European Review for Medical and Pharmacological Sciences 2019; 23: 105-112

Aclidinium bromide inhibits proliferation of osteosarcoma cells through regulation of PI3K/Akt pathway

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Abstract. – OBJECTIVE: Osteosarcoma is recognized as the most common primary malignant bone tumor, the 5-year disease-free survival rate in patients with metastatic or recurrent disease is below than 30%. Drug resistance and toxic side effects limit the therapeutic efficacy of osteosarcoma. Therefore, it is urgent to develop new drugs for osteosarcoma treatment. Muscarinic 3 (M3) acetylcholine receptor (AChR) has been demonstrated in nonneurocrest-derived malignancies such as colon, prostate, lung, and ovarian carcinomas. Hence, targeted regulation of M3 AChR may be a possible mechanism for treating tumors. Aclidinium bromide has anti-tumoral properties in several tumors, namely gastric cancer and glioma. In this study, we intended to investigate whether aclidinium bromide, a novel M3 AChR antagonist, had effects on osteosarcoma cells proliferation and migration.

PATIENTS AND METHODS: The viability of U2 OS cells was detected by cell counting kit-8 (CCK-8) assay. The migration and invasion capabilities were measured by transwell invasion and migration assays. The cell apoptosis rate was tested by Annexin V-fluorescein isothiocyanate (FITC)/Propidum iodide (PI) staining and flow cytometry. Key apoptosis-related and phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathway-associated were assessed by Western blot analysis.

RESULTS: Aclidinium bromide markedly decreased the OD value of U2 OS cells 48 h and 72 h after treatment. The number of positive crystal violet staining cells significantly decreased after treatment with aclidinium bromide. Treatment with aclidinium bromide significantly increased cell apoptosis rate, accompanied by the expression of anti-apoptotic protein Bcl-2 decreased, the expression of pro-apoptotic protein Active caspase-3 and Bax significantly increased in U2 OS cells treated with aclidinium bromide. Additionally, aclidinium bromide suppressed the PI3K/AKT signaling pathway in U2 OS cells.

CONCLUSIONS: Therefore, the current study reveals that aclidinium bromide might inhibit osteosarcoma cell growth by regulating the PI3K/AKT signaling pathway, which suggests aclidinium bromide is a potential chemotherapeutic agent for osteosarcoma.

Key Words

Osteosarcoma, Muscarinic 3 acetylcholine receptor, Proliferation, Migration, Invasion, Apoptosis.

Introduction

Osteosarcoma is known as the commonest primary malignant bone tumor, and the 5-year disease-free survival rate is lower than 30% due to high recurrence and aggressiveness[1]. Osteosarcoma is a high-grade neoplasm with the characteristic of rapid growth and early metastasis. The incidence of osteosarcoma in males is somewhat higher than females due to a longer duration of skeletal growth in males[2,3]. Osteosarcoma can occur in any bone of the body but approximately 75% of all cases occur in the distal femur and proximal tibia[4,5]. Despite the advancement of multiple therapeutic regiments, namely, surgery, radiation and belligerent chemotherapy[6,7], the effectiveness of treatment has been unsatisfactory. In addition, drugs, such as etoposide[8], astragaloside[9], cinobufagin and cisplatin[10] as well as drug resistance and toxic side effects, limit the efficacy of osteosarcoma treatment[11]. Therefore, it is urgent to develop new drugs for osteosarcoma treatment. Acetylcholine (ACh) has been known to contribute to sensory processing. Muscarinic and nicotinic receptors (mAChR and nAChR, respectively) have been identified as the best described ACh receptors. The activation of these receptors increases neuronal activity. Notably, this receptor system has been shown to be involved in the regulation of cell movement and proliferation in neuronal cells (benign or malignant), and gaining an under-

