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**Research Article**

# *In vitro* evaluation of the effects of potential GSK-3β inhibitors terbutaline and orciprenaline

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**Abstract:** Lung cancer is a type of cancer that is mostly diagnosed at an advanced stage and has a short survival time despite standard chemotherapy and targeted therapies. Terbutaline and Orciprenaline are bronchodilator agents that are potent and selective β2 receptor agonists. The purpose of this study was to investigate to evaluate the effects of Terbutaline and Orciprenaline on A549 human lung carcinoma cell line and Beas-2b human bronchial epithelial cell line. Cells were treated with 1, 10, 100, 200, 400 µM concentrations of Terbutaline and Orciprenaline. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay and xCELLigence real-time cell analyzer were used to determine their effects on cell viability. The cell index was monitored continuously by visualizing the impedance of the E-plate wells. Because of the roles of Glycogen Synthase Kinase 3β (GSK3β) in a diverse range of cellular processes like metabolism, cell proliferation, differentiation and survival and its key position at several signaling pathways, GSK3β inhibition by Terbutaline and Orciprenaline was also investigated. The results showed that Terbutaline and Orciprenaline inhibits GSK-3β. The overall results led to the conclusion that Terbutaline and especially Orciprenaline may have potential therapeutic effects in lung carcinoma.

**Özet:** Akciğer kanseri, çoğunlukla ileri evrede teşhis edilen ve standart kemoterapi ve hedefe yönelik tedavilere rağmen hayatta kalma süresi kısa olan bir kanser türüdür. Terbutalin ve Orsiprenalin, güçlü ve seçici β2 reseptör agonistleri olan bronkodilatör ajanlardır. Bu çalışmanın amacı, Terbutalin ve Orsiprenalin'in insan akciğer karsinom hücre hattı üzerindeki etkilerini sürekli izleme yoluyla araştırmaktır. Hücreler 1, 10, 100, 200, 400 µM konsantrasyonlarda Terbutalin/Orsiprenalin ile muamele edilmiştir. Bu bileşiklerin hücre canlılığı üzerindeki etkisini belirlemek için 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) testi ve xCELLigence gerçek zamanlı hücre analizörü kullanılmıştır. Hücre indeksi, E-plaka kuyularının empedansı görselleştirilerek sürekli olarak izlenmiştir. Glikojen Sentaz Kinaz 3β (GSK3β)'nın metabolizma, hücre çoğalması, farklılaşma ve hayatta kalma gibi çok çeşitli hücresel süreçlerdeki rolleri ve çeşitli sinyal yollarındaki kilit konumu nedeniyle, bu moleküllerin GSK3β inhibisyonu araştırılmıştır. Terbutalin ve Orsiprenalin'in GSK-3β'yı inhibe ettiği belirlenmiştir.

***Edited by:***
*Melike Sapmaz Metin*

***Key words:***
*Lung cancer*
*Cytotoxicity*
*Bronchodilator agents*
*xCELLigence*
*Glycogen synthase kinase*

## Introduction

Cancer is the most difficult disease to treat and the second leading cause of human death cases worldwide. These fundamental compelling negative features of cancer lead to emergence of a situation that necessitates the use of alternative and new methods and drugs in treatment applications (Barbaros & Dikmen 2015, Schabath & Cote 2019). Although surgical methods and radiotherapy provide successful treatment in tumor destruction, some cancerous cell remnants and disease recurrences due to metastasis emerge as an important problem. From this point of view, it is now suggested that chemotherapy should be used in combination with these methods in achievement of optimum treatment outcomes. However, considering the inevitable damage that chemotherapy causes on healthy cells of the body in addition to the cancerous cells, the interest in individual, tumor-specific treatments has been increasing day after day (Barbaros & Dikmen 2015, Zhou *et al*. 2020). Lung cancers tend to spread in early stages and have a high recurrence rate despite aggressive treatment with surgery, chemotherapy, radiotherapy or combinations of these modalities (Zhou *et al*. 2020). Therefore, new treatment approaches should be followed in lung cancer cases, and studies should be carried out not only to stop the cancer

but also to improve the respiratory functions of the patient during treatments (Schabath & Cote 2019).

