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ORIGINAL ARTICLE

Aclidinium Bromide holds promising inhibitory effects in A549 lung cancer cells potentials by regulating PI3K/AKT signaling pathway

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Summary

Purpose: *Muscarinic cholinergic receptors (MR) have been implicated to be overexpressed in lung cancer, and antagonists of MR have been proved to abrogate the growth of lung cancer cells. Aclidinium Bromide, identified as a muscarinic antagonist targeting muscarinic cholinergic receptor 3 (M3R) in the lung, is proposed as a maintenance treatment in patients with chronic obstructive pulmonary disease (COPD). In this study, we intended to investigate whether the Aclidinium Bromide held potential effects on lung cancer cells behaviors.*

Methods: *Human lung cancer A549 cell line was used and treated with Aclidinium Bromide. CCK-8 was used to assess cell proliferation, transwell was used to test cell invasion and Western blot assay determined the affected pathways. Apoptosis was detected by flow cytometry using Annexin V-FITC/Propidium iodide (PI) staining.*

Results: *The expression of Bcl-2 declined, and Bax and Caspase 3 increased in A549 cells treated with Aclidinium Bromide. Additionally, Aclidinium Bromide suppressed the PI3K/AKT signaling pathway in lung cancer A549 cells. CCK-8 showed that Aclidinium Bromide could suppress the growth of lung cancer cells A549. By transwell assay, Aclidinium Bromide displayed significant inhibition of cell invasion and migration capabilities. Aclidinium Bromide could induce cell apoptosis, which was detected by flow cytometry and western blot analysis of apoptosis-related markers levels.*

Conclusion: *Our results reveals that Aclidinium Bromide behaves as a novel M3R antagonist, and may inhibit lung cancer cell growth by regulating the PI3K/AKT signaling pathway, which sheds some light on that it might be a potential therapeutic agent for lung cancer.*

Key words: *Aclidinium Bromide, muscarinic antagonist, lung cancer, proliferation, migration, PI3K/Akt*

Introduction

As one of the most common malignancies, lung cancer causes approximately 1.4 million deaths per year worldwide [1,2]. At present, chemotherapy is still an indispensable treatment for lung cancer. Drug resistance and toxic side effects have become the biggest problem [3], therefore it is imperative to develop new drugs for lung cancer treatment.

In recent years, the role of non-neuronal cholinergic system in tumor formation and progression has been given more attention. Some recent studies proved that lung cancer cells are endowed

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with this non-neuronal cholinergic system [4]. Hua et al. analyzed R2HBJJ as a novel M3 receptor antagonist and showed that it can inhibit non-small cell lung cancer (NSCLC) cell growth and arrest the cell cycle in G0/G 1 [5].

Aclidinium Bromide is a novel, long-acting, muscarinic antagonist that inhibits the action of acetylcholine at M3 receptor in the lungs [6]. Aclidinium Bromide is used in the maintenance of bronchodilator therapy in patients with COPD [7]. However, the role of Aclidinium Bromide in lung cancer treatment remains unknown.

The aim of the present study was to illustrate the effect of Aclidinium Bromide on lung cancer cell behaviors and its potential mechanisms.

Methods

Cell culture

Human lung cancer A549 cell line was offered by Shanghai Institute for Biological Science (Shanghai, China). The Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 50 mg/ml streptomycin, at 37°C and 5% CO_2 incubator was used. When the confluence reached about 80%, the cells were treated with Aclidinium Bromide (10 μM) for 24 h, whilst the negative control group (NC) was treated with dimethylsulfoxide (DMSO).

Western blot

Total proteins in cells were extracted by RIPA lysis buffer (including protease inhibitor) after cells were treated with Aclidinium Bromide. Primary antibodies were as follows: AKT 1:1000 (Cell Signaling Technology [CST], Danvers, MA, USA, p-AKT 1:1000 (CST, USA), mTOR 1:1000 (CST, USA), p-mTOR 1:1000 (CST, USA), Bcl-2 1:1000 (CST, USA), Bax 1:1000 (CST, USA). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as internal control.