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standing of this new pathway may provide targets for therapeutic intervention[12,13]. As an important neurotransmitter and ligand to AChR in the central and peripheral nervous systems, acetylcholine can stimulate cell growth through binding of mAChR or nAChR. Researchers have shown that acetylcholine is synthesized in numerous some non-neuronal cell types, in addition to neuronal cells, including bronchial epithelial cells, glial cells, pulmonary vessel cells, and ovarian cells[14-22]. Noda et al[23] found that neural crest-derived tumors such as melanoma and small cell carcinoma of the lung express both mAChR and nAChR. AChR is overexpressed in neurocrest-derived tissues where it has been shown to be a critical determinant of cellular development and differentiation[24]. As such, these receptors and their ligand agonists may play an important role in the pathogenesis of presumable neurocrest derived tumors[24]. Particularly, muscarinic 3 (M3) AChR has been observed in nonneurocrest-derived malignancies such as colon, prostate, lung, and ovarian carcinomas[25-29]. However, whether this receptor can be targeted to control AChR to treat osteosarcoma has not been investigated. Aclidinium bromide is a novel, long-acting, muscarinic antagonist that inhibits the action of acetylcholine at the M3 receptor[24]. Aclidinium bromide is approved as a maintenance bronchodilator treatment in patients with chronic obstructive pulmonary disease (COPD)[30]. Aclidinium salts have been identified to have tumor-inhibiting properties *in vitro* models and *in vivo* against human tumor xenografts, such as lung cancer and colorectal adenocarcinoma[31,32]. Acridinium iodide provokes cell cycle arrest in response to DNA damage in the tumor cell line[33]. Acridinium methosulfate, identified as an anti-telomerase agent, is screened as a candidate for candidates for tumor stem cells ablation in osteosarcoma[34]. There are emerging data from many experiments, suggesting that aclidinium bromide is proved to inhibit gastric cancer and glioma cell proliferation and migration potentials[35,36]. However, the role of aclidinium bromide in osteosarcoma treatment remains unknown. The aim of the present study was to illustrate the effect of aclidinium bromide on osteosarcoma cells and its potential mechanisms.

Materials and Methods

Cell Culture

U2 OS cell lines obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) were grown in Roswell Park Memorial Institute (RPMI)-1640 (Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin; Sigma-Aldrich, St. Louis, MO, USA). The medium was changed every 3 days, and U2 OS cells were maintained at 37°C in a 5% CO_2 incubator. For evaluation of expression of protein, cells were seeded onto 6-well culture plates.

Cell Proliferation Assay

Cells were cultured in 96-well plates, when the cells reached about 80% confluence, then the medium of aclidinium bromide group was replaced by complete medium containing aclidinium bromide (10 μM, MedChemExpress Biotechnology Shanghai, China) for 24 h, and 0.1% dimethylsulfoxide (DMSO) was employed as vehicle control. Then, 10 μL cell counting kit-8 (CCK8, Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) was added into medium and incubated for another 1.5 h, then the resulted formazan precipitates were dissolved in DMSO, and the optic density at 450 nm was read immediately using a microplate reader.

Cell Invasion and Migration Assay

Cells were suspended by 100 μL Matrigel matrix (BD Bioscience, San Jose, CA, USA) and seeded on the top chamber of the 24-well inserts (8 μm pore size; Corning, Tewksbury, MA, USA). Serum-free medium was added to the lower compartments and cells were incubated for 0.5 h to hydrate basilar membrane. Cell suspension (1×10^5 cells/100 μL serum-free MEM media) was added to the upper compartments and complete culture solution (500 μL) were added to the lower compartments after incubation for 24 h. The cells that did not migrate through the pores were removed by scraping the membrane with a cotton swab. The cells were fixed for 30 min in 4% paraformaldehyde after passed through the filter, stained for 20 min with 0.1% crystal violet. Five random fields (100 ×) were captured under the microscope (Olympus, Tokyo, Japan). Finally, we counted the number of invasive or migratory cells and calculated the average values. Each experiment was conducted in triplicate.

Flow Cytometric Analysis

After the cells were treated with aclidinium bromide for 24 h, then the medium was removed and serum starved for 24 h. Evaluation of apop-

tosis was performed by an Annexin V-fluorescein isothiocyanate (FITC)/propidum iodide (PI) kit (Beijing 4A Biotech Co., Ltd) as described previously[37]. The result was evaluated by a flow cytometer (BD Bioscience) after the cells were labeled with Annexin-V and PI.