Glycogen synthase kinase 3β (GSK3β) is a serine/threonine protein kinase and attracted the attention of researchers for its roles in a diverse range of cellular processes like metabolism, cell proliferation, differentiation and survival and its key position at several signaling pathways that are crucial not only in cancer but also in various other human diseases (Cohen *et al.* 1978, Li *et al.* 2007).

Terbutaline is an agent that acts as a beta-adrenergic agonist, anti-asthmatic drug, bronchodilator and sympathomimetic agent. Terbutaline selectively binds and activates beta-2 adrenergic receptors, resulting in an increase in cyclic AMP production. Accordingly, it helps to relax the airway muscles, increases mucociliary clearance and decreases the release of inflammatory cell mediators (Peng *et al.* 2011, Sultan *et al.* 2023). Since Terbutaline is a highly functional substance in lung diseases, it is thought to be a promising agent in lung cancer as well (Peng *et al.* 2011, Zhou *et al*. 2020).

Orciprenaline, also known as Metaproterenol, is a potent and selective β2 receptor agonist in effect. It provides comfortable breathing by relaxing the muscles in the airways (DeNicola *et al.* 2001). Although Orciprenaline is structurally and functionally similar to Isoproterenol, it shows its effect only as a bronchodilator. In addition, it is advantageous compared to isoprenaline and adrenaline in terms of long duration of action, allowing oral administration and stabilization (Chahl & O'Donnell 1968).

There are limited research concerning the cytotoxic potential and mechanisms of action of the β2 receptor agonists Terbutaline and Orciprenaline. Carter *et al.* (2006) investigating whether agents that selectively bind to β2-adrenergic receptors directly affect the growth, survival and viability of prostate cancer cell line. They found that these agents caused antiproliferative effect *in vitro* suggesting that the sympathetic nervous system might indirectly affect prostate cancer progression through modulation of the immune system. Inbar *et al.* (2011) reported that Orciprenaline did not affect proliferation and viability rates in an *in vitro* study on CRNK-16 leukaemia cells and in an *in vivo* study with F344 rats.

In the present study, the cytotoxic effects of Terbutaline and Orciprenaline were evaluated by comparing the data obtained by testing Terbutaline and Orciprenaline on A549 human lung carcinoma cell line and Beas-2b human bronchial epithelial cell line *in vitro* using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay, Real-time cell analysis and GSK-3β kinase assay in order to determine their anticancer activity.

## Materials and Methods

A549 human lung carcinoma cell line and Beas-2b human bronchial epithelial cell line were purchased from the American Type Culture Collection. Ham's F-12, Roswell Park Memorial Institute medium (RPMI), 100 U/ml penicillin-100 µg/ml streptomycin were purchased from Sigma (St. Louis, USA). Fetal bovine serum (FBS) (Biological Industries, Cromwell, USA), 96-well E-plate (ACEA Biosciences, San Diego, USA) and GSK3β Kinase Assay kit (Promega, Madison, WI, USA) were used. MTT was purchased from Research Products International (RPI). Terbutaline, Orciprenaline and Pemetrexed were purchased from Medchem Express (NJ, USA).

### *Cell Cultures*

A549 human lung carcinoma cell line in Ham's F-12 and Beas-2b cell line in RPMI were grown containing 10% heat inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% $CO_2$.

### *Cell Proliferation Assay*

MTT Assay

A MTT assay was performed to test the effects of Terbutaline and Orciprenaline on the metabolic activity of the two cell lines. A549 (12500 cells/well) and Beas-2b (10000 cells/well) cells were plated in 96-well plates containing Ham's F-12 medium. Cells were treated with 1, 10 and 100 µM concentrations of Pemetrexed, Terbutaline and Orciprenaline. Pemetrexed was used as the positive control. Following the first treatment, the concentrations were increased to 100, 200, 400 µM. After addition 5 mg/ml MTT solution for 2 h exposure, the supernatant containing excess of MTT that did not attach to the cells or wells was disposed. After removing the incubation medium, formazan crystals were dissolved in 100 µL Dimethyl sulfoxide (DMSO) (Horáková *et al.* 2001). MTT reduction was quantified by measuring the absorbance at 570 nm (BioTek Synergy HT).

