Antineoplastic Effects of Erufosine on Small and Non-Small Lung Cancer Cells Through Induction of Apoptosis and Cell Cycle Arrest

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Research Article

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Abstract

Background: Lung cancer (LC) is one of the most common types of cancer with a high mortality rate. Depending on molecular and histological properties, LC is divided into non–small-cell and small-cell lung cancer. Not only surgery but also radiotherapy, chemotherapy, or combination treatment are used for patients. However, the survival rate of LC is still very low. Erufosine (ErPC3) is a novel promising antineoplastic agent and inhibits the translocation of AKT to the plasma membrane by dephosphorylating AKT.

Methods and Results: In the current study, the cell-type dependent effects of ErPC3 on cell viability, apoptotic situation, cell cycle distribution, related gene expression, and migration capacities of A549 and DMS 114 were investigated. As results, ErPC3 exhibited cytotoxic and pro-apoptotic properties against both cells, while DMS 114 was more affected. ErPC3 accumulated the cells in G2/M phase and blocked cell cycle. Proliferation markers were downregulated, while pro-apoptotic markers were upregulated in ErPC3 treated cells. Besides, ErPC3 displayed anti-migratory effect on A549 and DMS 114 compared to the control group according to scratch assay.

Conclusion: These findings promise a treatment approach and drug development against LC. The obtained results from the recent study lead it necessary to carry out more detailed studies about ErPC3.

Introduction

Lung cancer is the second most commonly diagnosed cancer type (11.4%) and has an 18% ratio among cancer-related death [1]. There are two different subtypes of lung cancer, non–small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) according to their molecularly and histologically characteristic [2]. LC generally occurs as NSCLC type (85%) and mostly derived from cigarette smoking [3]. However, 52% of lung cancer patients, especially women, are non-smokers according to recent reports [4]. In addition, genetic factors, aging, and environmental pollution may also cause the development of lung cancer [5–7]. SCLC is a more lethal type due to its aggressiveness and poor prognosis [8]. Most lung cancer patients cannot have surgery; they only take radiotherapy, chemotherapy, or combination treatment. These limited opportunities result in a short survival rate of five years [9]. In addition, LC diagnosis is delayed due to insensible symptoms and changes in epidemiology [10].

Variable processes activate apoptosis; inhibiting molecules such as Bcl-xl, Bcl-2, and the IAP family of proteins or promoting molecules such as Bak, Bax, and caspases have critical roles in apoptosis [11]. These pathways are often targets of cancer treatments. Besides, PI3K/AKT/mTOR pathway is upregulated in many human cancers, including lung cancer, hence, it has been recognized as a promising target, which could offer therapeutic potential through small molecule-based therapies [12]. This pathway contributes to tumorigenesis, tumor progression and drug resistance [13]. It is possible that AKT is a critical point and play role in lung cancer development due to serving active Akt in lung cancer precursors

[14]. In NSCLC, active AKT frequently is found and possibly related to higher progression and lymph node metastasis [15–17]. Therefore high AKT activity decreases the survival rate of patients [16]. AKT activation may derive from mutations, increased expressions of AKT isoforms (AKT1, AKT2 or AKT3) and their upstream regulators, and decreased negative regulators [17, 18]. Moreover, the expression level of AKT also relates to the chemoresistance of SCLC. The recurrence and developing drug-resistant contribute to the poor prognosis in SCLC patients [19].

Alkylphosphosphocholine (APC) differs from traditional chemotherapeutics, due to its interaction with cell membrane rather than DNA [20]. APCs are antineoplastic compounds with anti-apoptotic and cytotoxic activities [21]. Erufosine (erucylphospho-N,N,N-trimethylpropanolamine, ErPC3) is a novel membare-targeted agent and inhibits the translocation of AKT to the plasma membrane by dephosphorylating AKT on Ser473 protein [22, 23]. Antineoplastic effects of ErPC3 on various cancer types such as prostate cancer [23], leukemia [24], colorectal cancer [25], breast cancer [26], glioblastoma [27] and oral squamous carcinoma [28] have been proven. Besides, ErPC3 displayed pro-apoptotic activity via caspase-dependent signaling [29].

In the current study, the antineoplastic activity of ErPC3 on Human non-small cell lung cancer (NSCLC) cell line, A549 and small cell lung cancer (SCLC) cell lines, DMS 114 were evaluated. The comparative study was held to investigate the cell-type dependent effect of ErPC3 on cell viability, apoptotic situation, cell cycle distribution, related gene expression, and migration capacities of both cells.

Materials And Methods

Cell Lines and Reagents

Human non-small cell lung cancer (NSCLC) cell line, A549 and small cell lung cancer (SCLC) cell lines, DMS 114 were used for the current study. The cells were cultured in Dulbecco's Modified Eagle Medium with 4.5 g/L D-glucose (DMEM, Invitrogen, Gibco, UK) containing 10% fetal bovine serum (FBS, Invitrogen, Gibco, UK) and 1% of penicillin/streptomycin/amphotericin (PSA, Invitrogen, Gibco, UK) and incubated at 37 ºC in a 5% CO_2 humidified incubator. When the cells reach enough confluency (~ 80%), they were passaged by using 0.25% trypsin/EDTA (#25200-056, Invitrogen, Gibco, UK). Prof. H. Eibl from Max Planck-Institute of Biophysical Chemistry, Göttingen, Germany provided Erufosine (ErPC3). ErPC3 was prepared at a concentration of 20 mmol/L in saline and kept at 4°C for long-term storage.