Western Blotting

The cells were treated with aclidinium bromide for 24 h, then, cells were collected and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). The protein concentration was detected using a bicinchoninic acid (BCA) kit (Beijing ComWin Biotech Co., Ltd, Beijing, China). Subsequently, the equal proteins (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, Hercules, CA, USA) and then transferred onto a polyvinylidene difluoride (PVDF) membrane[38]. After blocked with 5% non-fat dry milk for 1 h, the proteins were incubated with various antibodies overnight at 4°C, including Bcl-2, Bax, Active caspase-3, protein kinase B (AKT), phosphorylated- (p-) AKT, mechanistic target of rapamycin (mTOR), p-mTOR, phosphorylated-serine-threonine protein kinase (p-P70S6K), GAPDH (1:1000, Cell Signaling Technology, Danvers, MA, USA). Secondary anti-mouse or anti-goat horseradish peroxidase (HRP) antibodies (1:5000, Beyotime Institute of Biotechnology, Shanghai, China) were added for 1 h, then we visualized the bands by enhanced chemiluminescence (ECL, Thermo Fisher Scientific) reagents and developed by ChemiDoc MP imager (Bio-Rad Laboratories, Hercules, CA, USA). The band density was quantified by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis

All data were analyzed with the SPSS version 18.0 software (SPSS, Chicago, IL, USA). Data were expressed as means ± standard deviations (SD). Statistical significance of difference between two groups was determined by *t*-test. $p<0.05$ was regarded as statistically significant.

Result

Aclidinium Bromide Inhibited the Proliferation of U2 OS Cells

To investigate the role of aclidinium bromide in the proliferation of U2 OS cells, we performed the CCK-8 assay. As shown in Figure 1, compared with control group, aclidinium bromide could significantly decrease the OD value of U2 OS cells 48 h and 72 h after treatment ($p<0.05$). This result suggested that aclidinium bromide inhibited the proliferation of U2 OS cells.

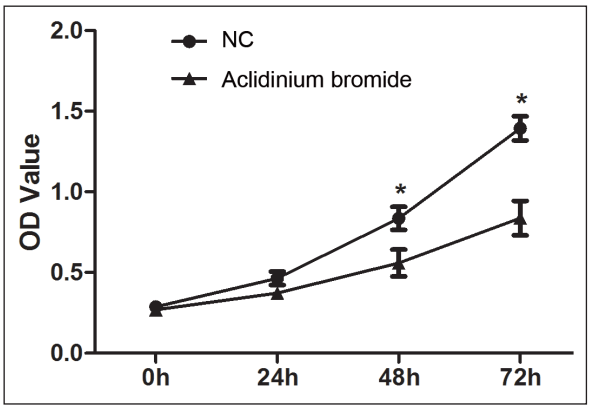

Figure 1. The proliferation of U2 OS cells treated with aclidinium bromide. * indicates $p<0.05$. Data was expressed as means ± SD. * indicates $p<0.05$.

Aclidinium Bromide Suppressed the Invasion and Migration of U2 OS Cells

Then, we observed the effect of aclidinium bromide on U2 OS cells invasion and migration. In invasion assay, the number of positive crystal violet staining cells significantly decreased after treated with aclidinium bromide ($p<0.05$) (Figure 2). Similar findings were shown in migration assay that the number of positive cells significantly decreased in aclidinium bromide treated group ($p<0.05$) (Figure 2). These results suggested that the ability of invasion and migration in aclidinium bromide treated group significantly decreased.

Induction of Apoptosis in U2 OS Cells by Aclidinium Bromide Exposure

To detect the effect of aclidinium bromide on U2 OS cells apoptosis, the Annexin-V-FITC/PI staining methods was used. Representative dot plots of Annexin-V-FITC/PI staining are shown in Figure 3A. Under control conditions, most numbers of cells were intact (quadrant (Q) 3, Annexin-V-FITC-negative/PI-negative) (96.79%). After treated with aclidinium bromide for 24 h, the populations of intact cells were decreased (90.99%), while the number of early apoptotic (Q4, Annexin-V-FITC-positive/PI-negative) and late apoptotic (Q2, Annexin-V-FITC-positive/PI-positive) (16.94% > 5.33%) cells were significantly increased ($p<0.05$). In addition, compared with