### *Real-Time Cell Analysis (RTCA)*

Cytotoxic effects of Terbutaline and Orciprenaline were monitored with xCELLigence RTCA system which measures electrical impedance and exhibits cell index. The system was used according to the manufacturer's (ACEA Biosciences) instructions with minor modifications. A549 and Beas-2b cells were seeded in 96-well E-plates. Due to real-time impedance measurement across biosensors frequent measurements have been made as it provides sensitive detection of cellular status from low cell counts to confluence. Cells were treated with 1, 10 and 100 µM, and then with 100, 200, 400 µM concentrations of Pemetrexed, Terbutaline and Orciprenaline. The experiments were run for 72 hours. The RTCA integrated software enabled the calculation of $IC_{50}$ (half maximal effective concentration) values.

### *GSK-3β Kinase Assay*

The GSK-3β Kinase Assay was performed using a custom commercial kit in accordance with the manufacturer's protocols. Enzyme, substrate, ATP and inhibitors were diluted in Kinase Buffer (40mM Tris [pH 7.5], 20mM $MgCl_2$, 0.1mg/ml BSA, 200µM DTT, 5% DMSO). Inhibitor, enzyme and substrate/ATP mix were added to the wells of a plate which was incubated at room temperature. After adding ADP-Glo™ Reagent, Kinase

Detection Reagent was added, waiting for the incubation period. The luminescence was recorded and the inhibition values (%) were calculated. The experiment is based on the principle that if there is inhibition, the remaining GSK-3β without inhibition uses the ATP and substrate added to the medium, luciferase converts luciferin to oxyluciferin with the help of ATP remaining from this reaction, and the resulting radiation is measured. The more ATP remaining from the uninhibited enzyme, the greater the inhibition and radiation.

*Statistical Analysis*

$IC_{50}$ (half-maximum inhibition concentration) values were calculated via the RTCA integrated software. Statistical analysis was performed using the GraphPad Prism Software Version 9.5.1 with $p < 0.05$ values considered statistically significant when compared to the control group. Descriptive statistics were calculated and reported as means and standard deviations. Statistically significant values were compared using one-way ANOVA and Dunnett's post-hoc test.

## Results

*Cell Viability Assays*

Monitoring of Terbutaline and Orciprenaline Effects in Real-Time Using RTCA

Pemetrexed started to show its effect after 60$^{th}$ hour and a significant specific cytotoxic effect was observed at 10 µM and 100 µM concentrations (Fig. 1a). Therefore, Pemetrexed was used in these two concentrations in further studies. Terbutaline had no cytotoxic effect at 1, 10, 100 µM concentrations (Fig. 1b). 400 µM concentration of Terbutaline decreased the cell viability (Fig. 1c), showing that Terbutaline does not show cytotoxic effects on Beas-2b cells (Fig. 1d).

Orciprenaline had no cytotoxic effect at 1, 10 µM concentrations (Fig. 2a). 100, 200 and 400 µM concentration of Orciprenaline decreased the cell viability (Fig. 2b). Orciprenaline did not show cytotoxic effect on Beas-2b cells at 400 µM concentration but showed a slight cytotoxic effect at 100 and 200 µM concentrations (Fig. 2c).

RTCA data was calculated from 24$^{th}$ and 48$^{th}$ hour cell index values and $IC_{50}$ values were calculated. $IC_{50}$ value of Terbutaline was 103.2 µM and the $IC_{50}$ value of Orciprenaline was 67.3 µM (Table 1) and sigmoidal concentration response curves are given (Fig. 3). These curves allow $IC_{50}$ values to be calculated using the sigmoidal concentration-response equation, which generates the curve that best fits the experimental data points according to mathematical functions.