Cell Viability Assay

To determine the effects of ErPC3 on the cell viability of A549 and DMS 114, MTS assay (3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (#G3582,CellTiter96 AqueousOne Solution; Promega, Southampton, UK)) was performed for 48 h. The cells were seeded onto 96-well plate at a concentration of $5*10^3$ cells/well. Next day, the cells were exposed to different concentrations of ErPC3 such as 6.25, 12.5, 25, 50, and 100 µM. 24 and 48h after administrations, MTS reagent was added onto the wells and incubated at 37 ºC in a 5% CO2 humidified incubator for 1h. The

plates were measured at 495nm by ELISA microplate reader (Biotek, Winooski, VT). 25 µM ErPC3 was selected for subsequent experiments according to MTS results.

Annexin V & Dead Cell Assay

Apoptotic situations of A549 and DMS 114 under 25 µM ErPC3 administration were evaluated by Muse® Annexin V & Dead Cell Kit (Merck Millipore, USA and Canada). The cells were seeded onto 6-well plate at a concentration of $0.1*10^6$ cells/well. Next day, the cells were exposed to growth media with and without 25 µM ErPC3. 48h after administration, the cells were collected from the wells and treated with the kit according to the manufacturer's instructions and analyzed with Muse® Cell Analyzer (Merck Millipore, USA and Canada).

Cell Cycle Assay

Muse® Cell Cycle Kit (Merck Millipore USA and Canada) was used for evaluating the cell cycle distribution of A549 and DMS 114 under 25 µM ErPC3 administration. The cells were cultured into 6-well plate at a concentration of $0.1*10^6$ cells/well. After day, normal growth media with and without 25 µM ErPC3 was added on the wells. 48h later, the cells were centrifuged and fixed with 70% cold-ethanol for 2h. The kit was applied to the groups according to the manufacturer's instructions and analyzed with Muse® Cell Analyzer.

Quantative Polimerase Chain Reaction (qPCR)

To evaluate the expression levels of apoptosis and proliferation-related genes of A549 and DMS 114 under 25 µM ErPC3 administration, qPCR analysis was carried out. AKT, BAX, BCL-2, CAS3, and CAS7 were selected as marker genes. The cells were seeded onto 6-well plate at a concentration of $0.1*10^6$ cells/well. Next day, the cells were administered with growth media with and without 25 µM ErPC3. 48h after administration, the pellets were obtained from the wells, and total RNA isolation were done by the kit (Roche, USA) according to the manufacturer's instructions. Later, isolated RNAs were used as samples in cDNA synthesis reaction by Transcriptor High Fidelity cDNA Synthesis Kit (Roche, USA) according to the manufacturer's instructions. Finally, SYBR Green (Thermo-Fischer, USA), gene specific primers and synthesized cDNA were mixed for qPCR analysis. The reaction was performed by running iCycler RT-PCR system (CFX Real Time System; Bio-Rad, Singapore).

Western Blot Analysis

The expressions of target proteins -Akt and p-Akt- of the 25 µM ErPC3 treated A549 and DMS 114 were evaluated by western blot analysis. The cells were cultured onto 6-well plate at a concentration of $0.1*10^6$ cells/well. Next day, the cells were exposed to growth media with and without 25 µM ErPC3. 48h after administration, the cell pellets were collected and total proteins were isolated by using Radio immunoprecipitation assay (RIPA) buffer (#sc-24948, Santa Cruz, USA). To calculate the protein concentrations, Bicinchoninic acid (BCA) assay (#23227, Pierce, Rockford, USA) was performed. For electrophoresis, Any kDTM Mini-PROTEAN®TGXTM precast gels (#456–9033, Biorad, USA) was used. After the running step, the gels were transferred to nitrocellulose membranes (122 – 0115, Biorad,

Germany). The blocking step was carried out with 5% nonfat dry milk powder (36-6404, Biorad, USA). The membranes incubated with primary antibodies of Akt (#ab9272, Abcam Cambridge, MA, USA) and p-Akt (#ab9271, Abcam Cambridge, MA, USA), at 4°C over-night. Next day, the membranes were washed with 1× Tris-buffered saline and Tween-20 solution (TBS-T) and incubated with anti-rabbit (#7074, Cell Signaling, Beverly, MA, USA) secondary antibodies at RT for 1 h. Finally, the membranes were photographed with a ChemiDoc MP imaging system (BioRad, USA) by using Enhanced chemiluminescence (ECL) substrate (# 1705060, BioRad, USA). The bands were normalized by using the expression of GAPDH (#8884, Cell Signaling, Beverly, MA, USA).