Cell proliferation

A549 cells were digested and counted when cultured at the logarithmic growth phase preparing cell suspension. DMSO 0.1% was used as the control group; 10 μM Aclidinium Bromide were put into the experimental group to be compared to the control group, then

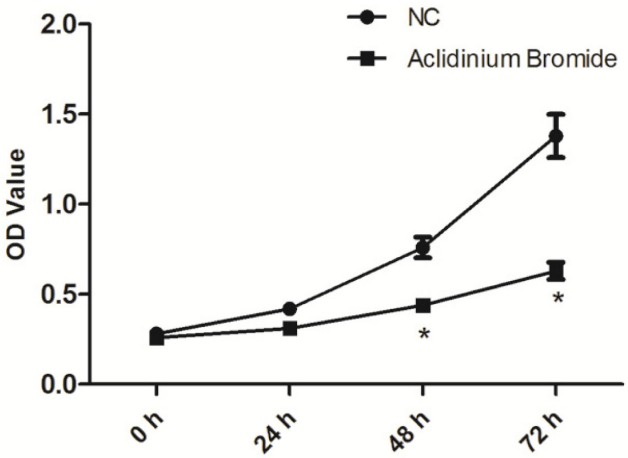

Figure 1. Aclidinium Bromide inhibits A549 cell proliferation in the CCK-8 assay. The results are the mean ± SD. *P<0.05 compared with normal control (NC) group.

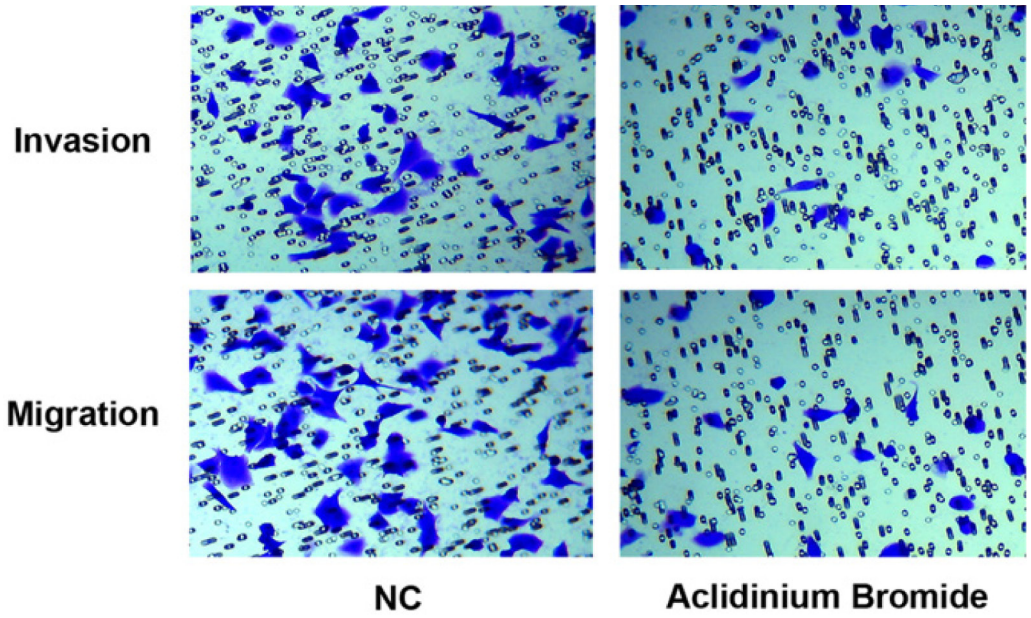

Figure 2. Aclidinium Bromide inhibits A549 cell invasion and migration. The images represent crystal violet stained A549 invaded or migrated cells, showing a significantly decreased number of invaded/migrated cells in Aclidinium Bromide-treated group in contrast with normal control (NC) group. Images were captured using an inverted microscope with x100 magnification.

10 µl of CCK8 reagent was added to each well of a 96-well plate. The absorbance was measured at 450 nm wave length by microplate reader (Bio-Rad, Hercules, CA, USA).

Cell invasion and migration assay

Transwell assay was used to test cell invasion. In the upper chamber 1×10^5 cells were placed in 24-well plate, and 500 µl RPMI-1640 medium were added in the bottom chamber. Invasion ability was analyzed using inverted microscope (Olympus, Japan). The migration assay was similar to the invasion assay and the difference was that the number of cells was 5000.

Cell apoptosis assay

Firstly, staining with 5 µl Annexin V-Fluorescein isothiocyanate (FITC) (eBioscience, Rockford, IL, USA) was performed for 5min at room temperature in the dark. Then, cells were stained with 10 µl PI, and were assessed using an Accuri C6 flow cytometry (Bio-Rad, Hercules, CA , USA). Experiments were performed in triplicate.

Statistics

SPSS 18.0 was used to analyze the data. The comparisons between two groups were assessed by Student's t-test. Data are shown as mean±SD and $p<0.05$ was considered as statistically significant.