**Table 1.** $IC_{50}$ values for 48$^{th}$ h*****

| Compound | $IC_{50}$ |
|---|---|
| Terbutaline | 103.2 µM |
| Orciprenaline | 67.3 µM |

***** The $IC_{50}$ of Terbutaline and Orciprenaline were acquired based on the concentration-response curves of CI over 48 hours of exposure.

Effects of Terbutaline and Orciprenaline on the Cell Viability as revealed by MTT Assay

According to the MTT assay results, 200 µM Terbutaline ($p < 0,01$); 100 µM Pemetrexed ($p < 0.01$) and 100 µM ($p < 0.01$), 200 µM ($p < 0.05$), 400 µM ($p < 0.01$) Orciprenaline significantly reduced viability in A549 cells (Fig. 4a). 400 µM Terbutaline ($p < 0.05$) and 400 µM Orciprenaline ($p < 0.05$) reduced viability in Beas-2b cells and it was determined that these molecules did not have cytotoxic effects at other concentrations applied to the healthy cell line (Fig. 4b, Table 2).

*GSK-3β Inhibitory Activities*

When the GSK-3β inhibitory activities was assessed, the changes were statistically significant for 100-400 µM Terbutaline ($p < 0.05$), 200 µM Terbutaline ($p < 0.01$), 100-200-400 µM Orciprenaline ($p < 0.01$) and 10-100 µM Pemetrexed ($p < 0.05$) when the groups were compared to the control (Fig. 5 and Table 3).

**Table 2.** Analysis of Variance (ANOVA) table with statistics output. (**a)** Data of A549 cells and, (**b)** Data of Beas-2b cells.

| ANOVA table | | SS | DF | MS | F (DFn, DFd) | *p* value |
|---|---|---|---|---|---|---|
| **Treatment (between columns)** | **(a)** | 7549 | 9 | 838.7 | F (9, 30) = 3.839 | 0.0025 |
| | **(b)** | 7154 | 9 | 794.9 | F (9, 30) = 2.385 | 0.0358 |
| **Residual (within columns)** | **(a)** | 6554 | 30 | 218.5 | | |
| | **(b)** | 9997 | 30 | 333.2 | | |
| **Total** | **(a)** | 14103 | 39 | | | |
| | **(b)** | 17152 | 39 | | | |

SS: Sum of squares; DF: Degrees of freedom; MS: Mean sum of squares; The F statistic is the ratio of intergroup mean sum of squares to intragroup mean sum of squares; *p*: Significance probability.