Scratch Assay

Scratch assay was carried out as a model experiment to determine the migration capacities of A549 and DMS 114 under 25 μM ErPC3 administration. They were seeded onto 6-well plate at a concentration of $0.1*10^6$ cells/well. When the cells reached to ~ 100% confluency, scratch model was formed by using a sterile 200 μL pipette. Then, normal growth media with and without 25 μM ErPC3 was added on the wells. The wells were photographed by the Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) at 0 and 24h and closure area rates were calculated by five randomly selected points using Zen 2011 software.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's post hoc test were chosen for statistical analysis. GraphPad Prism (version 7.00; GraphPad Software, Inc., San Diego, CA, USA) were used for all analysis and $*p < 0.05$ was accepted statistically significant results.

Results

The highest doses of ErPC3 significantly decreased the cell viability of lung cancer cell lines but displayed more effectiveness on DMS 114 compared to A549

A549 and DMS 114 were exposed to different concentrations of ErPC3 to detect the effects of ErPC3 on the cell viability of the cells for 48h. After 24h administration, the highest doses of ErPC3 (25, 50, and 100 μM) significantly decreased the cell viability of both cells compared to the control group (Fig. 1). Interestingly, 6.25 and 12.5 μM ErPC3 significantly increased the cell viability of DMS 114. The next day, ErPC3 caused significant differences except 6.25 and 12.5 μM compared to the control group. While 25 μM ErPC3 decreased the cell viability of A549 to 79.34 ± 12.79%, the cell viability of DMS 114 was decreased to 42.13 ± 8.9% at the end of the 48h. To evaluate the cell type-dependent effects of ErPC3 on the non-small cell lung cancer (NSCLC) cell line and small cell lung cancer (SCLC) cell lines, 25 μM ErPC3 was selected for both cells in subsequent experiments.

Apoptotic cell rates of the lung cancer cell lines were increased after ErPC3 administration

Annexin V & Dead Cell Assay was done to evaluate apoptotic situation of A549 and DMS 114 when they were exposed to 25 μM ErPC3 for 48h. There was a significant difference between control and treated groups in the aspect of live cell and apoptotic cell ratios in both cells (Fig. 2). In A549, ErPC3 caused 25.10% apoptotic cell ratio while 9.35% was measured in the control group. Besides, higher apoptotic ratio (61.40%) was showed in ErPC3 treated-DMS 114.

ErPC3 increased cell ratio in sub G0/G1 and caused accumulation of A549 and DMS 114 in G2/M phase

Cell cycle analysis was carried out to detect the cell cycle distribution of A549 and DMS 114 under 25 μM ErPC3 treatment for 48h. The cells displayed different distributions compared to the control group. Both cells gave higher apoptotic pick in Sub G0/G1 compared to the control groups. Besides, the cell ratios of ErPC3-treated A549 and DMS 114 in G2/M phase were significantly increased compared to the control group (Fig. 3).

ErPC3 administration caused overexpression in apoptosis-related genes and downregulation in the proliferation-related gene, BCL-2, for both cells. Besides, ErPC3 did not change gene and protein expression levels of AKT, while protein levels of p-Akt was down expressed compared to the control group

To observed the levels of apoptosis and proliferation-related genes in A549 and DMS 114 under 25 μM ErPC3 treatment for 48h, qPCR analysis was performed. In A549, the expressions of AKT and BCL-2, proliferation-related genes, were evaluated. AKT expression was not changed, while BCL-2 (~ 0.5) was significantly down expressed under the administration. Besides, ErPC3 significantly increased expressions of BAX, CASP3, and CASP7, which were apoptosis-related genes, to 3.11, 3.18, and 1.93 folds compared to the control group, respectively. Similarly, ErPC3 did not cause any difference in AKT levels. However, it significantly decreased the expressions of BCL-2 to 0.21 in DMS 114, while BAX, CASP3, and CASP7 were significantly increased to 3.46, 1.27, and 1.66 folds compared to the control group.

According to western blot analysis, the protein expression level of Akt in both 25 μM ErPC3 treated cells was not changed compared to the control group. However, active form of Akt, p-Akt, was down expressed in A549 and DMS 114 to 0.48 and 0.35 folds compared to the control group, respectively.

The migration capacities of both cells were significantly decreased under ErPC3 administration in scratch assay

To evaluate the effects of ErPC3 on the migration capacities of A549 and DMS 114, scratch assay was done. According to the analysis of taken photos at 0 and 24h, the wound closure rate of A549 under the administration was ~ 47%, while ~ 80% was measured in the control group (Fig. 5A). For DMS 114, ~ 25% closure rate was observed in 25 uM ErPC3 treated group at the end of the 24h, while closure rate in the control group was ~ 54% (Fig. 5B).

