


The outcomes of an impaired powerhouse in KRAS mutant lung adenocarcinoma cells by Elesclomol

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Abstract

Objectives: Lung cancer stands out as the most common cancer type worldwide. The most common genetic alteration detected in adenocarcinoma patients is KRAS. KRAS mutated patients still cannot get benefit from precision medicine approaches and lack a targeted therapy. Elesclomol is an investigational agent for melanoma and other malignancies. In this study, we evaluated its effect on cellular apoptosis, survival, and metastasis mechanisms on KRAS mutant A549 and Calu-1 cell lines.

Methods: The cytotoxic effects of Elesclomol on A549 and Calu-1 cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability test. Cells were treated with IC₅₀ concentration and then apoptosis-related (Casp-3, Casp-9, Bcl-2, and Bcl-xL), survival-related (Akt, p-Akt, Erk, and p-Erk), and metastasis-related (E-cadherin, Vimentin, MMP-2, and MMP-9) protein expressions were determined by Western blot analysis. Elesclomol's effect on cell migration was evaluated by wound healing. Total oxidant, malondialdehyde (MDA), and glutathione (GSH) levels after Elesclomol treatment were assessed.

Results: Elesclomol not only induced apoptotic proteins but also inhibited metastatic protein expressions and migration in both cells. Also, p-Erk activity was diminished by Elesclomol treatment as a reflection of decreased proliferation. However, p-Akt was enhanced as a cellular survival mechanism. Although Elesclomol's effects on oxidative stress parameters were puzzling, it induced total oxidant status (TOS), and MDA in Calu-1 cells.

Conclusion: Elesclomol might provide an alternative treatment approach for patients with KRAS mutant lung adenocarcinoma and other solid tumor malignancies that harbor KRAS mutations. This would enable the development of biomarker-driven targeted therapy for KRAS mutant adenocarcinoma patients.

KEYWORDS

adenocarcinoma, apoptosis, elesclomol, KRAS, metastasis, non-small-cell lung cancer

1 | INTRODUCTION

Lung cancer stands out as the most common cause of cancer death worldwide.¹ Difficulties in lung cancer

treatment mostly arise from the heterogeneity of the disease which multiple genetic mutations exist and their mechanisms are poorly understood.² The progress in the “genomic era” has exhibited several genomic alterations that drive the initiation and progression of non-small-cell lung cancer (NSCLC). Despite being the most commonly mutated oncogene in NSCLC, KRAS mutations remain elusive as a prognostic and predictive marker, such that there is still no approved agent for mutant KRAS in NSCLC.

In addition to having a prominent role in energy metabolism, mitochondria is at the crossroads of cell death and survival which has prominent cellular functions required for homeostasis.³ It not only acts as an oxygen sensor but also as adenosine triphosphate (ATP) and reactive oxygen species (ROS) producer in the cell.⁴ As the major source of ROS is the consequence of the mitochondrial respiratory chain reactions, increased ROS production induces apoptosis, thus it makes mitochondria an attractive target for cancer chemotherapy.⁵ Triggering mitochondrial oxidative stress might offer new routes to target cancer cells for therapeutic interventions.⁶

Elesclomol is an experimental drug compound that has been used in a variety of clinical trials. The underlying mechanism for its anticancer activity is due to unfunctioned mitochondria, disrupted cellular energy production and increased ROS levels.⁷ Elesclomol binds to Cu^{2+} and enables the reduction reaction of Cu (II) to Cu (I). This reduction disrupts mitochondrial oxidative phosphorylation where it induces oxidative stress⁸ via ROS accumulation.⁹

There are also studies that point out Elesclomol does not work through a specific cellular protein target instead, it targets a set of mitochondrial processes.¹⁰ It might be critical to figure out the mechanism of action for Elesclomol to have better insights of biomarker-based stratification of patients as responders in the clinic and to improve the efficacy of pro-oxidative therapy in human cancers.¹⁰ Therefore, in this study, we aimed to enlighten the plausible mechanisms of Elesclomol during important cellular processes like apoptosis and metastasis on KRAS mutant human lung cancer cells A549 and Calu-1 in vitro.

