**Propranolol Treatment Reduces A549-Derived Lung Cancer Spheroids via Intrinsic Apoptosis**

Menderes Yusuf Terzi¹,²

¹Department of Medical Biology, Faculty of Medicine, Hatay Mustafa Kemal University, Hatay, Türkiye
²Department of Molecular Biochemistry and Genetics, Hatay Mustafa Kemal University, Graduate School of Health Sciences, Hatay, Türkiye

**ABSTRACT**

**Objective:** Propranolol (PRO), a non-selective beta-adrenergic receptor inhibitor, has been recently discovered to possess anti-tumorigenic effects in cancer patients. Therefore, we aimed to investigate the in vitro effects of PRO in A549-derived lung cancer spheroids in terms of cell viability, spheroid formation, cell cycle regulation, cell differentiation, and apoptosis.

**Materials and Methods:** The effect of 24-hour PRO treatment on A549 cell viability was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. A sub-cytotoxic PRO concentration (125 µM) was employed to evaluate its impact on the clonogenicity of A549-derived cancer spheroids after seven days of incubation. Messenger Ribonucleic Acid (mRNA) levels of cell cycle regulators including cyclin-dependent kinase inhibitor 1A (p21) and G2 checkpoint kinase (WEE1), apoptotic markers such as caspases 3, 8, 9 (CASP3, CASP8, CASP9), and stem cell differentiation markers, namely POU class 5 homeobox 1 (octamer-binding transcription factor 4 (OCT4)), prominin 1 (CD133), and adenosine triphosphate (ATP) binding cassette subfamily G member 2 (ABCG2) were measured using reverse transcription quantitative polymerase chain reaction (RT-qPCR) after a 24-hour treatment of cancer spheroids with PRO.

**Results:** PRO treatment reduced cell viability and inhibited the clonogenicity of cancer spheroids by activating intrinsic apoptotic markers CASP3 and CASP9, leading to cell cycle arrest via increased p21 expression. PRO did not significantly alter stem cell differentiation markers.

**Conclusion:** The proliferation and clonogenic activity of lung cancer spheroids can be effectively suppressed with PRO, primarily through inducing intrinsic apoptosis following p21-mediated cell cycle arrest. While short-term PRO exposure did not affect the gene expression levels of stem cell differentiation markers, the notable decrease in both cell viability and spheroid formation efficiency suggests the potential of PRO as a therapeutic drug in lung cancer treatment.

**Keywords:** Propranolol, A549 spheroid, lung cancer, apoptosis, cell differentiation.
INTRODUCTION

The reduced therapeutic success of traditional cancer treatments over the long term, often leading to tumor relapses, can result in deaths post-chemotherapy and radiotherapy. This has incited researchers to develop novel and more effective strategies targeting cancer stem cells for permanent tumor eradication by triggering apoptosis. The β-adrenergic signaling pathway has previously been demonstrated to be implicated in the tumorigenesis of several cancer types, modulating numerous cellular events such as angiogenesis, inflammation, invasion/migration, Deoxyribonucleic Acid (DNA) repair, and apoptosis. It is now evident that the microenvironment around solid tumors provides strong survival signals to tumor cells via beta-adrenergic receptors. In this context, β-adrenergic receptor antagonists, also known as β-blockers like propranolol (PRO), have emerged as prominent alternative adjuvant therapeutic agents in cancer treatment by blocking tumorigenesis, metastasis, and angiogenesis.

PRO is a non-selective β-adrenergic receptor blocker drug that has been commonly prescribed for the treatment of cardiovascular diseases for decades. In a retrospective clinical study with non-small cell lung cancer (NSCLC) patients, it was reported that cancer patients receiving PRO concurrently with radiotherapy exhibited higher survival rates without metastasis or tumor relapses compared to those not on PRO medication. In parallel with the clinical studies, a previous in vitro cell culture model also demonstrated the anti-proliferative action of PRO on peripheral lung adenocarcinoma. Agonistic chemicals that activate β-adrenergic receptors, such as nicotine and mental stress, were shown to exert anti-apoptotic effects on NSCLC cells by activating β-receptors, effects that could be reversed using β-receptor antagonists.