Discussion

According to the 2020 report, new Lung cancer (LC) cases and death rates were estimated as 2.2 million and 1.8 million, respectively [1]. Despite understanding the reasons for the development of LC such as smoking, genetics, and immune system's effects and the discovery of new treatment options, lung cancer still has a critical level for cancer-related death [30]. LC has been increasing since 2011 in developing countries in parallel with tobacco smoking [31]. Conversely, lower tobacco smoking rates decrease the incidence and mortality of lung cancer in the United States. Not only tobacco smoking but also gender, age, race, geography, and socioeconomic status also influence LC development and outcomes [30]. As small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), primary lung cancer is histologically and molecularly divided into different groups. 85% of all cases are NSCLC consisting of subtypes such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [32].

LC, especially SCLC, has high aggressiveness and chemoresistant among all malignant tumors. Tumor cell heterogeneity and cancer stem cells contributed to these properties [33, 34]. Classically, surgery and chemotherapy are the most preferred treatment options, however, the survival rate of lung cancer is very low with 5-year [35]. Many other factors also influence the poor prognosis of lung cancer such as age, sex, lung function, clinical and pathological stage, body constitution, comorbidity, and optimal treatment [36]. Depends on the high migratory capacities of lung cancer, many patients cannot even have an operation and chemotherapeutic agents alone are used in therapy [37]. The discovery of better treatment options for both lung cancer types is urgently needed.

25 μM and higher doses of ErPC3 significantly decreased the cell viability rate of both cells compared to the control group at 24 and 48h. Interestingly, 25 μM ErPC3 caused different effects between the cells at 48h. While the cell viability of A549 was decreased to ~ 81%, DMS 114 was decreased to ~ 42%. DMS 114 is a type of SCLC, the most aggressive and chemoresistant subtype of LC [38]. However, 25 μM ErPC3 displayed more toxicity on DMS 114 than A549. Besides, apoptotic situations of A549 and DMS 114 under 25 μM ErPC3 administration overlapped with cell viability analysis. Previous studies have shown that ErPC3 displayed pro-apoptotic effects on various cancer types, including prostate cancer [39], acute myeloid leukemia [24, 40], chronic lymphocytic leukemia [40], acute T-lymphocytic leukemia [41], oral squamous carcinoma [28], human glioblastoma [27]. According to cell cycle analysis, ErPC3 arrested A549 and DMS 114 in G2/M phase. Besides, higher apoptotic pick was measured in Sub G0/G1 phase for both ErPC3-treated cells compared to the control group. In previous studies, ErPC3 displayed pro-apoptotic effects on different cancer cells through blocking pass from G2/M to G0/G1 phase [42, 43].

Not only lung cancer subtypes but also other cancer types such as breast cancer [44], melanoma [45], gastric carcinoma [46] and hematologic malignancies [47] exhibit chemoresistant activity by using particular pathways which are responsible for many cellular processes. For instance, the PI3K/Akt/mTOR pathway is play an important role in the resistant mechanism. This pathway is a favorable goal for the development of anti-cancer agents [48]. AKT expressions of the A549 and DMS 114 under the 25 μM ErPC3 was not changed while active form of Akt (p-Akt) was downregulated compared to the control group. ErPC3 inhibits the translocation of AKT to the plasma membrane by dephosphorylating AKT on Ser473 protein [22, 23]. Besides, BCL-2 expression, an anti-apoptotic marker [39], was decreased in 25 μM

ErPC3-treated group for both cells. The typical target in cancer therapy is the activation of the cascade of proteolytic enzymes, caspases. Moreover, Bax activation is also a marker of cell death [49]. When A549 and DMS 114 were treated with ErPC3, expressions of Caspase 3 and 7 and Bax were significantly decreased compared to the control group. Cancer cells spread away from their primary site, and migration capacities of the cells are related to aggressiveness. The anti-migratory effects of anti-carcinogenic drugs indicate antineoplastic activity. In the current study, migration capacities of 25 μM ErPC3 treated A549 and DMS 114 were significantly decreased compared to the control group. Similar results were also reported by the previous studies in different cell types [39, 50].

Overall, LC incidence and mortality rate is increase day by day worldwide. There are two different main types NSCLC and SCLC, with different characteristics. Used treatment options including surgery, radiotherapy, chemotherapy, or combination are not fully successful. ErPC3 has a novel promising agent, according to the current study. The cell-type dependent antineoplastic activity of the ErPC3 on the A549 and DMS 114 has been demonstrated. In further studies, *in vivo* LC model should be conducted to support the anti-cancer effects of ErPC3 have on an organism.

Declarations

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Author Contributions

Hüseyin Abdik: Investigation, Methodology, Validation, Visualization, Writing - original draft.

Ethics declarations

Conflicts of interest

The authors declare no conflicts of interest.

Ethical approval

This study does not require ethical statement.