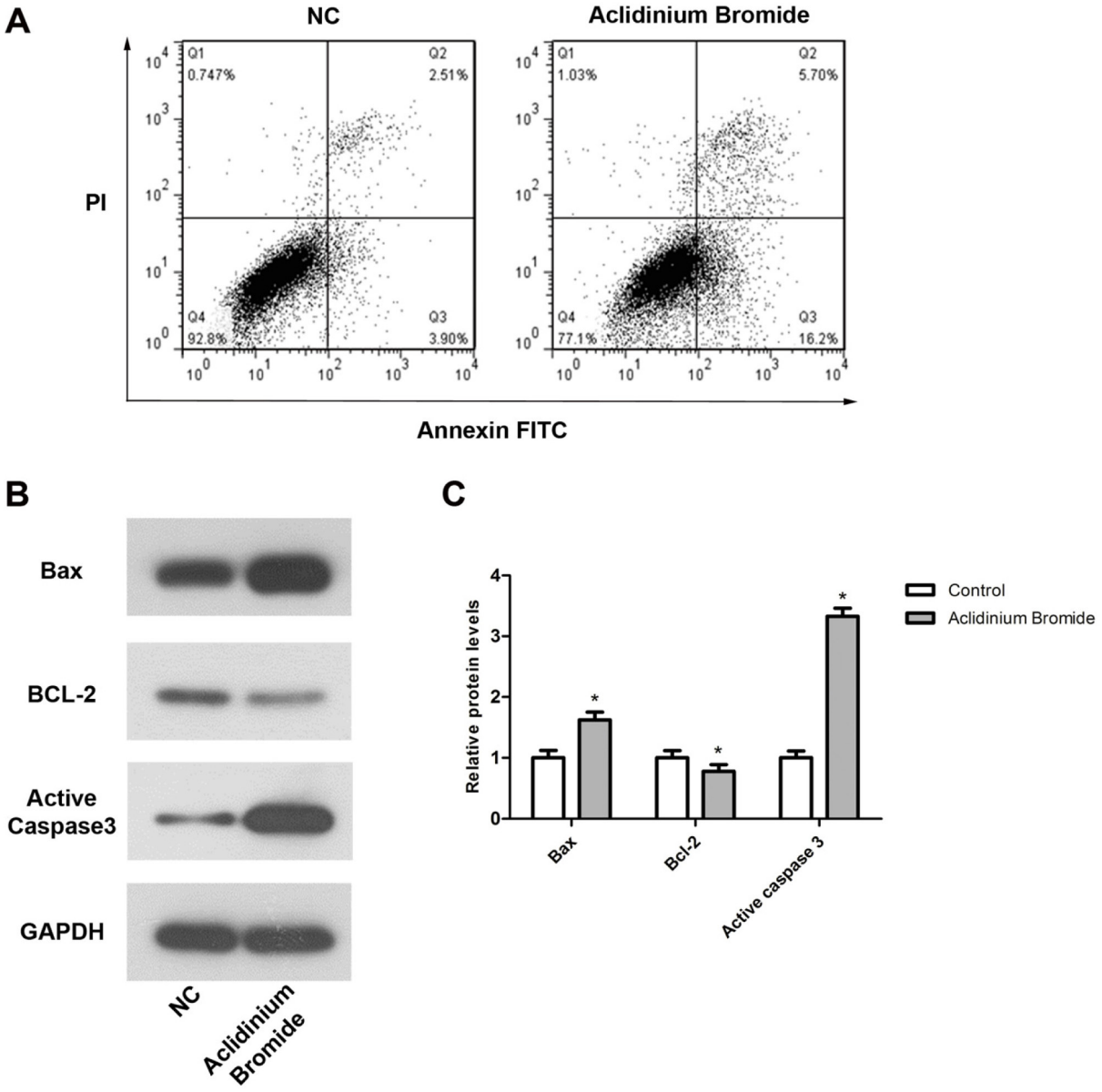

Figure 3. A549 cell apoptosis. **A:** Flow cytometric analysis was used to test A549 cells apoptosis and showed that A549 cell apoptosis rate was decreased by Aclidinium Bromide. **B:** Western blot and the band intensities were quantified and showed that anti-apoptosis protein Bcl-2 was increased while pro-apoptosis proteins Bax and active caspase-3 were decreased by Aclidinium Bromide. **C:** Relative protein levels of apoptosis-related proteins showing a statistically significant elevation of Bax and active caspase-3, and reduction of Bcl-2 in Aclidinium Bromide-treated group compared to normal control (NC). Data are expressed as mean ± SD. *P<0.05 compared with normal control (NC).

