Anticancer Activity of Selective and Non-Selective Beta Adrenoreceptor Blockers against Non-Small Cell Lung Cancer Cell Lines

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Abstract: Beta adrenoblockers is a large class of drugs mainly used to manage abnormal heart rhythms. Over the last couples of decades, the anticancer effects of these compounds has been extensively studied. There is much evidence about their activity in non-small cell lung, pancreatic, breast, colorectal, prostate and ovarian cancer. However, the mechanism of beta blockers anticancer activity is still not known, and more detailed studies are needed.

The aim of our study was to evaluate the anticancer activity of beta adrenoblockers in non-small cell lung cancer cell lines A549 and H1299. In order to find the relationship with their selectivity to beta adrenoreceptors, in our study we used selective (atenolol, betaxolol, esmolol, metoprolol) and non-selective (pindolol, propranolol and timolol) beta blockers. The effect on cell viability was evaluated by MTT assay and the activity on cell ability to form colonies was tested by clonogenic assay. The type of cell death was evaluated by cell double staining with Hoechst 33342 and Propidium iodide. The most active adrenoblockers against both tested cancer cell lines were propranolol and betaxolol. They completely inhibited lung cancer cell colony formation at 90% of EC₅₀ (half maximal effective concentration) value. Most tested compounds induced cell death through apoptosis and necrosis. In A549 cell lines apoptosis was mainly induced while in H1299 cell line compounds induced both apoptosis and necrosis. There was no correlation established between beta adrenoblocker anticancer activity and their selectivity to beta adrenoreceptors.

Keywords: beta adrenoblocker, anticancer, non-small cell lung cancer, clonogenic, apoptosis, necrosis

1. Introduction

Lung cancer is the most common type of cancer and a leading cause of death worldwide, accounting for an estimated 9.6 million deaths [1]. Despite progress in diagnostics and treatment, lung cancer therapy remains problematic. Resistance to drugs is the main reason decreasing effectiveness of therapy [2] and 5-year survival rate is less than 18% [3]. Catecholamines norepinephrine and epinephrine, also called noradrenaline and adrenaline, are neurotransmitters simultaneously released from sympathetic nervous system and adrenal gland as a response to physiological and psychological stress, otherwise called flight-of-fight response. They regulate the activity of organs and cells, related to stimulation of sympathetic nerve system. According to scientists, elevated concentration of catecholamines promotes growth of lung adenocarcinoma micro metastasis [1, 7]. Conversely, beta adrenergic receptor antagonists (beta
adrenoblockers) by binding to the beta adrenoreceptors stop binding of norepinephrine and epinephrine at these receptors, thereby decreasing their stimulation and risk of the growth of cancer.

Beta adrenoblockers is a large class of drugs mainly used to manage abnormal heart rhythms. Over the last couples of decades the anticancer effects of these compounds has been extensively studied. The first evidence about beta adrenoreceptor involvement in lung cancer development occurred in 1989 [4]. According to recent studies, beta blockers also possess anticancer activity in pancreatic, breast, colorectal, prostate and ovarian cancer [2,3,5]. The researchers concluded that stimulation of beta adrenoreceptors by catecholamines leads to increase of extracellular concentration of cyclic adenosine monophosphate, which promotes proliferation of cancer cells [6]. Catecholamines are also involved in immune system functioning. These neuromediators promote resistance of cancer cells and tumor formation and growth by decreasing the amount and activity of lymphocytes and natural killer cells [7]. In animal models, antagonistic effect of non-selective beta adrenoreceptors blockers on beta-2 adrenoreceptors reactivated functioning of lymphocytes but did not improve survival outcomes [8]. However, beta adrenoblocker in combination with COX-2 inhibitors improved survival rates of mice [9]. It was proven that through the activation of beta adrenoreceptors COX-2 becomes active and therefore cancer cell growth and invasion is promoted through arachidonic acid pathway [12, 13].