Cancer stem cells (CSCs), which can be obtained from heterogeneous cancer spheroids in vitro, are characterized by higher tumorigenicity, metastatic rate, and drug resistance compared to their adherent progeny. CSCs can express prominent stem cell and drug transporter genes, thereby evading conventional cancer therapies and contributing to tumor relapses. To increase the success of cancer therapies, research to develop alternative and/or combinatorial adjuvant therapies is warranted. In this perspective, the present study aims to investigate the apoptotic, anti-proliferative, and cell-differentiating effects of PRO on lung adenocarcinoma cell line-derived cancer spheroids. For this purpose, the Messenger Ribonucleic Acid (mRNA) levels of prominent apoptosis, cell cycle, and cancer stem cell markers, i.e., caspases 3, 8, and 9 (CASP3, CASP8, and CASP9), cyclin-dependent kinase inhibitor 1A (p21), G2 checkpoint kinase (WEE1), prominin 1 (CD133), POU class 5 homeobox 1 (octamer-binding transcription factor 4 (OCT4)), and adenosine triphosphate (ATP) binding cassette subfamily G member 2 (ABCG2) were measured with reverse transcription quantitative polymerase chain reaction (RT-qPCR). Additionally, the clonogenic effect of PRO on tumor spheroids was analyzed using a spheroid formation assay.

MATERIALS AND METHODS

Cell Culture of Human Lung Cancer Cells and Spheroids

We obtained the A549 NSCLC cell line from the cell culture stock of the Medical Biology Department at Hatay Mustafa Kemal University, which was previously purchased from the American Type Culture Collection. The lung cancer spheroids were produced as previously described. Briefly, A549 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (HyClone, USA) with 10% Fetal Bovine Serum (FBS) (Gibco, Brazil) and 1% antibiotic mixture in an incubator at 5% CO2 and 37 °C. Upon reaching confluency, the cells were suspended and sub-cultured in serum-free DMEM containing human basic fibroblast growth factor (5 ng/mL, Life Technologies, Maryland, USA), human epidermal growth factor (10 ng/mL, Invitrogen, California, USA), and B27-Supplement (1%, Life Technologies, New York, USA) in ultra-low attachment Tissue Culture 25 (T25) flasks (Corning, Maine, USA) to cultivate lung cancer spheroids. After five-seven days, spheroid cells were passaged by replenishing the growth medium every third to fourth day. The spheroids were used for further experiments after the second passage.

Cell Viability Analysis with MTT Assay

We utilized the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to assess cell viability of A549 cancer cells post-PRO treatment at concentrations of 320, 160, 80, 40, 20, 10, and 5 μM, as described in a previous study. For this, 96-well cell culture plates were seeded with a density of 10^4 cells/mL (100 µL per well). After reaching confluency, the test group was incubated with PRO for 24 hours in a 37°C incubator. Thereafter, the media were removed and cells were treated with 1 mg/mL MTT (100 µL) (Sigma Aldrich, USA) in Dulbecco’s Phosphate-Buffered Saline (DPBS) (Sigma Aldrich, Germany) for approximately 2–3 hours at 37 °C. After incubation, the MTT solution was replaced with a Dimethyl Sulfoxide (DMSO) solution for a 3–5 minute incubation. The color change was measured at 590 nm (reference wavelength 670 nm) in a spectrophotometer (Multiskan Go, Thermo Fisher, Finland). Cell viability was calculated as a percentage of the control group. The half-maximal inhibitory concentration (IC50) of PRO was calculated using GraphPad Prism software (version 8.0.2). The sub-cytotoxic PRO concentration of 125 μM was used as the test concentration for further experiments.
Spheroid Formation Assay

We performed a spheroid formation assay to analyze the effect of PRO on clonogenic sphere formation efficiency. For this purpose, we followed a previously established protocol. Briefly, $10^4$ cells/mL were seeded into 6-well ultra-low attachment culture plates containing 125 µM PRO or just serum-free medium as a control for seven days. Spheroids with diameters greater than 80 µm were counted using a phase-contrast microscope (Nikon Eclipse TS100, Tokyo, Japan). The spheroid formation efficiency was calculated as a percentage of the initial seeded cell number.