2 | MATERIALS AND METHODS

2.1 | Cell culture and chemicals

A549 and Calu-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO), L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Thermo Fisher Scientific, Inc, Waltham, MA). Cells were grown in a humidified incubator

in 5% CO_2 at 37°C. Elesclomol was kindly gifted from ApexBio Technology LLC (Houston, TX). Elesclomol was dissolved in dimethyl sulfoxide (DMSO).

2.2 | Cell cytotoxicity assay

The antitumor effects of Elesclomol treatment on the viability of NSCLC cells were determined by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549 and Calu-1 cells were seeded at 3×10^3 density per well into a 96-well plate in 100 μL of medium and cultured overnight. Elesclomol concentrations (0.625–20 μM) were added to the medium and the cells were further cultured for 24 and 48 hours. After incubation, MTT solution (5 mg/mL in phosphate-buffered saline [PBS]) was added to each well and then the plate was incubated for 4 hours at 37°C. The crystal dissolving buffer was added to solubilize formazan crystals and the plates were gently shaken on an orbital shaker for 5 minutes. The absorbance ratio was measured using SpectraMax M3 (Molecular Devices, San Jose, CA) microplate reader at 570 nm. Each treatment was repeated at least four times. The mean absorbance of four wells was used as an indicator of relative cell growth

2.3 | Total oxidant status, malondialdehyde, and glutathione after Elesclomol treatment

The total oxidant status (TOS) of Elesclomol treated and control A549, and Calu-1 cells were determined by using Rel Assay Diagnostics (Gaziantep, TR) according to the manufacturer's instructions. The absorbance of the samples was measured with a microplate reader at a wavelength of 530 nm. We also investigated Elesclomol's effect on oxidative and antioxidative stress by determining glutathione (GSH) and malondialdehyde (MDA) levels. MDA, a product of lipid peroxidation was studied by the measurement of the formation of thiobarbituric acid reactive substances.¹¹ The absorbance of the samples was measured with a microplate reader at a wavelength of 535 nm. GSH levels were studied via modified Ellman method.¹² The absorbance of the samples was measured with a microplate reader at a wavelength of 412 nm.

2.4 | Wound-healing assay

The cells were seeded at 70% confluency per 24-well plate and cultured for 24 hours. The cells were scratched using a p100 and then the plate was rinsed with PBS to remove cell debris. Elesclomol treated and untreated cells were incubated for 24 and 48 hours at above-mentioned culture conditions. Migrating cells and the scratched surface were observed during the experiment. The wells were photographed using

Zeiss optical microscopy (Zaventem, Belgium) at different time points. The wounded area was calculated by using the ImageJ software.

2.5 | Western blot analysis

A549 and Calu-1 cells were seeded at 2×10^5 at the six-well plate and cultured for 24 hours. Untreated and $10 \mu\text{M}$ Elesclomol treated cells were cultured for 24 hours. The cells were washed with PBS and scraped into RIPA lysis buffer containing 1 mM PMSF and the lysate was sonicated. Samples were centrifuged for 15 minutes at 13 500 rpm at 4°C and the supernatants were collected. Proteins were quantified by using BCA Assay Kit (Thermo Pierce, Rockford, IL). Protein lysates ($20 \mu\text{g}$) were heated for 5 minutes at 95°C in LDS nonreducing sample buffer (Pierce, Rockford, IL) and then loaded on 10% Tris-glycine gels, transferred to PVDF membranes (Pierce, Rockford, IL) at 250 mAmp for 2 hours. Membranes were blocked with 5% Bovine Serum Albumin (BSA) in Tris-buffered saline-Tween 20 (TBST; 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) for 1 hour at room temperature and incubated overnight at 4°C with the antibodies for Caspase-3 (#PA5-16335), Caspase-9 (#PA1-12506), Bcl-2 (#PA5-20068), Bax (#PA5-11378), Bcl-XL (#PA5-17805), FoxM1 (ab180710), Vimentin (CST #5741S), p-Erk (CST #4370S), Erk (CST #9102S), Akt (CST #2938), p-Akt (CST #4060), MMP-2 (sc13594), MMP-9 (sc13520), E-Cad (CST#3195S), and β -actin (PA1-183) were used at 1:1000 concentration. The secondary antibody was used at 1:10 000 dilution (ab97051). Blots were washed three times subsequently with TBS-T and visualized by Biovision ECL Western blot substrate (BioVision Inc, Milpitas, CA). Chemiluminescent signals of immunoblots were documented using Gel Logic 2200 Pro (Carestream Health; Rochester, NY).

