Prazosin showed no effect on the cell viability of MG63 cells at concentrations lower than 10 µM (*Figure 1A*), whereas it showed significant inhibitory effect at greater concentrations. Similar inhibitory effects were observed in 143B cells (*Figure 1B*). The half maximal inhibitory concentration (IC_{50}) concentration was 25.29 µM in MG63 cells and 35.28 µM in 143B cells, and 20 µM of Prazosin was used in MG63 cells and 25 µM was used in 143B cells in all the rest experiments as its appropriate effects. In order to substantiate the inhibition of Prazosin in the proliferation of MG63 and 143B cells, CCK8 assay was performed. As shown in *Figure 1C*, Prazosin significantly blocked the proliferation of MG63 cells when compared with NC cells (P<0.05), and Prazosin also inhibited the proliferation of 143B cells in a time-dependent manner (*Figure 1D*). Colony-forming assay was performed to further confirm the effect of Prazosin on clonogenic
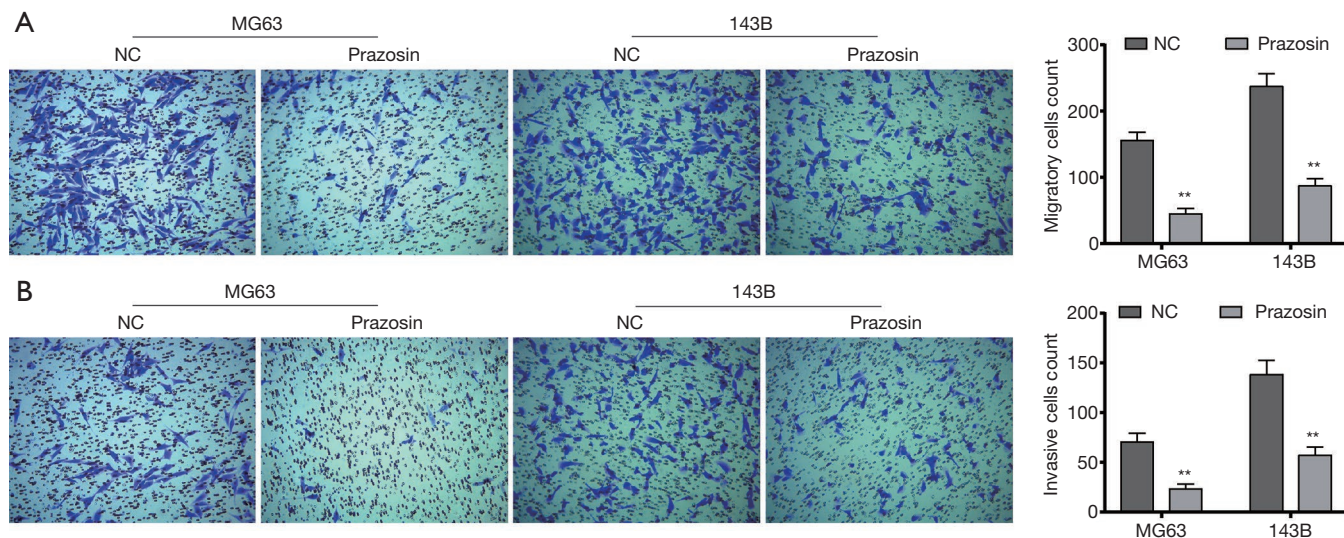
2000

An et al. Prazosin inhibits the progression of osteosarcoma

Figure 2 Prazosin inhibits the migration and invasion abilities of MG63 and 143B cells. (A) Prazosin (20 or 25 µM) inhibits the migration abilities of MG63 and 143B cells after 24 h treatment using Transwell assay; (B) Prazosin (20 or 25 µM) inhibits the invasion abilities of MG63 and 143B cells after 24 h treatment. Amplification, 100. Prazosin, Prazosin treated group. Data is expressed as the mean ± SD. **, $P < 0.01$ *vs.* NC group. SD, standard deviation; NC, negative control.

abilities of MG63 and 143B cells. Our data revealed a significant decrease in the number and size of colonies in Prazosin treated cells compared with NC group ($P < 0.05$, *Figure 1E*). Collectively, these data suggest that Prazosin inhibits the viability and proliferation of osteosarcoma cells *in vitro*.

