Tizanidine hydrochloride exhibits a cytotoxic effect on osteosarcoma cells through the PI3K/AKT signaling pathway

Xue-Wu Xing¹*, Yu-Fu Sun¹*, Jun Zhao², Zi-Xiang Pan¹ and Wen-Xue Jiang¹

Abstract

Objectives: α2-adrenergic receptors are reportedly involved in cancer cell proliferation, invasion, and apoptosis through regulation of diverse molecules, which implies that it contributes to tumor progression. However, the functional significance of α2-adrenergic receptors in osteosarcoma (OS) is unclear. Tizanidine hydrochloride (THC), an α2-adrenergic receptor agonist, is often used to alleviate symptoms of spasticity. This study investigated the functional implications of THC treatment on human OS cells and the underlying mechanisms of resulting changes.

Methods: The proliferation of U2 OS cells was assessed by Cell Counting Kit-8; the migration and invasion capacities of U2 OS cells were then analyzed by transwell assay. Moreover, apoptosis in U2 OS cells was evaluated by flow cytometry and western blot analyses. Additionally, expression levels of key proteins in the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathway were measured.

Results: THC inhibited the proliferation, migration, and invasion of U2 OS cells, but promoted apoptosis within these cells. Expression levels of p-AKT, p-mTOR, and p-P70S6K were reduced by exposure to THC, suggesting involvement of PI3K/AKT signaling in THC-induced cytotoxicity within OS cells.

Conclusions: THC may play a novel role in OS cell cytotoxicity, and these findings suggest a potent therapeutic strategy for OS treatment.

¹Department of Orthopedics, Tianjin First Central Hospital, Tianjin, P.R. China
²Department of Bone and Soft Tissue Tumor, Tianjin Medical University Cancer Institute & Hospital, Tianjin, P.R. China

*These authors contributed equally to this work.

Corresponding author:
Wen-Xue Jiang, Department of Orthopedics, Tianjin First Central Hospital, No. 24 Fu Kang Road, Tianjin 300192, P.R. China.
Email: jiangwenxuesp@sina.com
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Introduction
Osteosarcoma (OS) is a malignant tumor that comprises the most common primary sarcoma of bone; it mainly derives from the tibia or femur, but can also affect other bones in the body.1 OS primarily occurs in children and young adults and exhibits a bimodal age distribution.1 Chemotherapy is an important aspect of OS treatment,2 and biomaterials are a possible alternative approach to osteosarcoma treatment.3 However, survival remains low and drug resistance persists, despite treatment with multidrug combination chemotherapy. The 5-year survival rates for patients with OS are 60%–70%,1 whereas multidrug combination chemotherapy has increased 2-year survival rates to 90%. Therefore, novel, safe, and effective drugs are needed to increase 5-year survival rates in OS patients.

α2-adrenergic receptors are expressed in multiple tissues, where they perform specific biological functions.4 α2-adrenergic receptors have been shown to regulate the progression of several types of cancer, but their specific roles in these cancers are controversial.5,6 Some research has suggested that α2-adrenergic receptors promote tumor development. Szpunar et al.7 showed that α2-adrenergic receptor activation by the antidepressant desipramine promoted progression of several tumors in association with altered collagen structure.7 α2- and β2-adrenergic receptor activities have also been associated with breast cancer cell proliferation and accelerated tumor growth.8 Conversely, other research has suggested that α2-adrenergic receptor activity may contribute to the inhibition of cancer cell progression. Notably, α2-adrenergic receptor stimulation has been shown to inhibit cholangiocarcinoma growth through modulation of Raf-1 and B-Raf activities.9 Selective α2-adrenergic blockade by efavoxan also increases primary tumor size and distant metastasis under non-stress conditions through inhibition of sympathetic catecholamine release.10 Therefore, the roles of α2-adrenergic receptors in cancer progression remain controversial. α2-adrenergic receptors have been found in various bone cells or muscle cells, and are likely to be expressed by various subpopulations of neurons that interact with bone and muscle.11 α2-adrenergic receptors are expressed in human skeletal muscle that mediates vasoconstriction.12 However, the roles of α2-adrenergic receptors in OS pathogenesis are unclear.

Tizanidine hydrochloride (THC), an α2-adrenergic receptor agonist, is used to treat spasms, cramping, and tightness in muscle spasticity.13,14 It acts mainly at spinal and supraspinal levels to inhibit excitatory interneurons.15 THC can be used in adults and pediatric populations and its overall safety is excellent.16 In this study, we used THC to examine the relationships between α2-adrenergic receptor activity and proliferation, apoptosis, invasion, and migration in a human OS cell line.