2.6 | Statistical analysis

All experiments were performed at least three times. Statistical analysis was performed using SPSS 21.0 software (NY). Comparisons between normally distributed variables were conducted using one-way analysis of variance and non-normally distributed variables using the Mann-Whitney *U* test. $P < 0.05$ was considered as statistically significant.

3 | RESULTS

3.1 | Elesclomol's effects on NSCLC cell cytotoxicity

To determine the cytotoxic effects of Elesclomol on A549 and Calu-1 cells, cells were treated with 0.625 – $20 \mu\text{M}$ of Elesclomol for 24 and 48 hours. As shown in Figure 1A and

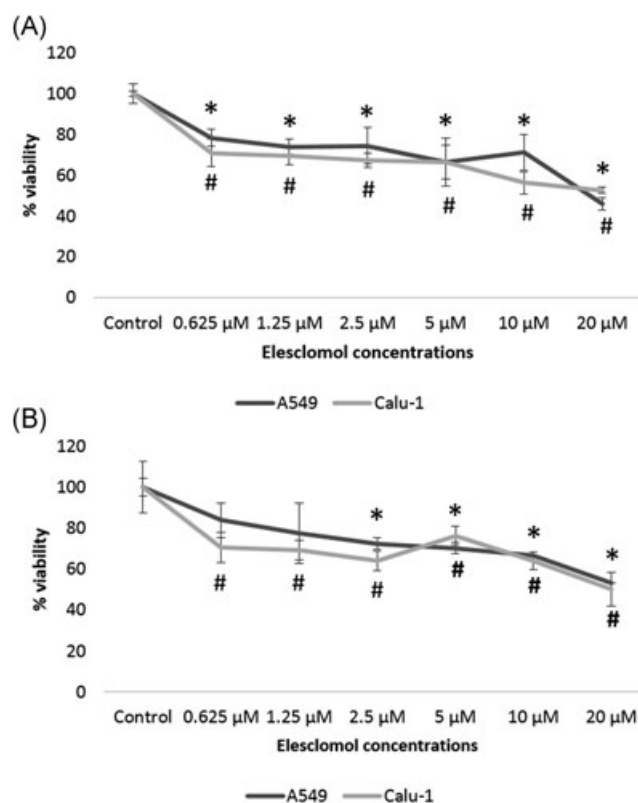


FIGURE 1 Cell viability of A549 and Calu-1 cells treated with 0.625 to $20 \mu\text{M}$ of Elesclomol at 24 (A) and 48 hours (B) ($*P < 0.05$ when compared to control group for A549 cells, $\#P < 0.05$ when compared to control group for Calu-1 cells)

1B, Elesclomol inhibited A549 and Calu-1 cell proliferation in a concentration-dependent manner at 24 hours at $10 \mu\text{M}$ (IC_{50}) concentration. It was determined that all tested doses significantly decreased cell viability at 24 hours in both cells (Figure 1A). In contrary, the decrease in cell viability at 0.625 and $1.25 \mu\text{M}$ was not statistically significant for A549 cells at 48 hours (Figure 1B).