RNA Isolation and Reverse Transcription Quantitative PCR (RT-qPCR)

The relative mRNA levels of the target genes were detected using the RT-qPCR method as previously described. Accordingly, we seeded $10^5$ spheroid cells per well (65x10^3 cells/mL) into 6-well plates and cultured them for seven days until spheroids formed. The spheroids were then treated with 125 µM PRO or just serum-free medium as the control for 24 hours. Thereafter, the spheroids were collected for total RNA isolation using the GeneJet RNA Purification Kit (Thermo Fisher, USA). After measuring RNA concentrations, 2 µg of RNA was converted into complementary DNA (cDNA) using the High Capacity cDNA RT Kit (Thermo Fisher Scientific, Lithuania) in a thermal cycler (Bio-Rad). The reaction conditions for reverse transcription were as follows: 10 minutes at 25 °C, 120 minutes at 37 °C, 5 minutes at 85 °C, and then held at 4 °C. The cDNAs were diluted (1:5) for subsequent gene expression analyses. For real-time detection of mRNA levels of CD133, OCT4, ABCG2, p21, WEE1, CASP3, CASP8, and CASP9, we employed the SYBR green method (Maxima SYBR Green, Thermo Fisher Scientific, Lithuania) in a Rotor Gene Q (Qiagen, Hilden, Germany) with the following cycling steps: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute, with a melting curve analysis by increasing 1 °C between 62 °C and 95 °C (Appendix 1, 2). To calculate relative gene expression, the raw data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers designed for qPCR (Thermo Fisher Scientific, Lithuania) are listed in Table 1, as determined using the NCBI Primer-BLAST Online Service. Relative gene expression differences were expressed as fold changes compared to the control group, based on the 2^ΔΔCt method (RT2 Profiler PCR Data Analysis, Qiagen, online service).

### Table 1. Primers used in qPCR analyses

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence</th>
<th>Amplicon (bp)</th>
<th>Anneal. (°C), cycle</th>
<th>Ref. Seq.</th>
</tr>
</thead>
</table>
| CD133       | F: 5′-GAGTCGAAAACCTGCGATATGCA-3′  
R: 5′-ACGCCCTTGCTTTGTTGTTG-3′ | 113 | 60, 40x | NM_006017.3 |
| OCT4        | F: 5′-TTCAGCCCAACGCACTCT-3′  
R: 5′-GGGTTCTGCTTGTGATCTC-3′ | 142 | 60, 40x | NM_002701.6 |
| ABCG2       | F: 5′-AACCTGTCTCAAGCCGATC-3′  
R: 5′-GTCGCCGGTGCTCCTTATC-3′ | 126 | 60, 40x | NM_004827.3 |
| p21         | F: 5′-CCGAAGTCAGTTCCCTTGG-3′  
R: 5′-CATGGGTTCTGACGGACAT-3′ | 112 | 60, 40x | NM_000389.5 |
| WEE1        | F: 5′-ACCAAGTTGCTTCTCCAAGA-3′  
R: 5′-CAGTGCCTATGGCTCAGGTC-3′ | 88 | 60, 40x | NM_003390.4 |
| CASP3       | F: 5′-CTTCTCACAAGATCCCCCTCAGA-3′  
R: 5′-TGTTCTTCTGAGCCCATGGTG-3′ | 102 | 60, 40x | NM_004346.4 |
| GAPDH       | F: 5′-GTCAAGCAGCTTGGGTGTATG-3′  
R: 5′-TGTAAGGGTGAAGGTAATGAAGG-3′ | 106 | 60, 40x | NM_002046.7 |
| CASP8       | PPH00359F 65 | 60, 40x |         |         |
| CASP9       | PPH00353B 114 | 60, 40x |         |         |