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

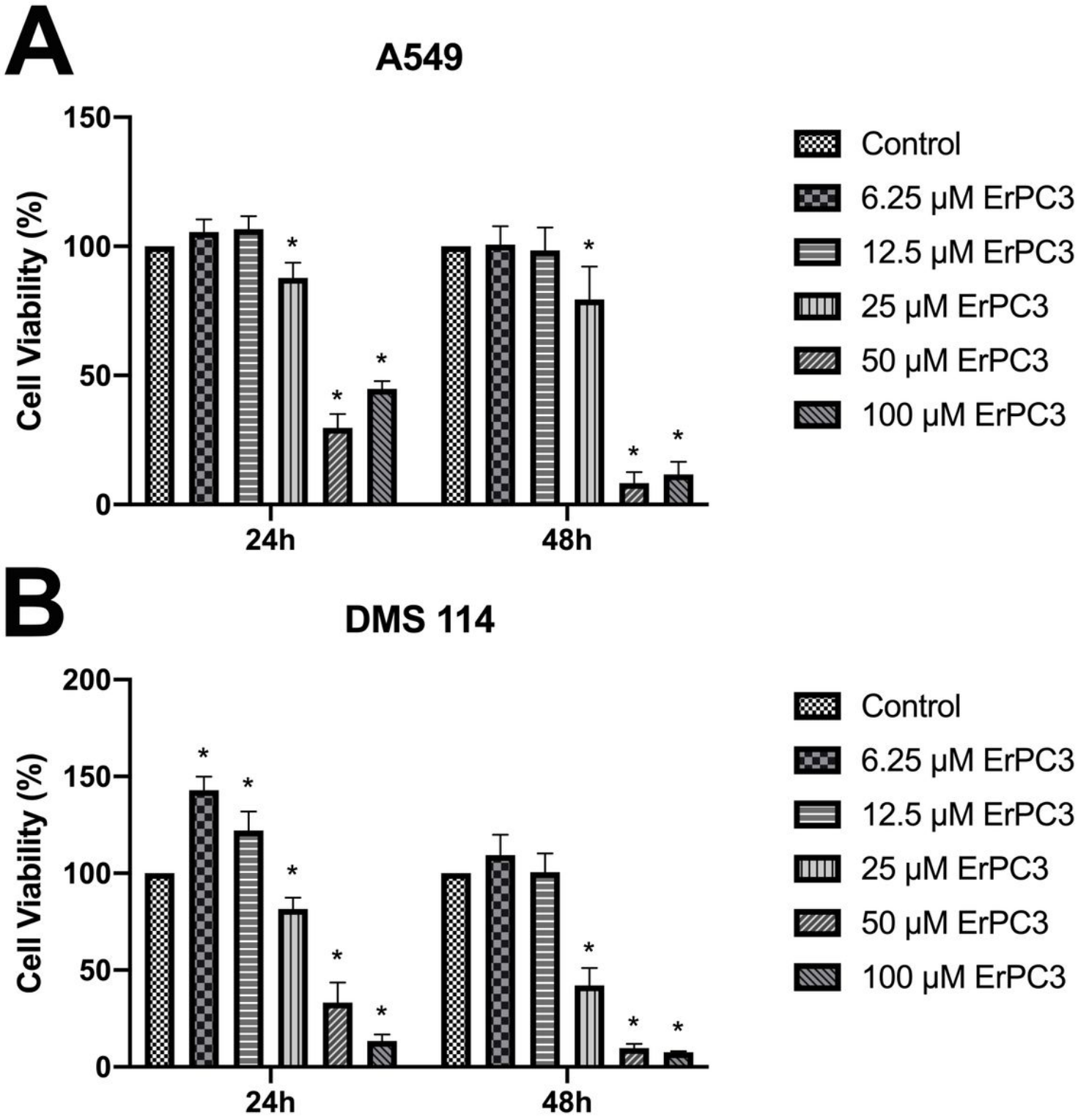

Figure 1

The effects of different concentrations (6.25-100 μM) of ErPC3 on the cell viability of A549 (A) and DMS 114 (B). (*$p < 0.05$).