3.2 | Elesclomol inhibited cell migration in NSCLC cell lines

We investigated the effect of Elesclomol on wound healing of A549 and Calu-1 cells for 24 and 48 hours. For A549, the wounded area was calculated as 57.44% in the control group, whereas it was 66.54% in $10 \mu\text{M}$ Elesclomol treated cells at 24 hours. At 48 hours the wounded area was calculated as 34.45% in the control group whereas it was 53.22% in $10 \mu\text{M}$ Elesclomol treated cells. For Calu-1 cells, the wounded area was calculated as 48.12% in the control group, whereas it was 64.63% in $10 \mu\text{M}$ Elesclomol treated cells at 24 hours. At 48 hours the wounded area was calculated as 26.83% in the control group, whereas it was 51.65% in $10 \mu\text{M}$ Elesclomol treated Calu-1 cells. These wounded area percentages were calculated by using ImageJ (NIH, Bethesda) (Figure 2).

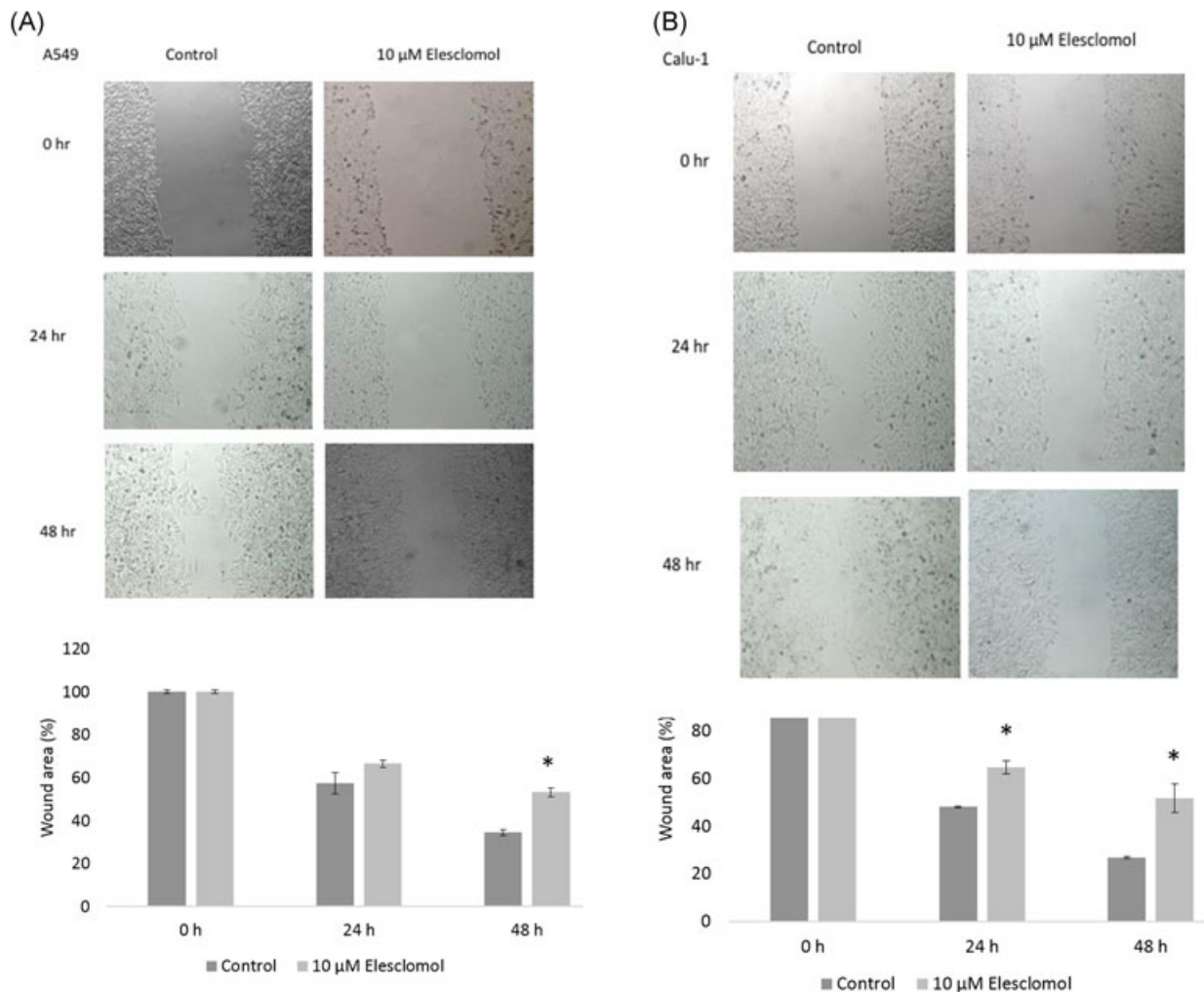


FIGURE 2 The effects of Elesclomol on wound healing of A549 (A) and Calu-1 (B) cells for 24 and 48 hours (* $P < 0.05$ when compared to control group for each time point)

3.3 | Elesclomol exposure to NSCLC cell lines induces apoptotic protein expressions in vitro