ABCG2: ATP binding cassette subfamily G member 2  
Anneal.: Annealing temperature  
bp: base pair  
CASP3, CASP8, CASP9: Caspases 3, 8, and 9  
CD133: Prominin 1  
F: Forward primer sequence  
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase  
OCT4: POU class 5 homeobox 1  
p21: Cyclin-dependent kinase inhibitor 1A  
R: Reverse primer sequence  
Ref. Seq.: NCBI reference sequence  
WEE1: G2 checkpoint kinase  
X: Times of cycle  
*: Qiagen RT2 qPCR Primer Assays (Maryland, USA)
**RESULTS**

We tested the anti-tumorigenic effects of PRO on spheroid cells derived from adherent A549 human adenocarcinoma cells *in vitro*. The results of the cell viability MTT assay, spheroid formation assay, and qPCR gene expression analyses are depicted in Figures 1–3 and summarized in Table 2.

**Table 2. Outcomes of MTT, spheroid formation assay, and qPCR analyses**

<table>
<thead>
<tr>
<th>PRO concentration, µM</th>
<th>320</th>
<th>160</th>
<th>80</th>
<th>40</th>
<th>20</th>
<th>10</th>
<th>5</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell viability, %, Mean±SD (n=5)</td>
<td>1.18±0.08</td>
<td>47.36±4.27</td>
<td>67.22±6.48</td>
<td>78.64±1.87</td>
<td>86.68±4.29</td>
<td>104.30±4.41</td>
<td>95.42±4.21</td>
<td>100.00±6.73</td>
</tr>
<tr>
<td>p, IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.0001*</td>
<td>163.2 µM</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Ordinary one-way ANOVA with Dunnett’s multiple comparisons test was used to compare multiple and two individual groups, respectively.

<table>
<thead>
<tr>
<th>Spheroid formation assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Spheroid formation, %, Median (min–max) (n=4)</td>
</tr>
<tr>
<td>p</td>
</tr>
</tbody>
</table>

Mann-Whitney U test was used to compare two individual groups.

<table>
<thead>
<tr>
<th>Relative gene expression analysis (qPCR)</th>
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<tbody>
<tr>
<td>PRO (125 µM)</td>
</tr>
<tr>
<td>Gene names</td>
</tr>
<tr>
<td>Fold change, 2&lt;sup&gt;-ΔΔCt&lt;/sup&gt;, Mean±SEM, (n=4)</td>
</tr>
<tr>
<td>p</td>
</tr>
</tbody>
</table>

Student’s t-test was used to compare two individual groups. RT² Profiler PCR Data Analysis online database was utilized for statistical analyses (Qiagen, https://dataanalysis2.qiagen.com/pcr).

ABCG2: ATP binding cassette subfamily G member 2; CASP3, CASP8, CASP9: Caspases 3, 8, and 9; CD133: Prominin 1; IC<sub>50</sub>: Half inhibitory concentration; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OCT4: POU class 5 homeobox 1; p21: Cyclin-dependent kinase inhibitor 1A; PRO: Propranolol; SD: Standard deviation; SEM: Standard error of mean; WEE1: G2 checkpoint kinase; *: P<0.05.

**Statistical Analysis**

All experiments were conducted at least three times. The Shapiro-Wilk test was used to analyze the data distribution. Multiple groups were analyzed with one-way Analysis of Variance (ANOVA) and Dunnett’s multiple comparison tests for the comparison of normally distributed data. The difference between two individual groups was determined using either Student’s t-test or the Mann-Whitney U test, depending on the data’s normality. P-values less than 0.05 were considered statistically significant. Statistical analyses and plots were prepared using GraphPad Prism software (version 8.0.2). For qPCR analyses, the RT² Profiler PCR Data Analysis online database was utilized (Qiagen, https://dataanalysis2.qiagen.com/pcr).