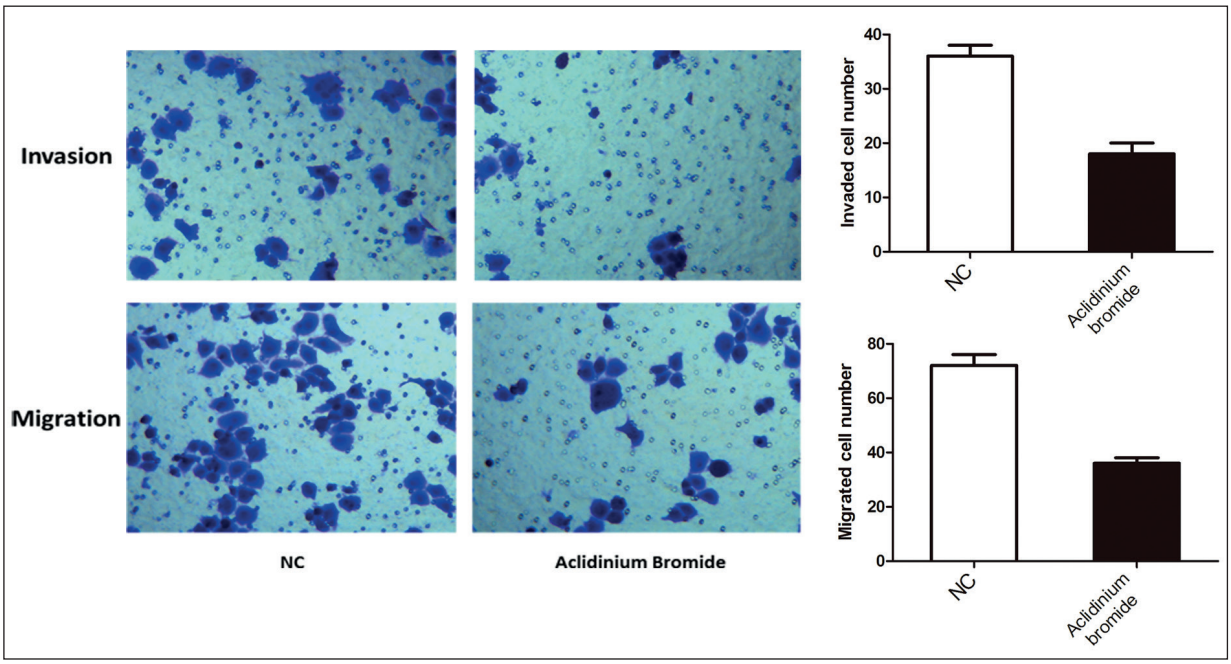

Figure 2. Aclidinium bromide significantly decreased the invasion and migration of U2 OS cells. The migrated and invaded cell number of U2 OS cells was quantified. Data was expressed as means ± SD. * indicates $p<0.05$.