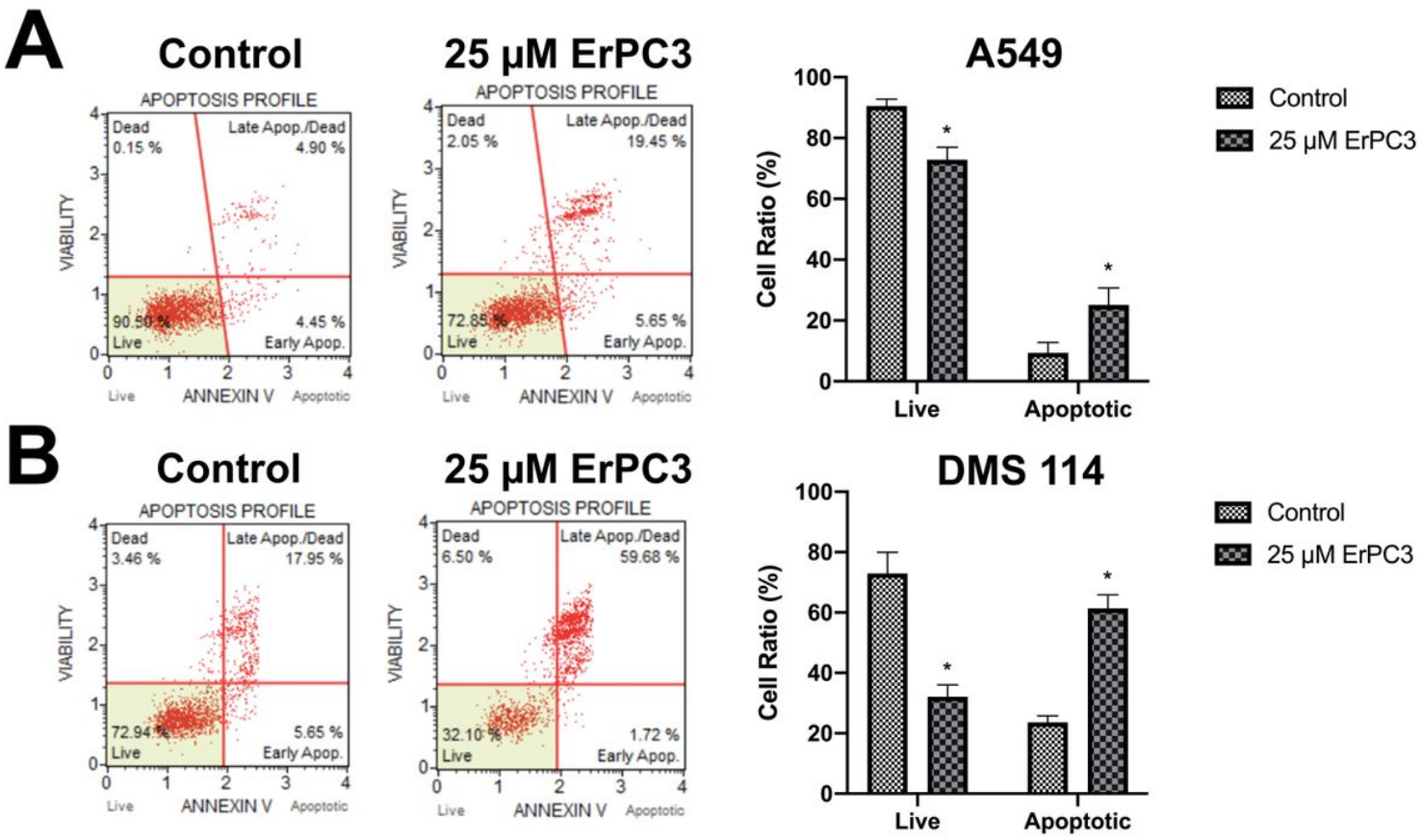

Figure 2

The apoptotic situation of A549 (A) and DMS 114 (B) under 25 μM ErPC3 treatment. The bars graph indicates the percentage of live and apoptotic cells. (*$p < 0.05$).