**Cytotoxic Effects of PRO on Lung Cancer Cells**

To observe the cytotoxic effects of various PRO concentrations (0–325 µM) on A549 cancer cells, an MTT assay was performed (Fig. 1). The analysis revealed that PRO concentrations ≥20 µM significantly reduced cell viability in A549 cells, with an IC<sub>50</sub> value calculated at 163.2 µM (p<0.05, Table 2). For further experiments, cancer spheroids in the test group were treated with 125 µM PRO, a sub-cytotoxic concentration.

**Reduction of Clonogenicity in Cancer Spheroids Following PRO Treatment**

Cultured spheroids derived from adherent A549 lung cancer cells at day seven, as depicted in Figure 2a, were used to perform a spheroid formation assay. This assay aimed to analyze the effect of PRO on the clonogenicity of cancer spheroids. After a 7-day exposure to 125 µM PRO, all spheroids greater than 80 µm in diameter were counted under a light microscope at a 4× objective (Figures 2b, c). The number of spheroids significantly diminished after PRO treatment compared to the untreated control group (p<0.05, Table 2).
Response of Cancer Spheroids to PRO via Intrinsic Apoptosis Ensuing Cell Cycle Arrest without Cellular Differentiation

To assess the effect of a 24-hour PRO treatment on mRNA levels of key stem cell differentiation, cell cycle, and apoptosis markers in cancer spheroids, the RT-qPCR method was utilized. As depicted in Figure 3 and Table 2, the relative expression levels of the target genes were expressed as fold changes (2^-ΔΔC_t) compared to the control group. The cell cycle regulatory gene p21 and the pro-apoptotic genes CASP3 and CASP9 were significantly upregulated after PRO treatment (p<0.05, Table 2), while there was no significant alteration in the G2 phase marker WEE1 and the initiator caspase of the extrinsic apoptosis pathway, CASP8 (p>0.05). Regarding cell differentiation markers (i.e., CD133, OCT4, and ABCG2), none showed significant changes upon PRO exposure (p>0.05, Table 2).

DISCUSSION

To date, various strategies have been employed to enhance treatment success rates and prevent metastases and relapses in cancer therapy. These include the use of natural products and adjuvant therapeutics, sensitization of cancer cells to chemotherapeutic drugs, and induction of immune system cells against tumor cells. However, due to the complexity of tumorigenesis and the heterogeneity of various cancer types, a leveraged treatment strategy that corroborates and/or replaces traditional cancer therapy has not yet emerged. This study investigates the effect of PRO, an anti-hypertensive β-blocker drug, on tumor spheroids derived from A549 adenocarcinoma cells. To the best of our knowledge, this is the first study to evaluate the cytotoxic activity of PRO on lung cancer spheroids.

