International Core Journal of Engineering Volume 7 Issue 2, 2021
ISSN: 2414-1895 DOI: 10.6919/ICJE.202102_7(2).0021

The Inhibitory Effect of Hexamethonium Bromide as a Possible α7-nAChRs Antagonist in Controlling Nicotine-Induced Cell Proliferation in Non-Small Lung Cancer Cells

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Abstract

Lung cancer is an urgent problem to the globe, and some of cases are highly connected to smoking according to the previous studies. Under this circumstance, nicotine plays a pivotal role in inducing and developing lung cancers especially in non-small lung cancer cells partially by elevating the expression of nAChRs, including α7-nAChRs. Therefore, inhibiting α7-nAChRs is a heated topic in treating lung cancers. One of the possible antagonist is called Hexamethonium bromide (C12H30Br2N2), which is a nicotine acetylcholine receptor antagonist. However, the specific mechanisms it triggers is still unknown. Hence, this paper reports possible results to three experiments that test hexamethonium's ability to inhibit non-small lung cancer cells proliferation, combine with α7-nAChRs as well as activate signaling pathways in the down-stream of α7-nAChRs.

Keywords

α7-nAChRs; Non-Small Lung Cancer Cells; Hexamethomium Bromide; Acetylcholine Receptor Antagonist; Nicotine-Induced Proliferation.

1. Introduction

Now lung cancer is one of the most common cancer all over the world. According to the research provided by IARC, it suggests that in 2012, the number of people suffering from lung cancer worldwide was 1.8 million, and the number of deaths was 1.6 million, ranking first in the global incidence of tumor suppression and mortality [1]. Now smoking is rendered as a factor that highly related to lung cancer. In developing countries, approximately 80% lung cancer cases have connection with smoking [2]. Until recently, scientists have detected 4000~5000 compounds in cigarette smoke, and about 60 compounds are carcinogens. Within the range of 60 compounds, nicotine is one of them to lead and promote lung cancer.

The various characteristics of nicotine are formed through its interaction with different types of nAChRs that is the abbreviation of nicotinic acetylcholine receptors. They are ligand-gated ion channels that consist of five transmembrane subunits (α1~α10, β1~β4, γ, δ, ε) [3]. The role of nicotine in the development of tumors is not only related to the increase in the number of new blood vessels, but it may be involved in the regulation of cells survival,apoptosis, proliferation and differentiation [4]. Research implies that reducing the activation of nAChRs provides treatments to lung cancer [5]. Besides, α-7 nAChR highly expresses in lung cancer cells. Whether in SCLC(small cell lung cancer) or NSCLC(non-small cell lung cancer) cell lines both exist a-7 nAChR [6]. According to one research study, α7 nAChR subunit was highly expressed in A549 and NCI-H1299 cells, about 16 and 24 times larger of those in normal cells respectively [7]. Nicotine induces fibroblast to produce fibronectin by stimulating the a7-nAChR signaling pathway, which leads to changes in the matrix composition of the lung cells [8]. And also treating small cell lung cancer (SCLC) or PNEC with nicotine can increase

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the expression of a7-nAChR causing calcium ion influx, activating Src, beta-arrestin, PKC, Raf-1, ERK1 / 2 and c-myc, leading to cell proliferation [9]. In this case, efficient a7-nAChR antagonist is in urgent need to cure lung cancer.

When it comes to hexamethonium bromide, apart from its common effect, researchers also propose that hexamethomium bromide is likely to be a kind of acetylcholine receptor antagonist, preventing the nicotine-induced inhibition of apoptosis [10]. They also estimate that hexamethonium bromide can combine with nAChRs, obstructing the specific ion channels and cutting off the signaling pathways that lead into the nicotine-induced proliferation [11] [12].However, until now there is only a few papers focusing on the relationship between hexamethomiun bromide and treating lung cancers. Given the fact that smoker who take into nicotine are most likely to catch SQ(squamous cell carcinoma), a kind of non-small cell lung cancer [8]. hence, this paper reports, when treated with hexamethomiun bromide, the possible changes in non-small cell proliferation and the activation of a7-nAChR in a condition that combined with nicotine, the major signaling pathways changes after combining with α7-nAChRs, as well as these results' implication with reasonable explanations.

2. Materials and methods

2.1 Chemicals and cells

2.11 First experiment: A549 cell line, nicotine, hexamethonium bromide, Nacl, as a negative control, TxlD , an alpha3 beta4 nAChRs antagonist, as a positive control. All solutions will be synthesized in the laboratory. Their concentration will range from 0.625 um, 1.25um, 2.5um, 5um, to 10um to form a titration.