Prazosin decreases the migration and invasion abilities of MG63 and 143B cells

It is well known that the invasion and metastasis properties of cancer cells are the main causes for poor prognosis in osteosarcoma. Therefore, we examined Prazosin's effect on these properties in MG63 and 143B cells using a Transwell assay *in vitro*. As shown in *Figure 2A*, Prazosin led to a significant decrease in the migration abilities of MG63 and 143B cells compared with NC group ($P < 0.01$). Meanwhile, the number of invasive cells was also significantly decreased by Prazosin treatment ($P < 0.01$, *Figure 2B*). Overall, Prazosin displays a significant anti-cancer role in the migration and invasion abilities of osteosarcoma cells.

Prazosin induces apoptosis in MG63 and 143B cells

Given that Prazosin was found to inhibit cell growth, cell apoptosis was assessed using flow cytometry to determine the effect of Prazosin on cell survival of osteosarcoma. Flow cytometric analysis showed that Prazosin significantly increased the rate of apoptosis induced by starvation in MG63 cells compared with NC group ($P < 0.05$, *Figure 3A*). Additionally, the rate of apoptosis in 143B cells was also promoted by Prazosin (*Figure 3A*). To investigate which apoptotic pathway was induced by Prazosin, western blotting was used to detect the expression level of Bcl-2, Bax and active caspase 3. As expected, the expression level of anti-apoptotic protein Bcl-2 was down-regulated by Prazosin compared with NC group ($P < 0.01$), while expression level of pro-apoptotic proteins Bax and active caspase 3 was simultaneously up-regulated ($P < 0.01$, *Figure 3B*). The above suggests that Prazosin induces the mitochondrial apoptotic pathway in MG63 and 143B cells.