Apoptotic protein expression levels were effected by Elesclomol treatment. It decreased caspase-3, caspase-9, Bcl-2, and Bcl-XL protein expression levels both for A549 and Calu-1 cells compared to control groups. Although Bax expression was increased at A549 cells, it was decreased at Calu-1 cells with Elesclomol treatment (Figure 3A). According to our results, it can be interpreted as Elesclomol triggers caspase-dependent pathway of apoptosis.

3.4 | Involvement of AKT and ERK activation in Elesclomol's antiproliferative effects on NSCLC cell lines

Treatment of A549 and Calu-1 cells with Elesclomol decreased p-Erk protein activation at 24 hours in both cell lines that indicates its antiproliferative effects. However,

both Akt expression and p-Akt activation were increased with 10 μM Elesclomol treatment in A549 and Calu-1 cells which can be conceived as a provocation of survival mechanism from apoptosis (Figure 3B).

3.5 | Antimetastatic effects of Elesclomol on NSCLC cell lines

We also investigated Elesclomol's effects on expression profiles of metastasis-related proteins (Figure 3C). Elesclomol treatment decreased MMP-2 and MMP-9 protein expression levels in A549 and Calu-1 cell lines at 24 hours compared to the control group. However, FoxM1 and β -catenin expressions were not changed within the groups. Although E-cadherin was increased with Elesclomol treatment at A549 cells, it was not changed at Calu-1 cells. Vimentin expression was increased at A549 cells, but it was decreased at Calu-1 cells inversely (Figure 3C).