In the first part of the study, the cytotoxic activity of PRO on A549 cells was determined using cell viability and spheroid formation assays. It was observed that PRO concentrations higher than 20 µM significantly reduced cell viability. As a sub-cytotoxic concentration, 125 µM PRO was selected for gene expression analysis and for assessing its cytotoxic and clonogenic effects on lung spheroids. We found that PRO at this concentration remarkably inhibited spheroid formation after a 7-day incubation period. In a similar study with the A549 cell line, findings parallel ours, showing that the half-effective concentration of PRO was around 120 µM, compared to ~160 µM in our study. Furthermore, a concentration of ~100 µM completely blocked the colony formation of cancer cells.13
In the second part, we evaluated the modulatory effect of PRO on prominent cell cycle, apoptosis, and cell differentiation markers. The attenuated cell viability in lung cancer spheroids was most likely driven by upregulated CASP9/CASP3-induced intrinsic apoptosis following PRO treatment, as there was no significant change in cell differentiation markers, namely OCT4, CD133, and ABCG2. In this study, the marked increase in p21 levels, but not WEE1, suggests that the spheroids were driven into apoptosis. In a previous study conducted by our group, PRO exhibited more notable cytotoxic effects in A549 cells compared to the non-synchronous healthy homologous cell line Bronchial Epithelial Airway Synthetic-2B (BEAS-2B), through p21-induced cell cycle arrest and caspase-dependent and independent apoptosis.5 Additionally, in another of our in vitro studies, an essential oil extract decreased the cell viability and clonogenicity of A549-derived cancer spheroids by downregulating mRNA levels of stem cell markers, but not through apoptosis, namely CD133, OCT4, and ABCG2, with the latter protein levels also being reduced.10 The fact that tumor relapses may arise from residual chemo-resistant cancer stem cells, which constitute a proportion of cancer spheroids in vitro, implicates drug transporter proteins as usual suspects. In the present study, the diminishing effect of PRO on cancer spheroids is more likely due to the induction of apoptotic pathways. Furthermore, the in vitro study mentioned earlier13 attributed the cytotoxicity of PRO on cancer cells to its anti-apoptotic effects, as ascertained with a dye exclusion assay. These results suggest that exposure of the same type of cancer spheroids to different agents might trigger alternative cell death-causing pathways. The branching vascularity due to activated angiogenesis is a well-known stage during tumorigenesis. PRO has been demonstrated to reduce increased angiogenesis by blocking the activated β-adrenoceptors in an in vivo mouse ovarian cancer model.14 Subsequent studies reported that the anti-tumorigenic effects of PRO arise not only from its anti-angiogenic actions but also from its apoptotic and anti-proliferative activities. For example, PRO reduced cell proliferation in an in vitro pancreatic cancer model by inducing the intrinsic apoptotic pathway, which corroborates our findings.15 A previous study showed that cancer cells treated with PRO can undergo cell cycle arrest at different cell cycle checkpoints, e.g., G0/G1, S/M, or G1/S.16 These findings, along with ours, suggest that PRO can exert its anti-proliferative and anti-apoptotic effects by arresting cancer cells at several cell cycle checkpoints, thereby reducing the cancer cells' potential for avoiding ultimate cell death. Lastly, the mechanisms underlying the anti-tumorigenic actions of PRO have been extensively studied in the literature through various preclinical and clinical cancer models. These studies attribute its effects to anti-proliferative, anti-angiogenic, and pro-apoptotic actions, as partly supported by our current study.17 Additionally, PRO has been shown to act as an anti-metastatic and anti-invasive drug by remodeling the extracellular matrix composition, primarily by inhibiting matrix metalloproteinases and reinforcing or restoring cell adhesion.17

Regarding the cytotoxic activity of PRO on cancer spheroids, there are limited studies in the literature. Among these, in vitro and in vivo studies conducted by Barathova’s group demonstrated that PRO exerted effects on 3D cancer spheroids and mouse xenograft models derived from colorectal cancer cells. It reduced cell viability and proliferation by hindering the adaptation of spheroids to hypoxic niches, inducing apoptosis,
and depleting the number of mitochondria. Additionally, Solerno et al. reported the anti-tumorigenic efficacy of PRO in both \textit{in vitro} and \textit{in vivo} osteosarcoma models. In these models, tumor growth was inhibited through reduced angiogenesis and induced G0/G1 cell cycle arrest and necrosis (but not apoptosis), using single or combined therapy strategies.