In preclinical studies, beta blockers have been shown to reduce the proliferation, migration, invasiveness, angiogenesis of cancer cells and tumor immune response [8,12,13]. The exact antitumor mechanism of action of this class of drugs remains unclear and it is therefore important to carry out more detailed studies in cancer cell lines.

According to the results of clinical trials, beta adrenoblockers increase the survival rate of patients suffering from breast, prostate, ovarian, colorectal, skin and lung cancer. In recent years, new evidence has shown that overall survival of patients, who received beta adrenoblockers combined with radiotherapy, increased by 22% comparing to the control group [14]. Despite the evidence of positive effect of beta adrenoblockers on patients’ survival some data shows that intake of beta adrenoblockers and other medicines, affecting metabolism of catecholamines, is associated with increased risk of development of cancer and higher mortality [16, 17].

Although anticancer activity of beta adrenoblockers has been observed for almost two decades, there are not enough studies done to clarify their activity in non-small cell lung cancer (NSCLC) cell lines. Considering the problem and prevalence of lung cancer treatment, we decided to investigate the anticancer activity of beta adrenoblockers in NSCLC lines A549 and H1299. In this work, their effect on cell viability, clonogenicity and the type of cell death was investigated. Also, for studies we used bot selective and non-selective beta blockers to explore possible relationship between their anticancer activity and selectivity to beta adrenoreceptors.

2. Results

Beta adrenoblockers reduce the viability of NSCLC cells

All tested compounds reduced NSCLC cell viability at the highest used concentration of 500 µM (Figure 1a). Propranolol and betaxolol were the most active compounds in both cell lines. Propranolol showed a stronger effect on viability of H1299 cell line, while betaxolol acted in the same way in both cell lines (p < 0.05) (Figure 1b).
Propranolol possessed the highest antiproliferative activity (EC\textsubscript{50} values were 119.3 ± 12.7 µM and 98.8 ± 10.3 µM in A549 and H1299 cell lines, respectively). Betaxolol activity was about twice lower compared to propranolol (EC\textsubscript{50} values were 251.3 ± 14.6 µM and 252.2 ± 7.6 µM in A549 and H1299 cell lines, respectively).

![Figure 1](a) Effect of beta adrenoblockers on NCLSC cell viability. (a) effect of all tested compounds 500 µM concentration on A549 and H1299 cell viability; (b) EC\textsubscript{50} values of propranolol and betaxolol. * p < 0.05, compared to control; # p < 0.05, compared activity between cancer cell lines.

**Beta adrenoblockers inhibit growth of cell colonies in concentration-dependent way**

Tested beta adrenoblockers showed different effect on NCLSC cell colony formation (Figure 2). Propranolol and betaxolol at a concentration of 90% of EC\textsubscript{50} value completely suppressed colony formation ability in both cell lines (p < 0.05) (Figure 3a and 3b). All compounds except for atenolol at the higher concentration inhibited growth of cells colonies.

![Figure 2](A549 and H1299 cell colonies after incubation with 90% of EC\textsubscript{50} concentrations of beta adrenoblockers.)

Slightly weaker than propranolol and betaxolol, the number and area of A549 cell colonies was reduced by metoprolol at the higher used concentration in this study. Non-selective beta blockers timolol and pindolol and selective beta blocker esmolol were found to possess lower activity compared to propranolol and betaxolol, but the similar one between them. The lowest A549 cell colony formation ability was established for selective beta blocker atenolol.
Similar trends have been identified in the study of the effect of beta blockers on H1299 cell line. All compounds except atenolol had a statistically significant reduction in the number of colonies and area occupied by these cells (p < 0.05). The most active were esmolol and metoprolol (p < 0.05).

Figure 3. Effect of beta adrenoblockers on NCLSC cell colony formation ability. Comparison of compound effect of 90% of their EC₅₀ value on (a) cell colony number and (b) area of colonies; and compound effect of 10% of EC₅₀ value on (c) cell colony number and (d) area of colonies. * p < 0.05, compared to control.