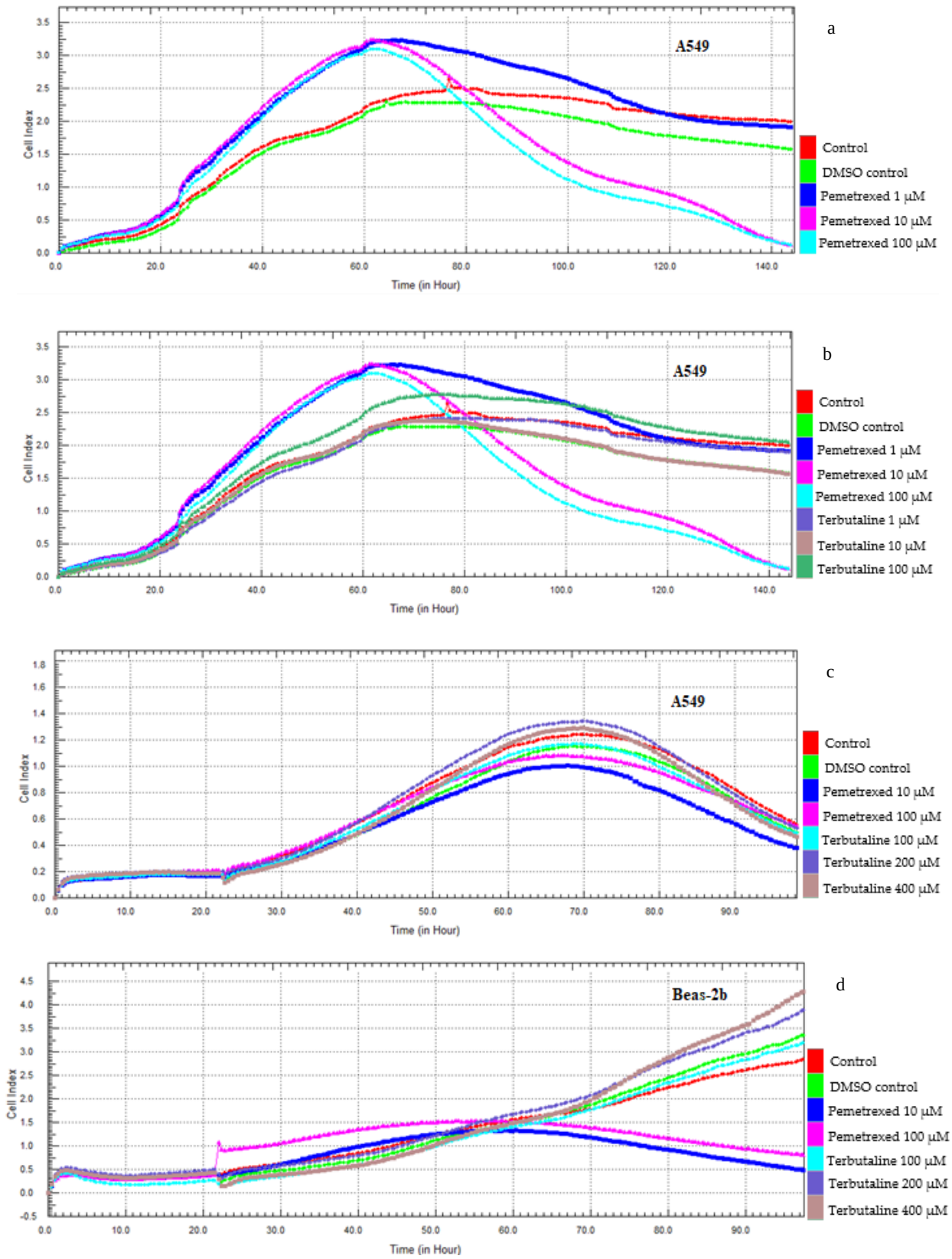


**Fig. 1.** Dynamic monitoring of effects of Terbutaline to A549 and Beas-2b cell proliferation. **a.** Effects of Pemetrexed on A549 cells, **b.** effects of Terbutaline (1, 10, 100 µM) on A549 cells, **c.** effects of Terbutaline (100, 200, 400 µM) on A549 cells, **d.** effects of Terbutaline (100, 200, 400 µM) on Beas-2b cells.