2.12 Second experiment: A549 cell line, nicotine, radioactive labeled hexamethonium bromide, C2 loop florescence protein, a florescent protein selectively combining α7-nAChRs without changing major subtypes' functions [13], a series of substances in inserting C2 loop florescence protein and cultivating modified A549 cell line.

2.13 Third experiment: A549 cell line, nicotine, hexamethonium bromide, a series of substances in flow cytometry including fluo4/AM, a series of substances in phosphorylated westernblots used to test the activation of phosphrylated Src.

2.2 Methods

2.21 The first experiment: Stock cultures of A549 cells will be maintained on RPMI 1640 medium, kept at 37`C with 95% air and 5% CO2, and supplemented with 5% fetal calf serum. Next, Figure 1 shows that fifteen different culture medium would be numbered separately as 1,2,3,4,5,6,7,8,9,10,11, 12,13,14,15. Instill 0.625um, 1.25um,2.5um,5um,10um hexamethonium bromide saline into number 1,2,3,4,5 culture medium with A549 cells respectively. Instill 0.625um, 1.25um,2.5um,5um,10um TXlD solution into number 6,7,8,9,10culture medium with A549 cells respectively. Instill 0.625um, 1.25um, 2.5um,5um,10um Nacl saline into 11,12,13,14,15 culture medium with A549 cells respectively. All of the culture medium would be instill 3 mg nicotine. Before and after 14 days of daily treatment, the cells will be counted using microscope, all of the experiments will be done at least three times and a inhibitory rate chart will be settled.

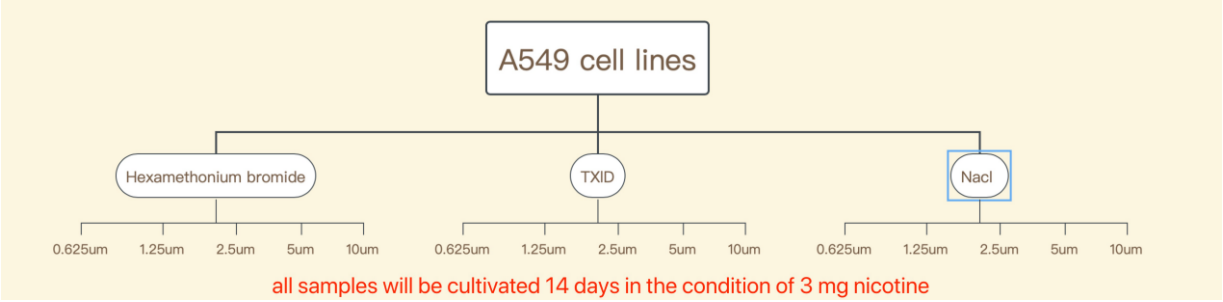

Figure 1. Setting of the first experiment

ISSN: 2414-1895 DOI: 10.6919/ICJE.202102_7(2).0021

2.22 The second experiment: Stock cultures of A549 cells will be maintained on RPMI 1640 medium, kept at 37`C with 95% air and 5% CO2, and supplemented with 5% fetal calf serum. Insert C2 loop florescence protein genes in RNA level, and send modified RNA back to naive, uncontaminated A549 cells to express the florescence protein in order to know the locations and amount of α7-nAChRs , then select A549 cells that express florescence stably. Cultivating selected A549 cells in the condition of 3mg nicotine for 14 days. At the same time, change hexamethonium bromide from $C_{12}H_{30}Br_2N_2$ to $C_{12}H_{24}T_6Br_2N_2$ as shown in Figure 2. Next, inject radioactive isotopes labeled hexamethonium bromide to the culture medium with the florescent modified A549 cells. All the results will be observed by laser scanning confocal microscopy and total internal reflectance fluorescence (TIRF) microscopy,nuclear detectors at the same scope. Then calculate the total number of radioactive spots, and total number of florescent spots, and the number of the overlapping spots, using the radioactive spots as a foundation to calculate the possible combining rate to a7-nAChRs of hexamethonium bromide under 95%CI.