Only betaxolol at a concentration of 10% of EC₅₀ value inhibited growth of A549 cells colonies, while pindolol and propranolol also decreased a size of colonies, comparing to the control group (p < 0.05) (Figure 3c and 3d). None of the compounds at lower concentration had effect on growth and size of H1299 cells colonies (p > 0.05).

Beta adrenoblockers mainly cause apoptosis

Most tested compounds induced cell death through apoptosis and necrosis. In A549 cell lines apoptosis was mainly induced while in H1299 cell line compounds induced both apoptosis and necrosis (Figure 4).

All the tested compounds induced apoptosis in A549 cell line even at concentration of 10% of calculated EC₅₀ value (p < 0.05). No statistically significant difference was found between apoptotic
effect of beta adrenoblockers at concentration of 10 and 90% of calculated EC50 value on A549 cells (p > 0.05). Only atenolol at both concentrations and betaxolol at lower concentration did not induce apoptosis in H1299 cell line (p > 0.05).

Figure 4. Effect of beta adrenoblockers on NCLSC cell death type. Number of apoptotic cells in (a) A549; (b) H1299 cancer cell lines; and number of necrotic cells in (c) A549 and (d) H1299 cell lines. * p < 0.05, compared to the control

No statistically significant difference was found between beta adrenoblockers effect on cell apoptosis in both cell lines.

Beta adrenoblockers mainly induced necrosis in H1299, but not in A549 cell line. All of the tested compounds with the exception of timolol induced necrosis in H1299 cell line (p < 0.05). Metoprolol, pindolol and propranolol almost did not cause necrosis of A549 cells and at lower concentrations did not have effect on H1299 cells (p > 0.05).

3. Discussion

Effect of beta adrenoblockers on cell viability is common subject of different scientific studies. Propranolol after 72 h of incubation inhibited cell viability of lung cancer A375 and melanoma P8 cell lines at concentrations 77.30 and 60.30 μM, respectively [17]. Similar results were obtained in
myeloma U266 cell line [18]. Difference between calculated EC\textsubscript{50} values can be explained by the fact that expression of receptors varies between different types of cell lines. In general, it is thought that non-selective beta adrenoblockers possess stronger effect on cell viability than beta-1 selective compounds [19]. Atenolol was from 7 to 50 times less active than propranolol in breast MCF-7, colorectal HT-29 and hepatocellular HepG2 cell lines. Similar results were achieved in this study. Atenolol was 6 times less active than propranolol. The amount of living cells after exposure to atenolol was 62.26% in H1299 and 65.12% in A549 cell line, comparing to 4.13% and 4.73%, comparing to a propranolol. Propranolol is non-selective beta adrenoblocker whilst atenolol is selective. Moreover, propranolol possesses membrane stabilizing activity. However, this tendency was not noticed in examining activity of the rest five substances used in the experiment. One of the most active compounds – betaxolol – is selective and propranolol is non-selective beta adrenoblocker.

We found that antiproliferative activity of beta adrenoblockers is not correlating with their selectivity to the receptors and might be depend on the compound lipophilicity and membrane stabilizing activity. Beta-2 adrenoreceptors in lung adenocarcinoma are responsible for lymphatic permeation and vascular invasion [20]. However, beta-2 adrenoreceptor expression in lung adenocarcinoma is not associated with worse survival outcomes in patients. In this study, only one of the non-selective beta adrenoblockers, propranolol, inhibited cell viability at concentration less than 500 μM. Betaxolol and propranolol possess the same selectivity to beta-1 adrenoceptors. However non-selective pindolol with the strongest beta-1 antagonistic activity of all the tested compounds was the least active compound in A549 cell line, but one of the most active compounds in H1299 cell line. Selective adrenoblockers esmolol and atenolol also were one of the most active compounds in H1299 cells, what might be a proof that expression of adrenoreceptors varies in cell lines themselves and selectivity of compounds is not the most important feature in order to predict anticancer activity of a substance. Zhang and the group suggested that activated k-ras gene mutation in cell lines might be responsible for the lower activity of beta-2 adrenoreceptor blockers [21]. This explains why propranolol was more active in H1299 cell line (p < 0.05), while betaxolol activity was the same in both cell lines (p > 0.05).