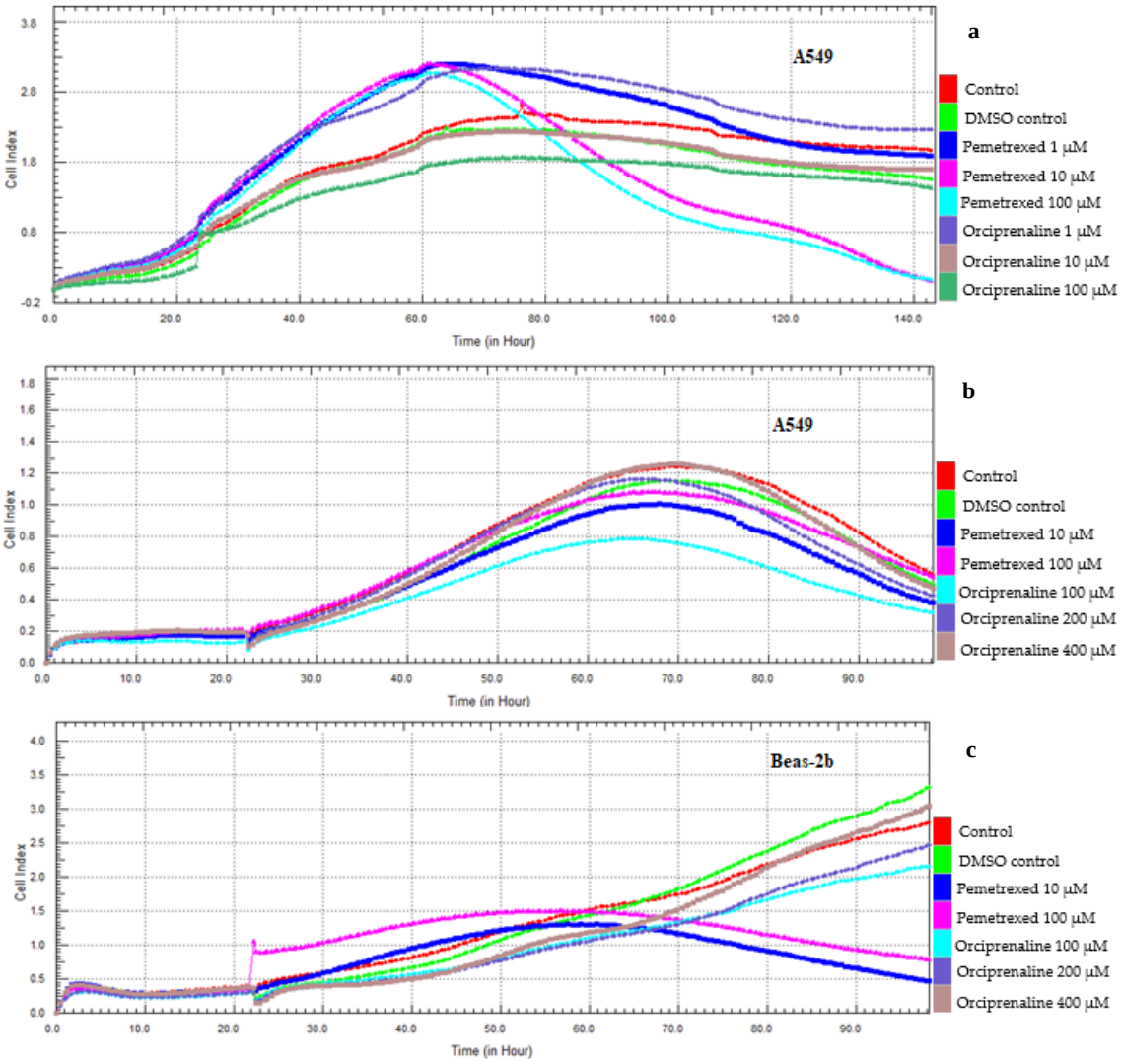


**Fig. 2.** Dynamic monitoring of effects of Orciprenaline on A549 and Beas-2b cell proliferation. **a.** Effects of Orciprenaline (1, 10, 100 µM) on A549 cells, **b.** effects of Orciprenaline (100, 200, 400 µM) on A549 cells, **c.** effects of Orciprenaline (100, 200, 400 µM) on Beas-2b cells.