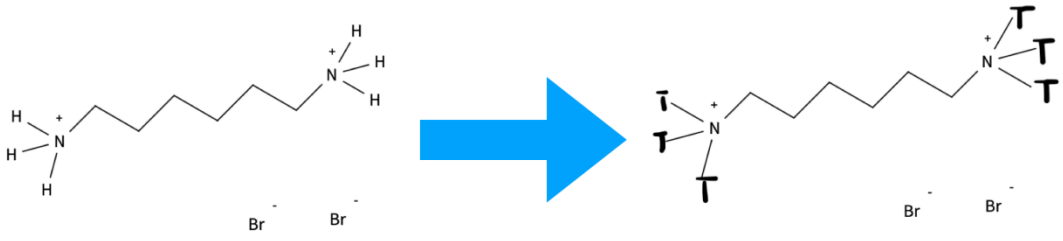

Figure 2. The structure of traceable hexamethonium bromide

2.23 The third experiment: Stock cultures of A549 cells will be maintained on RPMI 1640 medium, kept at 37`C with 95% air and 5% CO2, and supplemented with 5% fetal calf serum. Figure 3 suggests that twelve different culture medium would be numbered as 1,2,3,4,5,6,7,8,9,10,11,12. Instill 3mg nicotine in the number 1,2,3,4,5,6 culture medium ,Then instill 0.625um, 1.25um,2.5um,5um,10um hexamethonium bromide saline into number 2,3,4,5,6 culture medium with A549 cells respectively. After 14 days of daily treatment, the cells will be tested the activation of Src and beta-arrestin using phosphorylation western blots. As for number 7,8,9,10,11,12, each of them will be instilled 3mg nicotine. Then instill 0.625um, 1.25um,2.5um,5um,10um hexamethonium bromide saline into number 2,3,4,5,6 culture medium with A549 cells respectively. Next, load calcium indicator: fluo4/ AM to all of the culture medium and use software: Novocyte to test the influx of Ca2+.

3. Statistical analysis

The statistical significance of all numerical data gathered from negative and positive control, radioactive isotopes and florescence marking, phosphorylation western blots, flow cytometry will be using student's test.

4. Possible results

There are six possible results as shown in Figure 1.

4.1 hexamethonium bromide has no inhibitory effect. The comprehensive inhibitory rate of hexamethonium bromide is lower than that of saline group.

4.2 Hexamethonium bromide has inhibitory effect. The comprehensive inhibitory rate of hexamethonium bromide is higher than that of Nack saline group. Higher or lower than that of TXlD solution group.

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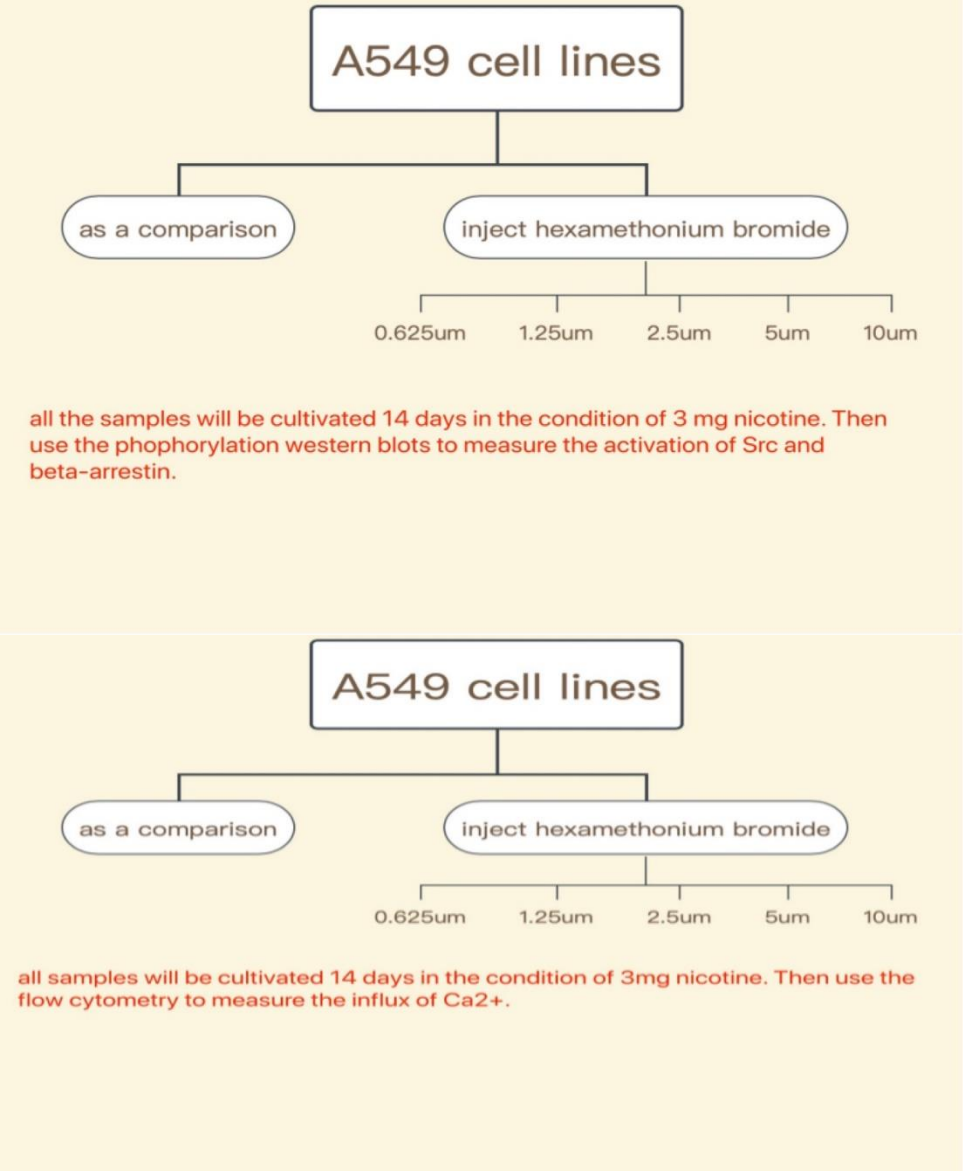