Beyond preclinical studies, the efficacy of PRO in cancer patients has also been extensively studied. In a clinical case study, an infant with pulmonary hemangioma was partially treated (reduced tumor size) with PRO without any side effects. An \textit{in vitro/\emph{in vivo}} study attributed this anti-tumorigenic activity of PRO in infantile hemangioma to the loss of cancer stemness feature via the blockade of Jagged1/Notch signaling, downstream of the $\beta$-adrenoceptor pathway. In another clinical study, a 61-year-old cardiac angiosarcoma patient with lung and liver metastases was treated with PRO as a single agent, resulting in reduced initial tumor size and metastases. In addition to its single anti-tumorigenic activities in cancer cases, PRO has been demonstrated to have chemo-sensitizing properties, serving as a potential alternative to traditional adjuvant therapies. A study by Pasquier et al. (2011) revealed that concomitant treatment of PRO with chemotherapeutics, especially when combined with paclitaxel, exhibited stronger anti-angiogenic and anti-proliferative effects compared to monotherapy in \textit{in vitro} models of several cancer types, particularly in breast cancer. Furthermore, they found that A549 the NSCLC cell line is among the most resistant to PRO treatment, with an IC$_{50}$ value of $\sim220$ $\mu$M. The significantly prolonged lifespan of animals with orthotopic breast cancer (2.5 times longer than the control group) following concurrent treatment with PRO and paclitaxel was another noteworthy result of their study. A recent study demonstrated the sensitizing effect of PRO to cisplatin in A549 cells by inducing apoptosis. The same study also showed that PRO could reverse the increased cell viability and cisplatin resistance observed after nicotine treatment in NSCLC cell lines. The anti-tumorigenic effects of PRO on NSCLC were evaluated in an \textit{in vitro} and retrospective clinical study, which revealed that PRO significantly sensitized NSCLC cell lines to both radiation and chemotherapeutic drugs. In the same study, advanced NSCLC patients (stage III) undergoing chemoradiation therapy in conjunction with PRO medication exhibited higher overall and metastasis-free survival rates compared to patients not receiving PRO. Conversely, one of the earliest studies in 1997 investigating the modulatory adjuvant therapeutic effect of $\beta$-adrenergic receptor agonists and antagonists found that the cisplatin sensitivity of NSCLC cell lines was significantly altered after co-administration with the agonist, but not with PRO. It can be speculated, considering the significant time span between the two studies (1997–2019), that the discrepancies may arise from differences in experimental settings, the status of the cell lines, and differences in the sensitivity of the molecular assays and materials used.

**Limitations**

The major limitations of the present study include the lack of analyses to measure the protein levels of target genes, absence of data regarding the effect of PRO on the metastatic ability of cancer spheroids, and the use of only a single type of lung cancer cell line. Additionally, the \textit{in vivo} effect of PRO could not be investigated using a xenograft animal model, which would have provided insights into the systemic or ectopic therapeutic effects of the drug. Nonetheless, to the best of our knowledge, this is the first experimental \textit{in vitro} study demonstrating the anti-tumorigenic actions of PRO, a commonly used anti-hypertensive drug, on A549 cell line-derived cancer spheroids. It is expected to contribute significantly to the literature.

**CONCLUSION**

In conclusion, the elevated levels of pro-apoptotic markers CASP3 and CASP9 may suppress tumor spheroid overgrowth by triggering intrinsic apoptosis following p21-mediated cell cycle arrest. The findings of this study suggest that although a short-term (24-hour) exposure of lung cancer spheroids to PRO did not alter the mRNA levels of stem cell differentiation markers, the marked decrease in both cell viability and spheroid formation efficiency positions PRO as a promising alternative option in cancer therapy.

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Appendix 1. Representative melting curves of target genes; CASP3, CD133, ABCG2, OCT4, and housekeeping gene GAPDH in A549-derived cancer spheroids analyzed with qPCR. ABCG2: ATP binding cassette subfamily G member 2; CASP3: Caspases 3; CD133: Prominin 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; OCT4: POU class 5 homeobox 1.
Appendix 2. Representative melting curves of target genes; WEE1, CASP8, CASP9, and p21 in A549-derived cancer spheroids analyzed with qPCR. CASP8, CASP9: Caspases 8, 9; p21: Cyclin-dependent kinase inhibitor 1A; WEE1: G2 checkpoint kinase.