Results

Aclidinium Bromide inhibits lung cancer cell A549 proliferation

We firstly detected the functional effect of Aclidinium Bromide on cell proliferation. We selected lung cancer A549 cell line, and used Aclidinium Bromide at a concentration of 10 μM. In this experiment, Aclidinium Bromide inhibited the cell proliferation with potency values of 10 μM, which was in the range of the potency of this muscarinic antagonist in NSCLC cell growth [8]. What was found was that the optical density (OD) at 48 and 72 h in Aclidinium Bromide group was significantly lower than in normal control (NC) group (Figure 1, $p<0.05$). This suggests that Aclidinium Bromide could effectively inhibit A549 lung cancer cells proliferation.

Aclidinium Bromide inhibits the invasion and migration of lung cancer A549 cells

Next, we analyzed the effect of Aclidinium Bromide on the invasion and migration abilities of lung cancer cells A549 via transwell assay. The cells were stained with crystal violet, which reflected cell invasion and migration abilities. As demonstrated in Figure 2, the Aclidinium Bromide-treated group exhibited fewer cells in cell invasion and migration assay, respectively, compared to the control group. These results indicate Aclidinium Bromide could inhibit effectively lung cancer cell A549 invasion and migration.

Aclidinium Bromide promotes lung cancer A549 cell apoptosis

As indicated in Figure 3A, the apoptosis rate in Aclidinium Bromide-treated group was increased (5.7% > 2.51%, $p<0.05$). Corresponding to the flow cytometric analysis, the anti-apoptotic protein Bcl-2 decreased, while active caspase 3 and Bax increased (Figure 3B). These results imply that Aclidinium Bromide could promote lung cancer A549 cell apoptosis.

Aclidinium Bromide suppresses the PI3K/AKT pathways in lung cancer A549 cells

To explore the possible mechanism of Aclidinium Bromide effect on PI3K/AKT pathway, the expression of AKT, mTOR and P70S6K was initially detected by western blot in A549 cells. The results are demonstrated in Figure 4A and B, suggesting that Aclidinium Bromide significantly decreased