Figure 3. The setting of the third experiment

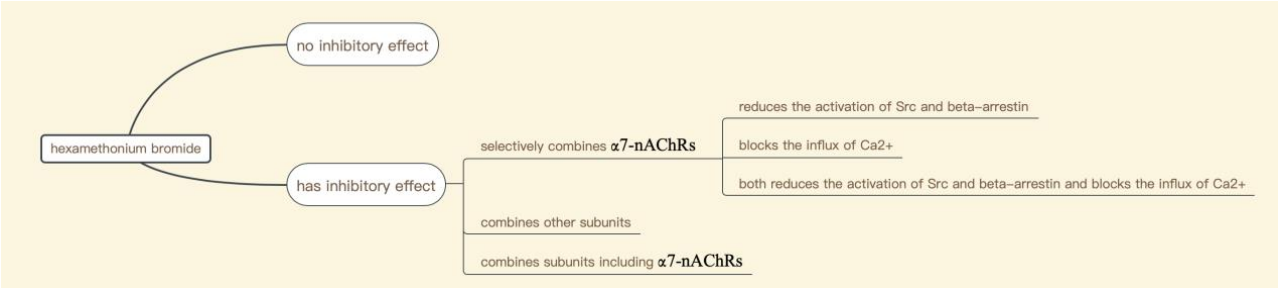

Figure 4. Six possible results

4.2.1 Hexamethonium bromide combines not only with α7-nAChRs, but also other subtypes. Under 95%CI, less than 95% but more than 5% points are overlapped in two pictures in the same scope.

4.2.2 Hexamethonium bromide does not combine with α7-nAChRs. Under 95%CI, less than 5% points are overlapped in two pictures in the same scope.

4.2.3 Hexamethonium bromide combines only with α7-nAChRs. Under 95%CI, more than95% points are overlapped in two pictures in the same scope.

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4.2.3.1 Hexamethonium bromide only blocks beta-arrestin-dependent Src pathway signaling. The expression of Scr and beta-arrestin are lower in the intervention group than that in the comparison group. The influx of Ca2+ of intervention group is basically at the same level compared with that in comparison group.

4.2.3.2 Hexamethonium bromide only blocks the influx of Ca2+. The expression of Scr and beta-arrestin are basically the same in the intervention group than that in the comparison group. The influx of Ca2+ of intervention group is at a lower level compared with that in comparison group.

4.2.3.3 Hexamethonium bromide blocks both beta-arrestin-dependent Src pathway signaling and the influx of Ca2+.`The expression of Scr and beta-arrestin are lower in the intervention group than that in the comparison group. The influx of Ca2+ of intervention group is basically at the lower level compared with that in comparison group.

5. Discussion

Previous studies have reported that hexamethomium bromide can reverse the nicotine-induced proliferation, blocking the specific ion channels and cutting off several signaling pathways. To confirm whether hexamethomium bromide has the capacity to suppress the abnormal NSCLC proliferation and apoptosis, and to test which subunit is hexamethomium bromide likely to combine with to sever the channel of influx of Ca2+ or beta-arrestin-dependent Src pathway signaling or both of them, a comprehensive study of hexamethomium bromide's effects on nAChRs in NSCLC is designed.

There are mainly six possible results to this study.