Prazosin inhibits activity of the Akt/mTOR pathway in MG63 and 143B cells

As everyone knows, the Akt/mTOR pathway plays a pivotal role in regulating cell growth and survival, being particularly involved in progression of osteosarcoma. In the present study, we examined the expression levels of crucial proteins in the Akt/mTOR pathway to confirm whether the pathway is involved in the anticancer effects
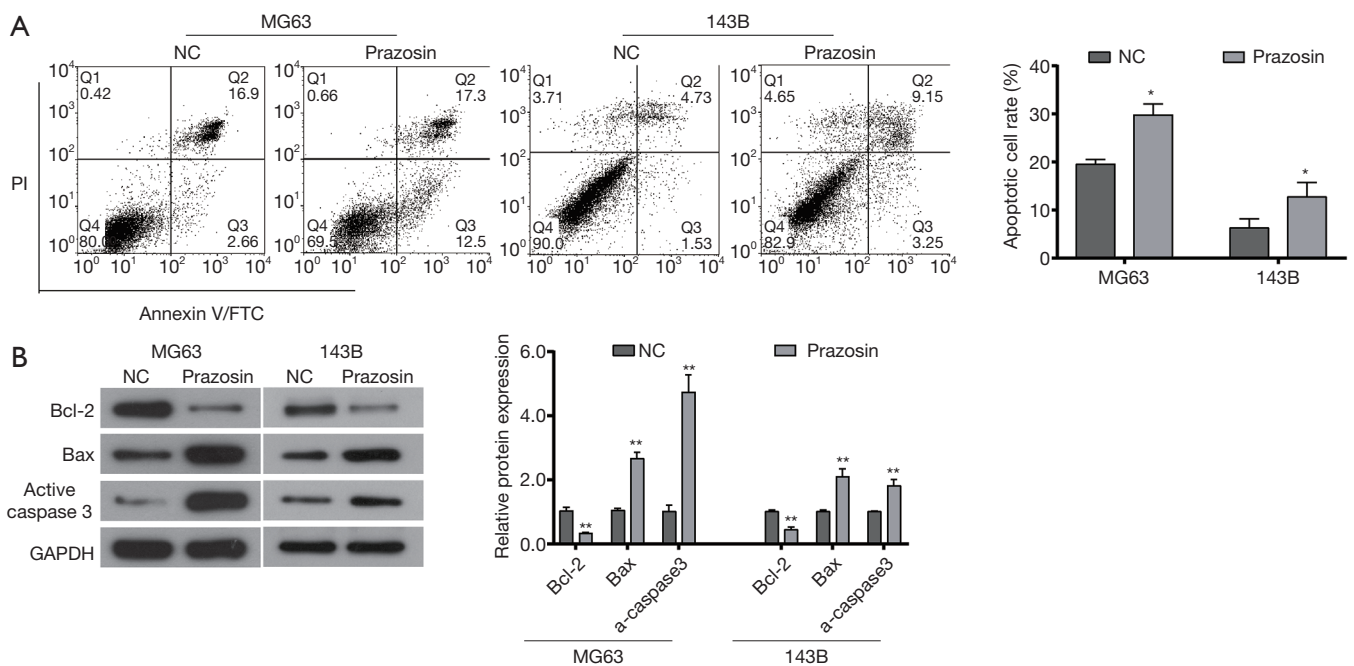
Figure 3 Prazosin induces apoptosis in MG63 and 143B cells. (A) After MG63 and 143B cells were treated with Prazosin (20 or 25 µM), the rate of apoptosis significantly increased; (B) after MG63 and 143B cells were treated with Prazosin (20 or 25 µM), Western blotting was used to examine expression of apoptosis-related proteins. Prazosin, Prazosin treated group. Data is expressed as the mean ± SD. *, P<0.05, **, P<0.01 *vs*. NC group. SD, standard deviation; NC, negative control.
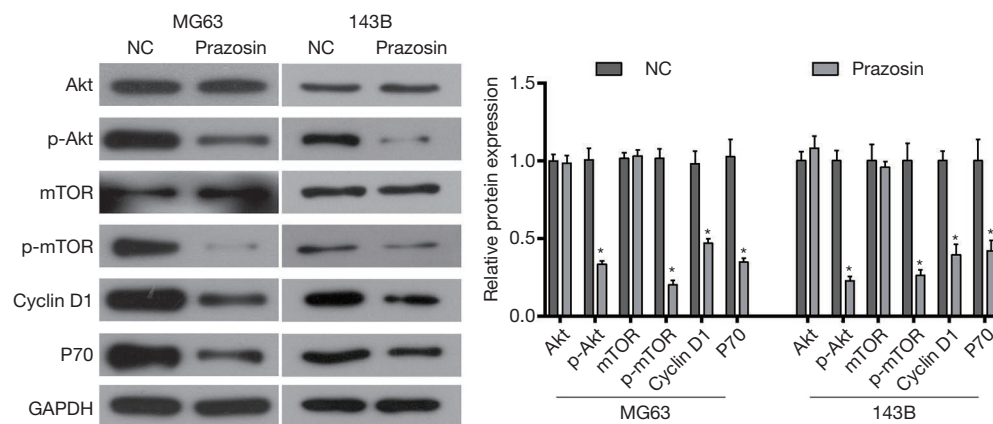

Figure 4 Prazosin suppresses the PI3K/Akt pathway in MG63 and 143B cells. After MG63 and 143B cells were treated with Prazosin (20 or 25 µM), the expression level of phosphorylation levels of Akt (p-Akt) and mTOR (p-mTOR) and cyclin D1, P70 were significantly decreased. Prazosin, Prazosin treated group. Data is expressed as the mean ± SD. *, P<0.05 *vs*. NC group. SD, standard deviation; NC, negative control.

of Prazosin. Our data showed that the phosphorylation levels of Akt (p-Akt) and mTOR (p-mTOR) were significantly attenuated by Prazosin in both MG63 and 143B cells. Furthermore, it showed that the expression

levels of downstream proteins p70S6k (P70) and cyclin D1 were also decreased (P<0.05, *Figure 4*). This data suggests that the Akt/mTOR pathway might be involved in the anti-tumor effects of Prazosin.

2002

An et al. Prazosin inhibits the progression of osteosarcoma

Discussion

Metastasis is an important factor affecting the prognosis of patients with osteosarcoma, especially the occurrence of lung metastasis (14). Therefore, blocking tumor metastasis and invasion is a necessary strategy for osteosarcoma therapy. It has been well-documented that Prazosin enhances the apoptosis rate in a number of tumor cells, displaying anti-growth activity in prostate cancer (12,13,15), glioblastoma (16), and MTC cells (9). In the present study, we have tested and identified Prazosin as a novel anti-cancer agent in osteosarcoma cells. We have shown that Prazosin possesses a significant inhibitory effect on cell viability of osteosarcoma MG63 and 143B cells in a dose- and time-dependent manner *in vitro*. Furthermore, we've shown that Prazosin inhibits both cell migration and invasion, and also induces apoptosis, further displaying an anti-tumor role in the progression and motility of osteosarcoma cells.