Methods

Ethical approval
This article does not contain any studies with human participants or animals.
performed by any of the authors. Therefore, ethical approval was not required.

**Cell culture**

Human U2 OS cells and normal human osteoblasts (hFOB cells) were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). U2 OS cells and hFOB cells were grown in Roswell Park Memorial Institute medium (RPMI-1640; Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 0.1 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 100 U/mL penicillin (Sigma-Aldrich). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ within a humidified cell incubator. Upon reaching confluence, the cells were washed three times with phosphate-buffered saline (PBS), detached with 0.25% trypsin (Solarbio, Beijing, China) and seeded in six-well plates at 1 × 10⁶ cells/well for experiments. When the cell density reached 80%, cells in the experimental group were treated with THC (10 μM) for 24 h, while cells in the control group were treated with dimethylsulfoxide (DMSO) for 24 h.

**Western blot**

After cells had been treated with THC or DMSO for 24 hours, the six-well plates were placed on ice and radio immunoprecipitation assay (RIPA; Beyotime Inc., Jiangsu, China) lysis buffer with protease inhibitor (Beyotime Inc.) was added to the cells. Total protein was then extracted by shaking for 15 minutes and scraping of each well; the total protein concentration of the resulting supernatant was measured by using a bicinchoninic acid kit (Beyotime Inc.). Extracted proteins (20 μg) were mixed with loading buffer and boiled at 95°C for 5 minutes for denaturation. The denatured proteins were separated by polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene fluoride membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membrane was then blocked with 5% non-fat milk for 1 hour, followed by incubation with primary antibodies of relevant proteins (described below) overnight at 4°C. On the next day, the membrane was washed with Tris-buffered saline + Tween (three times, 5 minutes each); the membrane was then incubated with horseradish peroxidase-conjugated secondary antibody (described below) for 1 hour. Signals were enhanced by ECL reagent (Pierce Biotechnology Inc., Rockford, IL, USA) and then quantified using Quantity One software (Bio-Rad); GAPDH was used as an internal control for blotting detection. Western blots were performed with the following rabbit primary antibodies diluted at 1:1000: anti-Bax (Cat. No. 2774), anti-Bcl-2 (Cat. No. 4223), anti-active caspase-3 (Cat. No. 9661), anti-protein kinase B (AKT) (Cat. No. 9272), anti-p-AKT (Cat. No. 9271), anti-mTOR (Cat. No. 2972), anti-p-mTOR (Cat. No. 2971), and anti-p-P70S6K (Cat. No. 9208) (all from Cell Signaling Technology, Danvers, MA, USA), and GAPDH (1:5000, ProteinTech Group, Rosemont, IL, USA); and a polyclonal antibody horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000, Cat. No. 10285-1-AP, ProteinTech Group).

**Cell proliferation assay**

Cells were seeded in 96-well plates, 1000 cells/well in 100 μL media. Cells in the experimental group were treated with different concentrations of THC (0, 2, 10, and 50 μM) and cells in the control group were treated with DMSO. Cells were incubated at 37°C in an atmosphere of 5% CO₂ within a humidified cell incubator. Cell viability was assessed every 24 hours as
follows. Cell supernatant (100 μL) was mixed with 10 μL of CCK-8 reagent (Cell Counting Kit-8, Solarbio) and then incubated for 1.5 hours at 37°C in the cell incubator described above. The proliferation curve was plotted by the optical density (OD) values at 450 nm.

**Invasion and migration assay**

Cell invasion assays were performed using a 24-well transwell plate. One hundred microliters of Matrigel matrix gel (BD Bioscience, San Jose, CA, USA) was melted overnight, then diluted 1:6 with serum-free medium, added to transwell chambers within a 24-well plate, and cultured for 4–6 hours at 37°C in an atmosphere of 5% CO₂ within a humidified cell incubator. The upper medium was then removed, and the lower chamber was filled with 500 μL RPMI-1640 medium without FBS to hydrate the basement membrane. Cells were treated for 24 hours as described above (either experimental treatment or control treatment); then, the culture medium was removed and replaced serum-free medium. Cells were detached by using trypsin-EDTA, then centrifuged at 134 × g for 5 minutes. Centrifuged cells were resuspended in pre-cooled PBS and centrifuged again with the same protocol. They were then resuspended in 1 × binding buffer 1 to 5 × 10⁶ cells/mL. Cell suspension (100 μL) and Annexin V/FITC (5 μL) were mixed and incubated at room temperature for 5 minutes in the dark. PI dye (10 μL) and PBS (400 μL) were added for detection. Results were analyzed and processed with FlowJo software (version 7.6, FlowJo LLC, Ashland, OR, USA). In the resulting cytograms, the top right quadrant (Q2) represented the proportion of late apoptotic cells (Annexin V/FITC and PI double-stained), while the bottom right quadrant (Q4) represented the proportion of early apoptotic cells (Annexin V/FITC-stained).