Firstly: hexamethonium bromide has no inhibitory effect. Secondly: hexamethonium bromide has inhibitory effect which combines not only with α7-nAChRs, but also other subtypes. Thirdly, hexamethonium bromide has inhibitory effect which does not combine with α7-nAChRs. Fourthly, hexamethonium bromide has inhibitory effect which combines only with α7-nAChRs, and only blocks beta-arrestin-dependent Src pathway signaling. Fifthly, hexamethonium bromide has inhibitory effect which combines only with α7-nAChRs, and only blocks the influx of Ca2+. Sixthly, hexamethonium bromide has inhibitory effect which combines only with α7-nAChRs, and blocks both beta-arrestin-dependent Src pathway signaling and the influx of Ca2+.

The first result is not consistent with several previous studies investigating the relationship between hexamethonium bromide and nicotine-induced apoptosis. Considering the fact that hexamethonium is a common nAChRs antagonist, besides nAChRS play significant role in lung cancer cells proliferation, researchers are required to make it clear the reason why NSCLC is the exception in the further investigations.

The second result successfully demonstrates hexamethonium bromide's effect on inhibiting nicotine-induced proliferation, but for a various kinds of subunits including α7-nAChRs. This result shows that although hexmethomium bromide is a ganglion blocker agent, targeting nAChRs, it can not selectively combine with single subunit, which provides evidence for Wendy L.Heusch and Rhoda Maneckjees' claim in their review that hexmethomium bromide based on its original property is able to regulate nicotine-induced apoptosis and proliferation [14]. However, why hexmethomium bromide bonds with different nAChRs subunits and what concrete kinds of subunits are they require further experiments.

The third result successfully demonstrates hexamethonium bromide's effect on inhibiting nicotine-induced proliferation, but for a various kinds of subunits withoutα7-nAChRs. This result shows that although hexmethomium bromide is a ganglion blocker agent, targeting nAChRs, it still can not selectively combine with α7-nAChRs even if the expression of α7-nAChRs in A549 is 16 times more than that in normal cells [7]. However, only a limited amount in NSCLC studies have been done to investigate hexmethomium bromide's blocking effect. Therefore, with insufficient evidence showing distinct affinity between hexmethomium bromide with different subunits in NSCLC,more studies

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casting into the determine what types of subunits hexmethomium bromide attaches, and the reasons behind the phenomenon, should be conducted.

The forth result successfully presents that hexamethonium bromide's effect on inhibiting nicotine-induced proliferation. Besides, it selectively combines withα7-nAChRs with cutting off beta-arrestin-dependent Src pathway signaling. This result is one of the complete explaination why hexamethonium bromide inhibits nicotine-induced NSCLC proliferation. Nevertheless, why treating with hexamethonium bromide does not cause reduction extend of the Ca2+ influx requires further investigations.

The fifth result successfully shows that hexamethonium bromide's effect on inhibiting nicotine-induced proliferation. Besides, it selectively combines withα7-nAChRs with blocking the Ca2+ influx channel. This result is another complete explaination why hexamethonium bromide inhibits nicotine-induced NSCLC proliferation, corresponding with the statements proposed by Piyali Dasgupta and Srikumar P.Chellappan [14]. Whereas why hexamethonium bromide does not sever beta-arrestin-dependent Src signaling pathway needs further studies.

The sixth result successfully shows that hexamethonium bromide's effect on inhibiting nicotine-induced proliferation. Besides, it selectively combines withα7-nAChRs both blocking the Ca2+ influx channel and severing beta-arrestin-dependent Src signaling pathway. This result is a intact explaination of the whole mechanism. To be more specific, this result is the most likely to happen given the fact thatα7-nAChRs is crucial in lung cancers, and hexamethonium bromide inhibits the nicotine-induced NSCLC proliferation with its special properties in blocking Ca2+ influx and severing signaling pathways.

When it comes to the methods in the experiments, there is a shortage in the second experiment using radioactive labeling plus fluorescent labeling technologies. Researchers have to control the dosage of hexamethonium bromide to make it sure that no extra hexamethonium bromide is loafing around, all of it combines with nAChRs in the picture. Unless up to this standard can they regard the radioactive isotopes as a background, then calculate the combining degree using fluorescent α7-nAChRs under the same scope.

6. Conclusion

In conclusion, this study investigates the inhibitory effect of hexamethomium bromide as a possible α7-nAChRs antagonist in controlling nicotine-induced cell proliferation in non-small lung cancer cells, and the likely signaling pathways and mechanisms under α7-nAChRs. The result of our study is able to tell whether hexamethomium bromide is a nAChRs antagonist in non-small lung cancer cells, indicate whether hexamethomium bromide only applies to α7-nAChRs, and the specific mechanisms behind it. However, regardless of the experiment results, further investigations are required to study cell lines difference, concrete kinds of subunits except the singleα7-nAChR if necessary, specific several pathways hexamethonium bromide shut down and mechanisms it triggers.

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