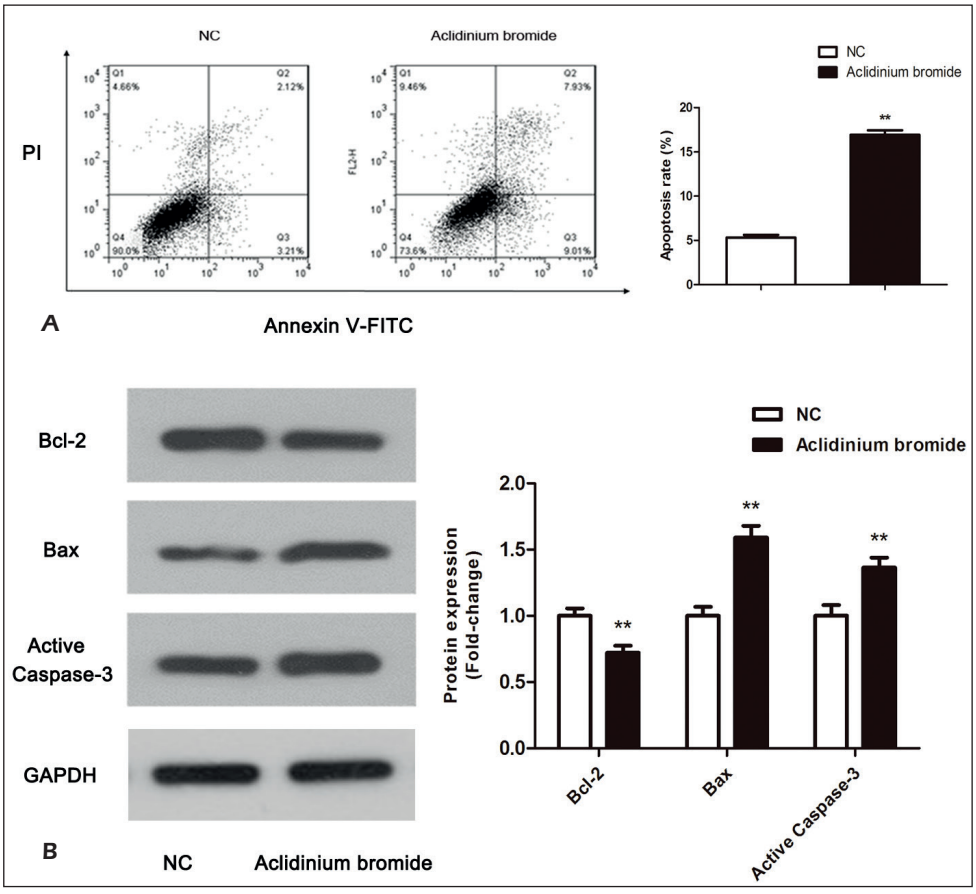

Figure 3. Apoptosis is induced by aclidinium bromide treatment in U2 OS cells. **A**, The proportions of living and dead cells were determined using flow cytometric analysis of Annexin-V/FITC and PI-labeled cells. Cells showing Annexin-V/FITC and PI double labeling (Q2) indicate those that have already died by apoptosis. Live cells were unlabeled with an Annexin-V/FITC and PI (Q3), while Annexin-V/FITC labeling (Q4) represents the population of early apoptosis. **B**, Western blot analysis of apoptosis protein expression in human U2 OS cells. Data was expressed as means ± SD. * indicates $p<0.05$.

control group, the expression of anti-apoptotic protein Bcl-2 in aclidinium bromide group was significantly decreased (p<0.05), and the expression of proapoptotic protein Bax and Active caspase-3 was significantly increased (p<0.05) (Figure 3). All these results suggested that aclidinium bromide could induce apoptosis in U2 OS cells.

Aclidinium Bromide Inhibited the Activation of PI3K/AKT Pathway in U2 OS Cells

It has been shown that phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway is closely related to the tumor formation (18-20); therefore, the expression of related proteins were analyzed with Western blot assay. These results showed that the level of p-AKT and p-mTOR, p-P70S6K was significantly decreased in aclidinium bromide treated U2 OS cells (p<0.05). Meanwhile, p-P70S6K protein was significantly decreased in aclidinium bromide treated U2 OS cells either (p<0.05). Collectively, these results suggested that aclidinium bromide might inhibit the proliferation and metastasis of U2 OS cells through blocking the PI3K signaling pathway (Figure 4).

Discussion

In this study, we identified that aclidinium bromide could inhibit cell proliferation, invasion and migration in U2 OS cells. Meanwhile, we showed that cell apoptosis was significantly promoted by aclidinium bromide. Finally, we proved that the effects of aclidinium bromide on U2 OS cells might be related to the inhibition of the PI3K/Akt signaling pathway. The two principal acetylcholine receptors, consisting of mAChR and nAChR, make some differences in distribution, agonists and function. The mAChR is classified into five subtype G-protein-coupled receptors M1-M5 AChR. M1, M3, and M5 facilitate cell proliferation and M2 and M4 initiate cell inhibition[39]. AChR is overexpressed in neurocrest-derived tissues, where it has been shown to be a critical determinant of cellular development and differentiation[24]. As such, these receptors and their ligand agonists may play an important role in the pathogenesis of presumable neurocrest-derived tumors such as primitive neuroectodermal tumors (PNET)[24]. Aclidinium bromide is a muscarinic antagonist and has a high binding affinity for the M3 receptor. However, the role of aclidinium bromide in osteosarcoma treatment remains unknown. Therefore, in this study, we investigated the role of aclidinium bromide in osteosarcoma. The results showed that aclidinium bromide significantly inhibited the proliferation of U2 OS cells, suggesting that aclidinium bromide inhibits the proliferation and growth of U2 OS cells. In addition, aclidinium bromide decreased the abilities of invasion and migration of U2 OS cells. Collectively, aclidinium bromide may be a novel drug for treating osteosarcoma in the future. Apoptosis is a vital pathway regulating cell death, which is also the most common cellular mechanism for