**Statistical analysis**

SPSS statistical analysis software (version 18.0, SPSS, Inc., Chicago, IL, USA) was used to analyze the experimental data. Measurement data are shown as mean ± standard deviation. Comparisons between two groups were performed by using t-tests, and comparisons among multiple groups were performed by using one-way analysis of variance. Differences with \( P < 0.05 \) were considered to be statistically significant.
Results

THC inhibits proliferation of U2 OS cells

To investigate the effect of THC on cell growth, the OD values of U2 OS cells and normal hFOB cells were analyzed by CCK8. hFOB cells and U2 OS cells were treated with different concentrations (0, 2, 10, and 50 μM) of THC for 72 hours. We found that only the OD value of 50 μM THC-treated hFOB cells was significantly decreased, compared with that of the 0 μM THC group \((P < 0.01, \text{ Figure 1a}).\) Figure 1b shows that the OD value of U2 OS cells decreased as the concentration of THC increased; moreover, the OD values at 10 μM and 50 μM of THC were significantly lower than that at 0 μM THC \((P < 0.01).\) These results indicated

![Figure 1](image)

**Figure 1.** Tizanidine hydrochloride inhibits the proliferation of U2 OS cells. (a) OD values of normal hFOB cells with different THC concentrations at 72 hours. (b) OD values of U2 OS cells with different THC concentrations at 72 hours. (c) OD values of U2 OS cells at different times. The results are shown as mean ± standard deviation. \(^*P < 0.05\) compared with NC, \(^{**}P < 0.01\) compared with NC. THC, tizanidine hydrochloride treatment (experimental group); NC, negative control (control group).
that THC could inhibit the growth of U2 OS cells in a dose-dependent manner, but that a toxic effect was only detected in normal hFOB cells at the maximal dose of THC. Thus, 10 μM THC was used for the following experiments: migration, invasion, apoptosis, and signaling pathway analysis. The OD values of U2 OS cells treated with 10 μM THC were also analyzed every 24 h; the OD value in the experimental group was lower than that in the control group after 48 hours of treatment (P<0.05), and was lowest after 72 hours of treatment (P<0.01 Figure 1c). Thus, THC could significantly inhibit the proliferation of U2 OS cells in a dose- and time-dependent manner.

**THC inhibits migration and invasion of U2 OS cells**

Given that THC inhibited the proliferation of U2 OS cells, we investigated the roles of THC in cell migration and invasion of U2 OS cells. Transwell assay analysis demonstrated that the number of invading U2 OS cells in the experimental group (16 ± 3) was significantly lower than that in the control group (48 ± 4) (P<0.01) (Figure 2). The number of migrating U2 OS cells in the experimental group (46 ± 5) was also significantly lower than that in the control group (72 ± 6) (P<0.01). These results suggested that THC could significantly inhibit the migration and invasion of U2 OS cells.

**THC promotes apoptosis in U2 OS cells**

Annexin V staining of U2 OS cells (i.e., annexin-V and FITC double-positive) demonstrated apoptosis in a proportion of adherent cells at 72 hours after treatment with THC. Flow cytometry analysis showed that the number of apoptotic cells was markedly increased in the experimental group (23.45% ± 1.4%), compared with that in the control group (6.10% ± 0.9%) (Figure 3a). These results suggested that THC could promote apoptosis in OS cells. To confirm that THC induced apoptosis, the pro-apoptotic proteins, active caspase-3 and Bax, as well as the anti-apoptotic protein, Bcl-2, were measured in U2 OS cells. The expression levels of both Bax and active caspase-3 increased, whereas the expression level of Bcl-2 decreased in U2 OS cells in the experimental group, compared with the levels in the control group (Figure 3b). Overall, these results demonstrated that THC could promote apoptosis in U2 OS cells.

**THC restrains PI3K/AKT signaling pathway of U2 OS cells**

Previous studies have shown that activation of the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway can positively regulate apoptosis. To determine whether THC induced apoptosis in U2 OS cells through the PI3K/AKT signaling pathway, we analyzed the expression of key signal proteins, including AKT, p-AKT, mTor, p-mTor, and p70S6K. We found that the expression levels of p-AKT, p-mTOR, and p-P70S6K were lower in the experimental group than in the NC group (Figure 4). These results suggested that THC exhibited a cytotoxic effect on OS cells, which was closely associated with regulation of the PI3K/AKT signaling pathway.