Effect of beta adrenoblockers on colony formation is not a common subject of scientific researches. Min and the group discovered that propranolol and atenolol at 10 μM concentrations suppress the growth and ability of A549 and H0CC-15 cells, treated by NNK, to form colonies [22]. In this study, 12 μM concentration propranolol reduced the size of A549 cell colonies, but atenolol even at 450 μM concentration did not have a statistically significant effect on colony growth. The deviation from expected results can be explained by the difference between cell lines and also methods of cultivation used in research. There is also evidence that propranolol in combination with radiotherapy and sumatinib reduces clonogenicity of stomach cancer and melanoma [17, 23].

Zhang and the group concluded that 100 μM concentration metoprolol does not cause apoptosis in pancreatic cell lines [21]. In this study metoprolol even at 50 μM concentration induced apoptosis in A549 and H1299 cell lines. The results of experiments may differ due to variation of expression of beta adrenoreceptors in cell lines and mechanism of action of drugs through metabolic pathways.

In another study propranolol at 50 μM concentration did not cause apoptosis of gastric adenocarcinoma BGC-823 and SGC-7901 cell lines, but in combination with radiotherapy after 48 h incubation it induced apoptosis, clonogenic survivability and cell viability [23]. In our experiment
propranolol induced apoptosis at 12 μM concentration, however, cells were incubated with solutions of compounds 72 h. Ability of beta adrenoblockers to cause apoptosis may be time-dependent.

In order to evaluate impact of beta adrenergic receptors on type of cell death, the effect of beta-2 selective adrenoblocker butoxamine, non-selective propranolol and beta-1 selective metoprolol were used to induce apoptosis in PC-2 pancreatic cancer cell line [24]. The apoptosis rate was the lowest after treatment with metoprolol, the highest – after treatment with butoxamine. According to the results of this study, it can be stated that apoptotic effect of beta adrenoblockers is mainly dependent on selectivity to beta-2 adrenoreceptors. It is worth noting that Zhang and others used only single compounds that possess specific selectivity to a certain type of receptors. In our study, lung cancer cell lines were treated with several different compounds possessing different selectivity towards beta adrenoreceptors, but no statistically significant differences between their effect were noticed. However, both NSCLC cell lines, A549 and H1299, possess K-Ras gene mutation that is thought to be responsible for lower sensitivity to non-selective beta adrenoblockers [21]. Moreover, different concentrations of compounds were used. It may be presumed that selectivity of beta adrenoblockers is important for anticancer activity in some specific cell lines, but not all of them in general.

4. Materials and Methods

4.1. Chemicals and materials

Atenolol (99% pure), betaxolol (96% pure), esmolol (98% pure), timolol (99% pure) and pindolol (99% pure) were purchased from Abcam (Cambridge, UK), metoprolol (98% pure) from Alfa Aesar (Massachusetts, USA) and propranolol (99% pure) from Acros Organic (New Jersey, USA). All tested compounds were dissolved in dimethylsulfoxide (DMSO, ≥99%, Ph. Eur.) which was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

TrypLE Express, Dulbecco’s modified Eagle high glucose medium (DMEM GlutaMAX), fetal bovine serum (FBS), penicillin/streptomycin solution (10,000 IU/mL), phosphate buffered saline (PBS, pH 7.4) were purchased from (Gibco, Carlsbad, CA, USA). Aqueous 16% paraformaldehyde solution (PFA), Hoechst 33342 (1 mg/mL) solution and Propidium iodide (1 mg/mL) solution were obtained from Thermo Fisher Scientific, UK.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, ≥ 97%) and crystal violet (≥90%) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Ethanol (96.6%) was obtained from Stumbras, LLC (Kaunas, Lithuania).