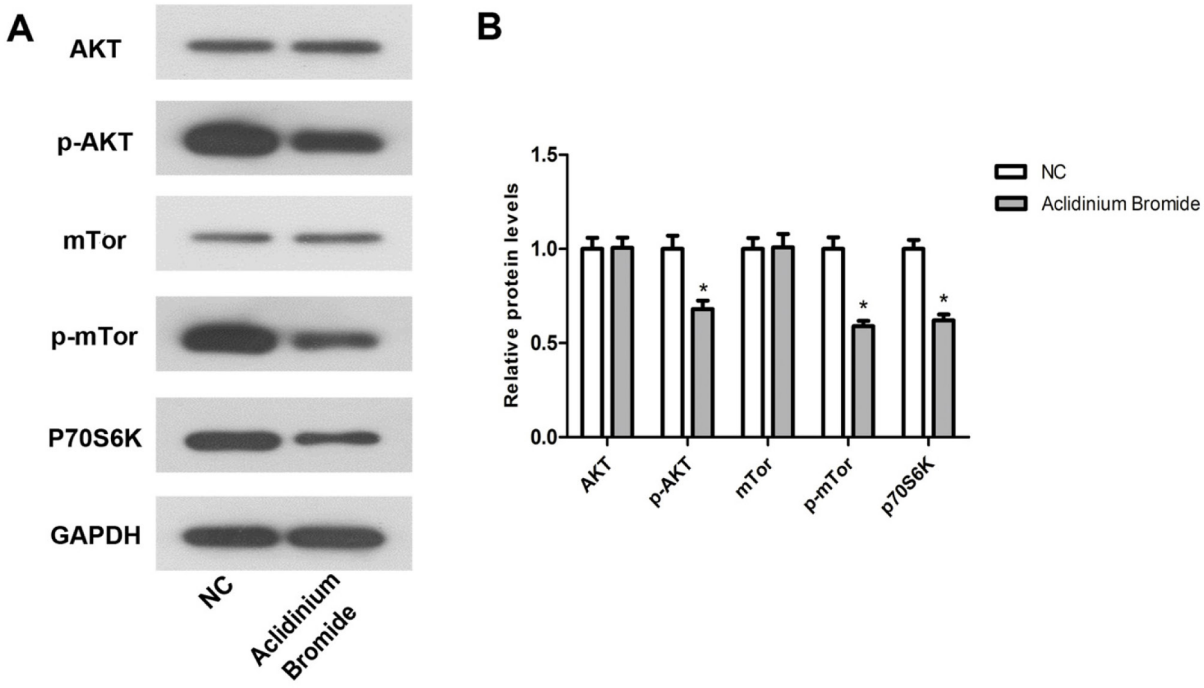

Figure 4. Effects of Aclidinium Bromide on the PI3K/Akt signaling pathway in A549 cells. **A:** Western blot was used to measure the expression levels of AKT, p-AKT, mTOR, p-mTOR, P70S6K, showing an obvious reduction in the levels of p-AKT p-mTOR and P70S6K, not AKT and mTOR in Aclidinium Bromide-treated group compared with normal control (NC) group. **B:** The relative protein levels of AKT, p-AKT, mTOR, p-mTOR, P70S6K compared with control group, showing a statistically significant decline of p-AKT p-mTOR and P70S6K, but not of AKT and mTOR in Aclidinium Bromide-treated group compared with normal control (NC) group. Data are expressed as mean ± SD. *P<0.05 compared with normal control (NC).

the expression of phosphorylated AKT and mTOR in A549 cells ($p<0.05$), but had no significant effect in the total AKT and total mTOR protein levels.

Discussion

The expression of the cholinergic autocrine loop does not uniquely occur in normal bronchial epithelial cells, but it manifests also in most lung cancers derived from airway epithelial cells [8,9]. Angelica et al. reported that muscarinic agonists stimulate human H508 and HT29 colon cancer cell invasion and migration [10-15]. It is also reported that stimulation of mAChR by cholinergic agonist carbachol for 20 h increased breast tumor cell death [16-18]. In colon, gastric and lung cancer, tumor growth has been associated with M3 subtypes of mAChR [19-21]. Song et al. selected M3 antagonist 4-DAMP and p-F-HHSiD, and observed that the two M3 antagonists significantly inhibited small cell lung cancer (SCLC) H82 cell line proliferation at 9 days [22]. The majority of reports show that M3 antagonists inhibit cell proliferation and evidence is particularly strong for their role in cancer progression. Since M3 antagonists inhibit cancer cell proliferation, Aclidinium Bromide, as a novel M3 antagonist, perhaps also inhibits lung cancer cell activity which provides a potential new drug development for lung cancer therapeutic management.

Aclidinium Bromide is approved as a maintenance bronchodilator therapy in patients with chronic obstructive pulmonary disease (COPD), and restrains the action of acetylcholine target M3 receptors in lungs, indirectly resulting in airway smooth muscle relaxation [23]. In this report, it is illustrated for the first time that Aclidinium Bromide could inhibit lung cancer cell proliferation. In our report, we used CCK-8 assay to analyze the cell proliferation treated with Aclidinium Bromide. Our results suggested that M3 antagonist Aclidinium Bromide inhibited the proliferation of NSCLC cell A549 *in vitro*. The anti-proliferative effect of Aclidinium Bromide may be related to its selective M3 receptor antagonistic activity. In contrast to Song's et al. report [22] our study elucidated M3 antagonist Aclidinium Bromide inhibited NSCLC cell growth but not SCLC cell growth. As a supplement to the study of Hua et al., treatment of NSCLC cells with the M3 antagonist Aclidinium Bromide in our study was A549 cell line, not H1299, H460 and H157 cell growth inhibition by muscarinic antagonist R2HBJJ in their report [24].

In this report, transwell assay showed that Aclidinium Bromide inhibited the migration and invasion of A549 lung cancer cell line. In earlier reports, the cell invasion and migration induced by M3 antagonists was rarely analyzed, such as with R2HBJJ a M3 antagonist [25]. Furthermore, Aclidinium Bromide could promote cell apoptosis and further increase the expression of pro-apoptotic protein caspase 3. This seems to be the case because, as shown in Figure 4, M3 antagonist Aclidinium Bromide decreases the phosphorylation of Akt and mTOR in A549 cells, while induces cell apoptosis and suppression of cell proliferation. Of note, Lu et al. have also shown that M3 antagonist 4-DAMP significantly inhibited the phosphorylation of Akt with decreased cell migration [26]. Thus, inflammatory factors secreted by lung tumors may also stimulate Akt phosphorylation through interaction with muscarinic receptors.

In conclusion, this study has shown that Aclidinium Bromide inhibits lung cancer growth, cell invasion and cell migration, and induces cell apoptosis through PI3K/AKT signaling pathway. Our study not only offers explanations for the possible mechanisms of Aclidinium Bromide for its antitumor competence but also establishes a foundation for further clinical research of Aclidinium Bromide in lung cancer treatment.

Authors' contributions

YH, GL and DM H conceived the study. DM H, DS and TL acquired, analyzed the data and wrote the manuscript. TL, YT G, WG and GH C interpreted the results and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflict of interests.

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