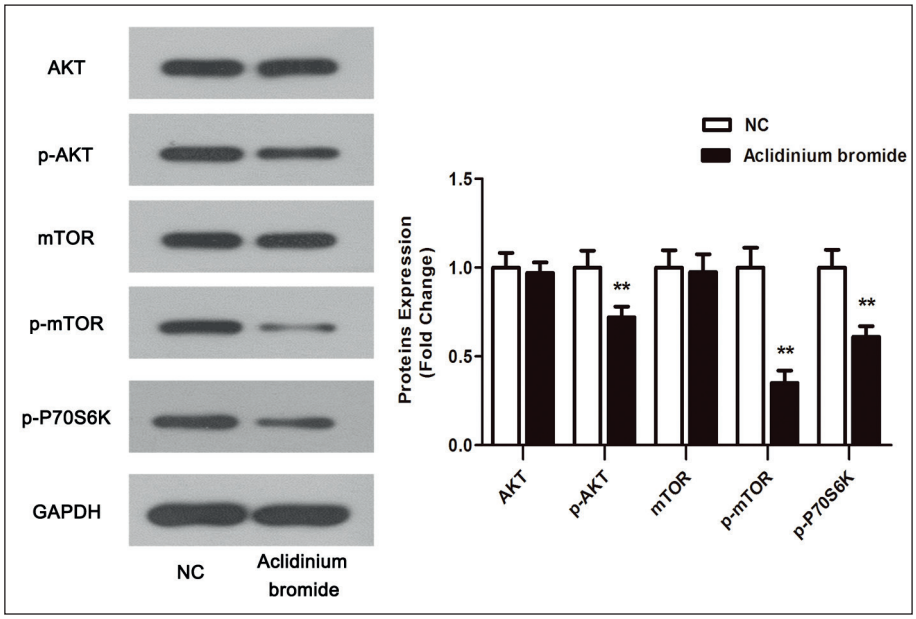

Figure 4. Western blot analysis of PI3K/AKT pathway protein expression in U2 OS cells treated with aclidinium bromide. Data was expressed as means ± SD. * indicates p<0.05.

naphthalimide inhibition in cancer cell[40,41]. Insufficient apoptosis could result in uncontrolled cell proliferation, which has been shown to be involved in cancer[42-44]. Ji et al[45] synthesized a triazolonaphthalimide, LSS-11, and found that it exhibited strong cytotoxicity by inducing cell cycle arrest and apoptosis in selected human colon cancer cell lines, which was accompanied by DNA damage response. In our study, we found that after treatment with aclidinium bromide for 24 h, the populations of intact cells were decreased, while the numbers of early apoptotic and late apoptotic cells were significantly increased. Meanwhile, the expression of anti-apoptotic protein Bcl-2 in aclidinium bromide group was significantly decreased, and the expression of proapoptotic protein Bax and Active caspase-3 was significantly increased after treatment with aclidinium bromide for 24 h. Therefore, we hypothesized that aclidinium bromide may inhibit U2 OS cells proliferation by promoting the cell apoptosis. The PI3K/AKT pathway is an important signaling pathway that contributes to cellular growth. For instance, the PI3K/AKT pathway is involved in survival in different cell types, which is activated by receptors of growth factor and stimulates cell growth, and differentiation[46-48]. A serine/threonine kinase AKT is located downstream of class I and class III PI3K[49,50]. In the context of infection, activation of this pathway is associated with an increase in proliferation, cell survival, and cell migration, as well as enhanced protein synthesis via mTOR activation[51-54]. AKT activation is known to regulate cell cycle progression, cell death, and cell growth. In our study, aclidinium bromide exposure inhibited the phosphorylation of AKT and its downstream protein mTOR in U2 OS cells. Moreover, p-P70S6K protein was significantly decreased in aclidinium bromide-treated U2 OS cells. p-P70S6K is closely related to cell proliferation and cell cycle[55]. p-P70S6K was reported to be an analogous suppression of the phosphorylation of mTOR[56]. It has been shown that the PI3K signaling pathway is closely related to tumor formation; therefore, the expression of related proteins was analyzed with western blot assay. These results showed that the level of p-AKT and p-mTOR was significantly decreased in aclidinium bromide treated U2 OS cells. Meanwhile, p-P70S6K protein was significantly decreased in aclidinium bromide treated U2 OS cells. Collectively, these results suggested that aclidinium bromide might inhibit the proliferation and metastasis of U2 OS cells through blocking the PI3K signaling pathway.

Conclusions

We provided a scientific basis for aclidinium bromide application to the treatment of osteosarcoma in the future. Aclidinium bromide inhibited U2 OS cells proliferation and growth by suppressing PI3K pathway to mediate apoptosis. However, *in vivo* experiments, which might prove its drug efficacy, will be better in the future.

Ethics Committee approval

Not applicable.

Conflict of Interests

The Authors declare that they have no conflict of interest.

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