All cell culture plastic ware was purchased from Thermo Fisher Scientific, Corning and Techno Plastic Products.

4.2. Cell culture

Human NSCLC cell lines A549 and H1299 were obtained from prof. Esteller Manel (The Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain). Both cell lines were cultured in Dulbecco’s Modified Eagle’s Medium GlutaMAX, supplemented with 10% FBS and 1% antibiotics. Cells were incubated at 37°C temperature in a humidified atmosphere containing 5% CO₂. All cell cultures routinely were grown to 70% confluence and trypsinized with 0.125% TrypLE™ Express solution before passage. They were used until passage 20.
4.3. Cell viability assay
Cell viability was evaluated by MTT assay. A549 and H1299 cells were seeded in a 96-well plate at a concentration 5,000 cells/well and incubated overnight. After 24 h, cells were affected by different concentrations of beta adrenoblockers. Medium without cells served as a positive control, and the cells treated with medium containing 0.5% DMSO was used as a negative control.

After 72 h, 20 µL of MTT 0.5 mg/mL solution was added into each well of a 96-well plate and cells were incubated at 37°C for 3 h. Then supernatant was removed and formed formazan crystals were dissolved in 100 µL of DMSO. The absorbance was measured at 570 nm and 630 nm reference wavelengths using a multi-detection microplate reader. Experiments were repeated three times independently and the results were presented as means ± SD.

Applying Hill fit to compound dose – cell metabolic activity (absorbance) curves, the half maximal effective concentration (EC50) values, reducing cell viability by 50%, were calculated.

4.4. Cell colony formation assay
1000 of A549 and H1299 cells in a volume of 1 ml were seeded in 12-well and then were treated with 100 µL of 10 or 90% of EC50 values of adrenoblockers. Medium containing 0.5% of DMSO served as a negative control. H1299 cells were incubated for 8 days, and A549 for 12 days at 37°C in an atmosphere containing 5% CO2. Then the colonies were rinsed twice with PBS and fixed with 4% paraformaldehyde solution in PBS for 15 min. Colonies were stained with 0.1% aqueous crystal violet solution for 15 min and washed twice with sterile deionized water. Pictures were taken using G:BOX gel documentation system (Syngene International Ltd, Bengaluru, India) and analysed using Genesys software (Syngene International Ltd). The number and percentage area of colonies were calculated.

4.5. Evaluation of type of cell death
Lung cancer cells were seeded in 24-well plates at a concentration 15,000 cells/well and incubated for 24 h at 37°C in an atmosphere containing 5% CO2. Then 10 or 90% of EC50 values of adrenoblockers were added to the wells. After 72 h 3 µL of aqueous solution of Hoechst 33342 (1 mg/mL) and 1 µL of aqueous solution of Propidium iodide (1 mg/mL) were added to each well. After 10 min, images of cells were taken by inverted fluorescent microscope (Olympus IX73). Apoptotic and necrotic cells were counted, and the percentage number of cells was calculated.

4.6. Statistical analysis
Statistical analysis was performed using Microsoft Office Excel 2007 software (Microsoft Corporation, Redmond, WA, USA), evaluating an average and standard deviation of, at least, 3 measurements. Student’s t-test was used, and p-values were calculated. A value of p <0.05 was considered as the level of significance.

5. Conclusions
Our results show that both selective and non-selective beta adrenoblockers, especially betaxolol and propranolol, reduce the viability of NSCLC cell lines H1299 and A549. Betablockers inhibit formation of cell colonies and induce apoptosis and necrosis. Anticancer activity of tested beta adrenoblockers is not related to the selectivity to beta adrenoreceptors.
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References


Sample Availability: Compounds tested in this research are commercially available.