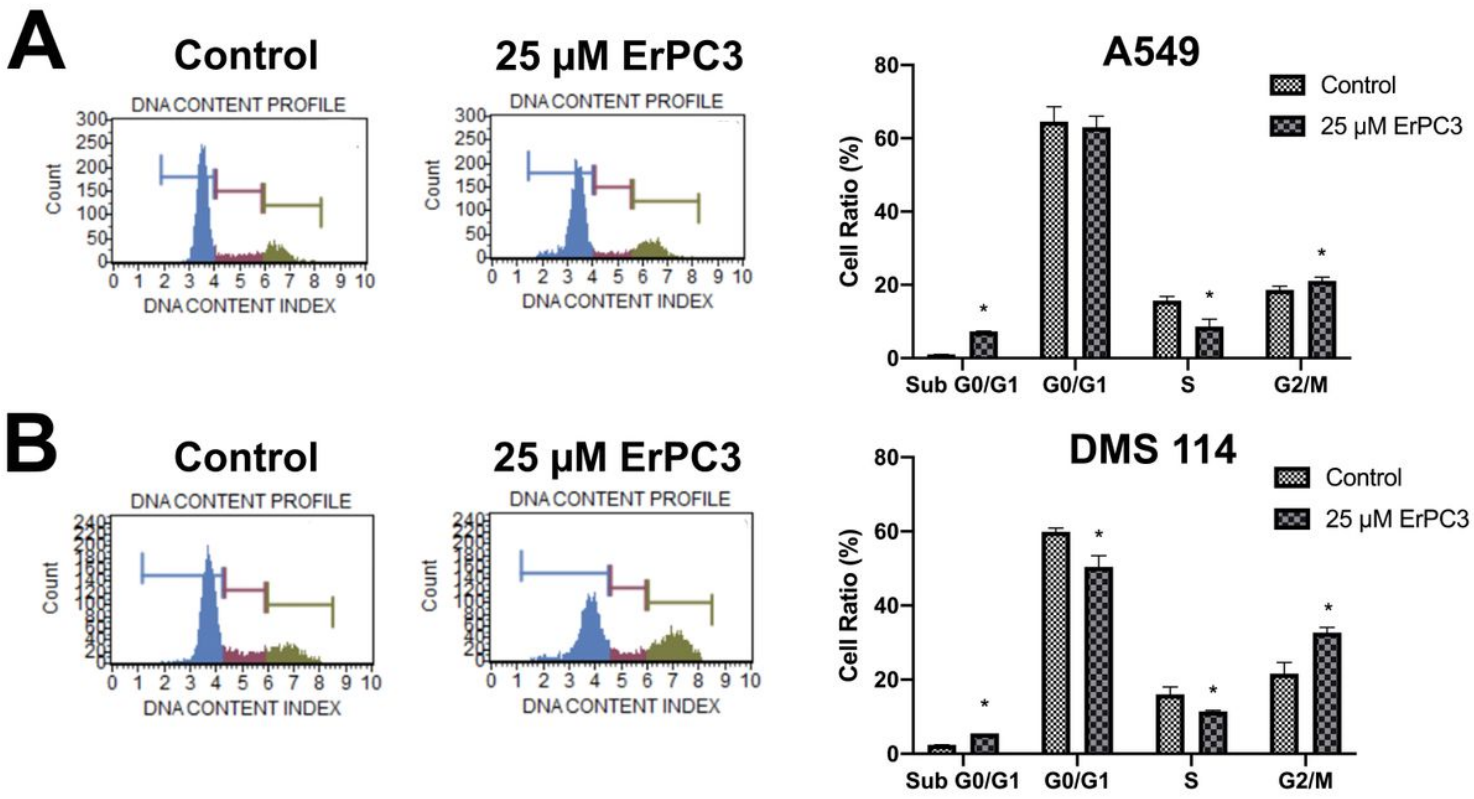

Figure 3

The cell cycle distribution of A549 (A) and DMS 114 (B) under 25 μM ErPC3 treatment. The bars graph indicates the percentage of cell cycle phase distribution. (*p < 0.05).