Dysregulated apoptosis is one of the common hallmarks of tumor cells, reducing apoptosis is a general mechanism of survival of osteosarcoma cells. In this study, our data confirmed a significant induction in apoptosis in Prazosin treated cells compared with the NC group. It has been shown that apoptosis involves numerous proteolytic events mainly mediated by the family of cysteine proteases, including the activated caspase 3, a pivotal executioner to trigger apoptosis (17). It has already shown that Prazosin induces apoptosis in prostate cancer PC-3 cells by triggering mitochondria mediated caspase cascades through targeting DNA (12). Our data confirm that Prazosin induces apoptosis by up-regulating the level of active caspase 3 in MG63 and 143B cells, suggesting that activating the caspase cascades is involved in the promotion of apoptosis induced by Prazosin. The Bcl-2 family also plays an essential role in initiation of apoptosis by triggering the mitochondrial pathway (18,19). In general, a decreasing ratio of Bcl-2, a pivotal anti-apoptotic protein located in the mitochondria, to Bax, a crucial pro-apoptotic protein mainly located in the cytoplasm, promotes apoptosis (20,21). In our study, a significant decrease in the expression level of Bcl-2 and concurrent increase in Bax were observed in MG63 and 143B cells exposed to Prazosin, indicating that apoptosis promoted by Prazosin is specifically triggered in the mitochondrial pathway. Autophagy, also known as type II programmed cell death, is another way of causing cell death. Prazosin also has been reported to be able to induce autophagy in prostate cancer (13,15) and H9C2 cells (22). As described above, Prazosin might be used as an effective anti-tumor agent for cancer therapy, and whether

Prazosin induces autophagy in osteosarcoma cells will be the focus of our further study.

Abnormalities in the PI3K/Akt/mTOR pathway are frequently observed in various cancers and are correlated with tumor cell growth and survival. Aberration of PI3K/Akt pathway is frequently observed in cancers including osteosarcoma (23). More specifically, growing evidence reveals that some anti-tumor agents, such as Ferulic acid (8), Lupeol (24) and Andrographolide (25), blocks the PI3K/Akt/mTOR pathway, exerting significant inhibitory effects on cell growth and survival of osteosarcoma and even enhancing the anti-cancer effects of cisplatin (26). Furthermore, Yang *et al.* report that the Akt/mTOR pathway is involved in Prazosin-induced autophagy in rat embryonic ventricular myoblast H9C2 cells (22). The Akt pathway is also observed to be inhibited by Prazosin in glioblastoma-initiating cells (16). Consistent with these previous studies, we have found that the phosphorylation levels of Akt and mTOR were down-regulated by Prazosin. Similarly, we found that expression levels of P70 and cyclin D1 were also decreased. P70, a downstream target protein of mTOR, modulates protein synthesis and is involved in cell proliferation and cell cycle. It has been demonstrated that P70 is over-activated in osteosarcoma tissues, being associated with the progression of osteosarcoma (27). Cyclin D1, a key regulator in the cell cycle, promotes cell proliferation. Cyclin D1 overexpression is frequently observed in tumors and associated with tumorigenesis (28). Given the above, the Akt/mTOR pathway may be involved in the anti-tumor effects of Prazosin on the proliferation, invasion, and survival of osteosarcoma cells.

Conclusions

In the current study, we highlight that Prazosin inhibits the cell growth and motility of MG63 and 143B cells. We have also shown that it promotes apoptosis in the mitochondrial pathway. In sum, as Prazosin inhibits cell survival and metastasis, our data suggests that it may serve as a potential anti-cancer agent in osteosarcoma therapy.

Acknowledgments

Footnote

Conflicts of Interest: All authors have completed the ICMJE

uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.09.03). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of The First Central Hospital of Baoding (No. 2019061). Individual informed consent was waived.

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An et al. Prazosin inhibits the progression of osteosarcoma

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