**Discussion**

To the best of our knowledge, this report is the first to show that THC dramatically inhibited the proliferation, invasion, and migration of human U2 OS cells. Furthermore, THC promoted apoptosis in U2 OS cells, and significantly inhibited the expression of key proteins in the PI3K/AKT signaling pathway in these cells. Our findings may provide a foundation for
novel research into understanding the mechanism by which THC affects OS cell characteristics; this may lead to discovery of an alternative agent for treatment of OS, both in monotherapy and in combination therapy.

THC is an $\alpha_2$-adrenergic receptor agonist, used to alleviate symptoms of spasticity associated with multiple muscle sclerosis, spinal cord injury, or neurodegenerative disease. It is also used in treatment of painful muscle spasm associated with musculoskeletal damage, as well as low back pain. We found that THC could inhibit U2 OS cell proliferation, invasion, and migration. The $\alpha_2$-adrenergic receptor is a member of the adrenergic receptor family, which contains $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, and $\beta_3$-adrenergic receptors. Notably, sustained adrenergic signaling leads to increased metastasis of ovarian cancer via increased prostaglandin E2 synthesis. Moreover, adrenergic signaling promotes angiogenesis through
endothelial cell-tumor cell crosstalk. β-adrenergic receptor antagonists may be useful for treatment of skin cancer, and have shown a large impact on cancer progression. Importantly, β2-adrenergic receptor antagonists suppress pancreatic cancer cell invasion by inhibiting cAMP response element binding protein, nuclear factor κB, and activator protein-1. In contrast, the α2 adrenergic receptor has been found to increase primary tumor size and distant metastasis of many cancers. Lamkin et al. reported that α2-adrenergic blockade by phentolamine significantly inhibited chronic primary tumor growth and metastasis to distant tissues. α2-adrenoceptor agonists (clonidine and dexmedetomidine) significantly enhanced proliferation of the mammary tumor cell line MC4-L5. In contrast to those findings, the α2-adrenergic receptor has been reported to inhibit progression of some types of cancer cells, as described in the Introduction. Consistent with the latter view, we found that the α2-adrenergic receptor agonist, THC, exhibited an inhibitory effect on human OS cells: notably, it suppressed proliferation, invasion, and migration of U2 OS cells.

Additionally, our study suggested that THC significantly inhibited the PI3K/AKT signaling pathway in U2 OS cells by

Figure 3. Tizanidine hydrochloride promotes apoptosis in U2 OS cells. (a) U2 OS cells were treated with THC labeled with Annexin V/FITC and PI, followed by flow cytometric analysis. (b) Western blot analysis of U2 OS treated with THC. The band intensities were quantified. The results were normalized to the GAPDH loading control. "P < 0.05 compared with NC, ""P < 0.01 compared with NC; results are shown as mean ± standard deviation (n = 3). THC, tizanidine hydrochloride treatment (experimental group); NC, negative control (control group).
decreasing p-AKT, p-mTor, and p-P70S6K expression. The PI3K/AKT pathway regulates a variety of cellular activities, such as proliferation, apoptosis, and tumorigenesis. Activation of AKT triggers mTor activation, and mTOR plays a crucial role in the proliferation of various cancers, such as breast cancer, glioma, OS, and colon cancer. Importantly, mTor could directly promote the proliferation of cancer cells by stimulating the activity of p-P70S6K. Thus, inhibition of p-mTor could suppress OS cell proliferation through suppression of p-P70S6K. In addition, PI3K/AKT can affect cell apoptosis through a variety of proteins, such as Bcl-2, Bax, and active caspase-3. Activation of the PI3K/Akt pathway can accelerate the role of Bcl-2 in cell apoptosis; conversely, the PI3K/Akt pathway can also deactivate Bax, thereby inhibiting cell apoptosis. Finally, inhibition of the caspase family can also induce cell apoptosis. Together with the findings of the above studies, our findings (i.e., that THC dramatically increased the expression levels of active caspase-3 and Bax, but decreased the expression of Bcl-2) suggest that THC inhibits cell growth by enhancing induction of apoptosis by the PI3K/AKT signaling pathway.

In conclusion, our findings suggest that THC can inhibit proliferation, invasion, and migration of U2 OS cells, possibly by promoting apoptosis through the PI3K/AKT signaling pathway. To the best of our knowledge, this is the first report to highlight the correlation between THC and OS, and might therefore support the development of a new treatment for OS.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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