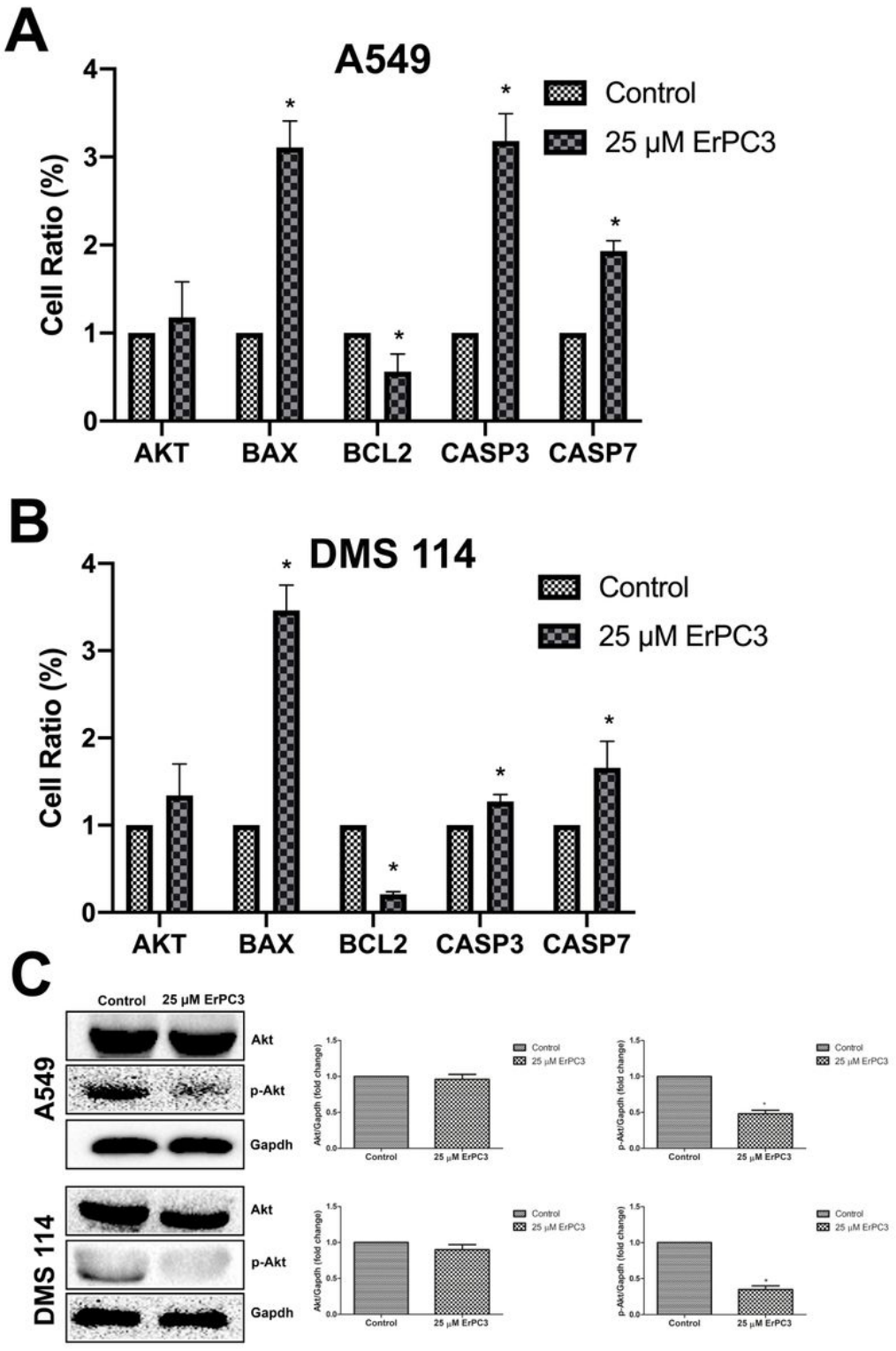

Figure 4

The effect of 25 μM ErPC3 on the gene expression level of related genes in A549 (A) and DMS 114 (B) after 48h treatment. The effect of 25 μM ErPC3 on the protein expression level of Akt and p-Akt in A549 and DMS 114 after 48h treatment by western blot analysis (C). (*p < 0.05).

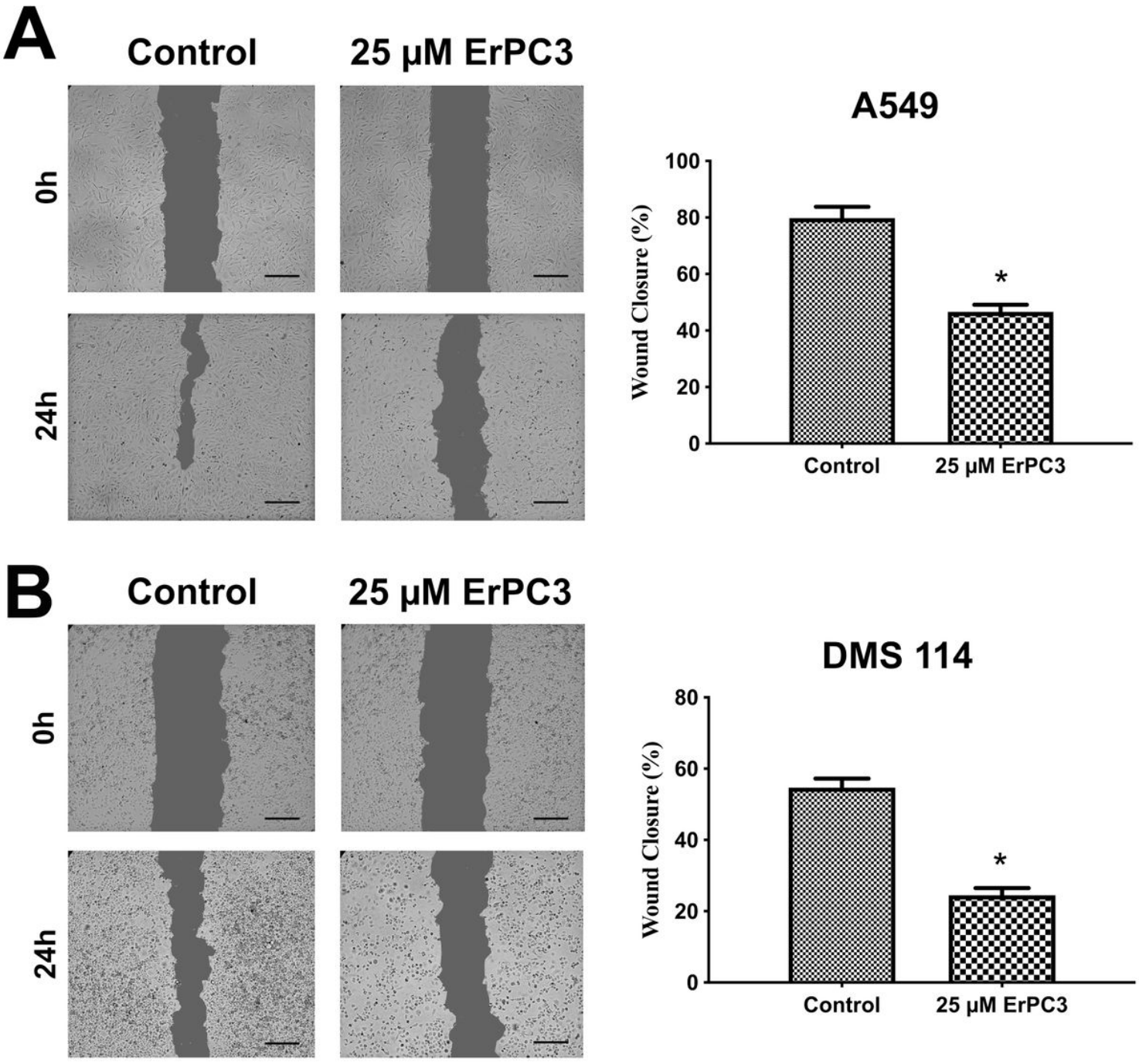

Figure 5

Scratch assay photographs of A549 (A) and DMS 114 (B) under 25 µM ErPC3 treatment at 0h and 24h. The bars graph indicates the percentage of wound closure. (*p < 0.05), (Scale bar 200 µm).