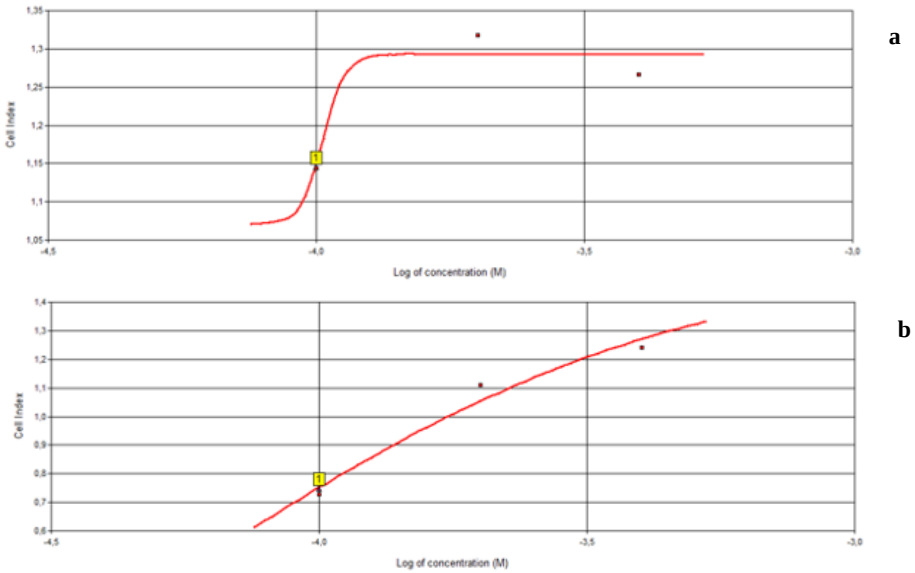


**Fig. 3.** Sigmoidal concentration response curve at 48 hours treatment of Terbutaline and Orciprenaline on A549 cell line. **a.** Terbutaline, **b.** Orciprenaline.

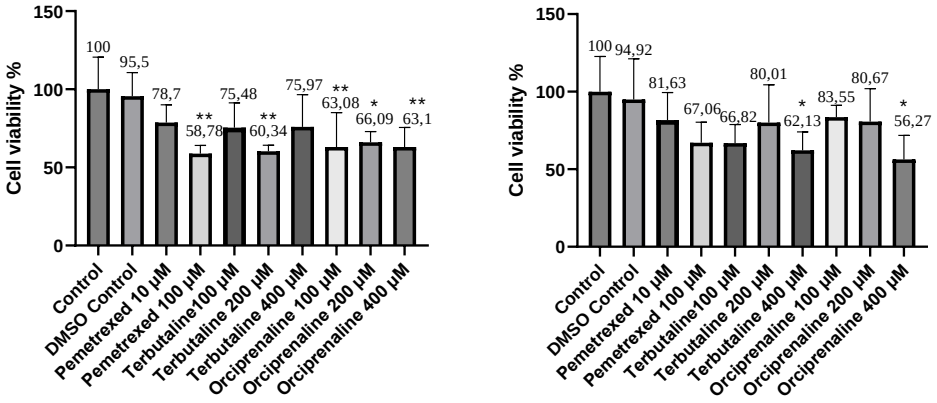


**Fig. 4.** Percent live cell presence values in **a.** A549 cells and, **b.** Beas-2b cells. The results are presented as the mean ± SD. *****$p < 0.05$ and ******$p < 0.01$ compared with the control (n=3).

**Table 3.** Analysis of Variance (ANOVA) table with statistics output.

| ANOVA table | SS | DF | MS | F (DFn, DFd) | *p* value |
|---|---|---|---|---|---|
| **Treatment (between columns)** | 0.01109 | 10 | 0.001109 | F (10, 22) = 1.014 | 0.4627 |
| **Residual (within columns)** | 0.02407 | 22 | 0.001094 | | |
| **Total** | 0.03516 | 32 | | | |

SS: Sum of squares; DF: Degrees of freedom; MS: Mean sum of squares; The F statistic is the ratio of intergroup mean sum of squares to intragroup mean sum of squares; p: Significance probability.

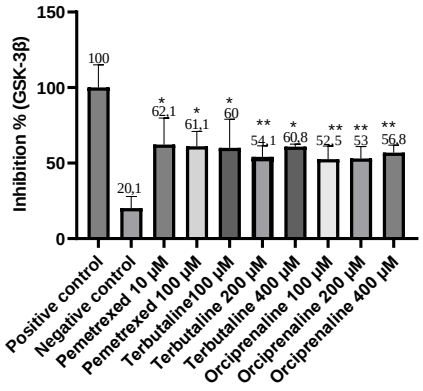


**Fig. 5.** GSK-3β inhibitory activities of Terbutaline, Orciprenaline and Pemetrexed (%). The results are presented as the mean ± SD. # $p < 0.05$ vs control (n=3). *****$p < 0.05$ and **$p < 0.01$ compared with the control.

## Discussion

RTCA is a system used for detecting cell proliferation and morphology change without using markers, making automatic measurements with real-time monitoring without requiring physiological contact, and imaging with high sensitivity and accuracy. The microelectronic cell sensor array integrated into the electronic plates allows measuring the electronic impedance of the electrodes, detecting and monitoring the changes on the electrodes.

Measuring the electronic impedance provides insight into the adhesion, proliferation and viability of cells. Changes in impedance due to cell attachment and spreading are expressed as a parameter called Cell Index (CI). In RTCA, the electronic impedance value increases as more cells are connected to the electrodes (Urcan *et al.* 2010, Uzunhisarcıklı & Yerer 2022).

In this study, Terbutaline and Orciprenaline were investigated for their cytotoxicity against cancer the cell line A549 and the healthy cell line Beas-2b. Since cytotoxicity is an important criterion for detecting the anticancer activity of a potential therapeutic agent, it was aimed to determine the effect of Terbutaline and Orciprenaline. The results were obtained by repeated xCELLigence measurements of dose-response curves. Cells were treated with Terbutaline and Orciprenaline while the index of cells increased proportionally to the number of cells. In biological activity studies, it was determined that both Terbutaline and Orciprenaline inhibited cell proliferation in A549 cell line and cytotoxic studies gave positive results, in which a better efficacy for Orciprenaline was determined. The results showed that the cytotoxic effect of Terbutaline and Orciprenaline on the Beas-2b cell line was low. 200 µM Terbutaline induced more effective GSK-3β inhibition than other applied concentrations. Similar results were obtained in terms of GSK-3β inhibition at all applied concentrations of Orciprenaline. These data raise the possibility that Terbutaline and Orciprenaline may act in a concentration-independent manner to reduce cell viability and target GSK-3β.

In a study that evaluated whether the activation of β2-adrenergic receptor signaling by Terbutaline, an β2-adrenergic receptor agonist, was involved in non-small cell lung cancer resistance to Apatinib therapy, Terbutaline increased the $IC_{50}$ of Apatinib, oral anti-

angiogenic drug that target Vascular Endothelial Growth Factor (Xu *et al.* 2022).

Studies on these Terbutaline and Orciprenaline investigated in cancer are not available in the literature, there are limited clinical studies, so it is important to elucidate the mechanism of action.

In this study, GSK3β inhibition, which is an important regulator enzyme in many disease pathogeneses including cancer, immune disorders, metabolic disorders and neurological disorders, was investigated and Terbutaline and Orciprenaline were found to inhibit GSK-3β. Some studies about tumors showing that GSK3β may have different effects on cell proliferation are presented below.

In a study on colon cancer cells inhibition of GSK3β activity with chemical inhibitors revealed that GSK3β has an unrecognized pathological role in cell survival and proliferation (Shakoori *et al*. 2005).

In most of the investigations of tumors such as MCF-7 breast cancer cells and hepatocellular carcinoma, GSK3β showed growth-inhibitory effect and therefore was predicted a tumor suppressor (Alao *et al.* 2006, Parekh & Rao 2007). In a study in NSCLC, O'Flaherty *et al.* (2019) investigated tumor growth inhibition using a paclitaxel combination and GSK3 inhibition. According to their findings, Paclitaxel and a GSK3 inhibitor worked in concert to prevent the progress of NSCLC cells both *in vitro* and *in vivo* (O'Flaherty *et al*. 2019). GSK3 inhibitors have been reported to have a potential role in cancer therapy by interfering with abnormal activation of signaling pathways in cancer cells, leading to cell cycle arrest, inhibition of proliferation and induction of apoptosis (Thapa *et al.* 2023).

In conclusion, Terbutaline and especially Orciprenaline may have potential therapeutic effects in lung carcinoma. It is considered that the information obtained about the cytotoxicity of Terbutaline and Orciprenaline will be beneficial for about future research.

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**Ethics Committee Approval:** Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

**Data Sharing Statement:** All data are available within the study.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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