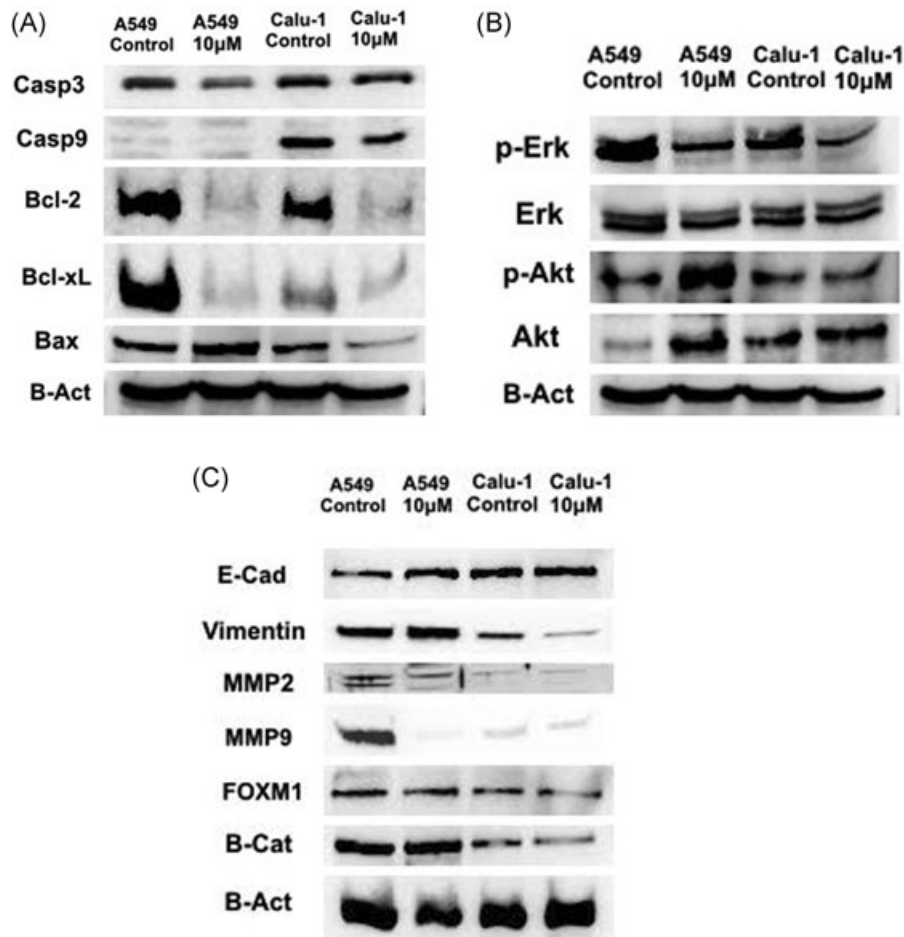


FIGURE 3 Elesclomol's effects on cellular apoptosis-(A), survival-(B), and metastasis-(C) related protein expressions on A549 and Calu-1 cells at 24 hours. Bax, BCL2-associated X; Bcl-2, B-cell lymphoma 2; Casp-3, caspase-3; Casp-9, caspase-9; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9

3.6 | The changes on oxidative stress regarding Elesclomol treatment in NSCLC cell lines

TOS levels were significantly decreased in Elesclomol treated A549 cells. However, Calu-1 cells demonstrated the opposite effect and increased TOS levels with Elesclomol treatment.

MDA levels in Elesclomol treated A549 cells were significantly lower when compared with the control group. In contrary, Elesclomol treatment significantly enhanced MDA levels in Calu-1 cells. This finding was also consistent with GSH levels that Elesclomol treated Calu-1 cells have significantly higher GSH levels than controls (Figure 4).

4 | DISCUSSION

The Warburg effect, which describes the mitochondrial dysfunction requirement for tumor development has been questioned and approved several times.¹³ However, the phenomena has switched over to mitochondria's important roles in cancer prevention and progression.¹⁴ In addition to its roles in cellular homeostasis, mitochondria

produce ROS, as an inevitable byproduct of oxidative phosphorylation, that can modulate the activity of several proteins and effect cellular survival.^{14,15}

Targeting mitochondria not only affects the energy-dependent cell survival but also initiates the apoptotic pathway. Modulating ROS levels has been proposed as a therapeutic strategy to selectively interrupt cancer cell metabolism.¹⁶ Elesclomol is found to be a potent growth inhibitor of cancer cells that specifically targets the mitochondria.¹⁷ It causes the selective mitochondrial ROS induction and therefore induces oxidative stress in cancer cells.⁶ In the present study, we aimed to highlight the underlying cytotoxic mechanisms of Elesclomol by evaluating its effects on proliferation, apoptosis, oxidative stress, and metastasis.

Treatment of KRAS mutant NSCLC cells with 10 μM Elesclomol caused a straight halt in cell viability even at 24 hours. In contrast, Hasinoff et al⁸ determined the growth inhibitory effects of Elesclomol yielded IC₅₀ value as 14.3 nM at human erythroleukemic K562 cells. Also, Wangpaichitr et al¹⁸ showed that Elesclomol selectively kills cisplatin-resistant cells 4-10 times more than their parental counterparts. The diversity of IC₅₀ concentrations

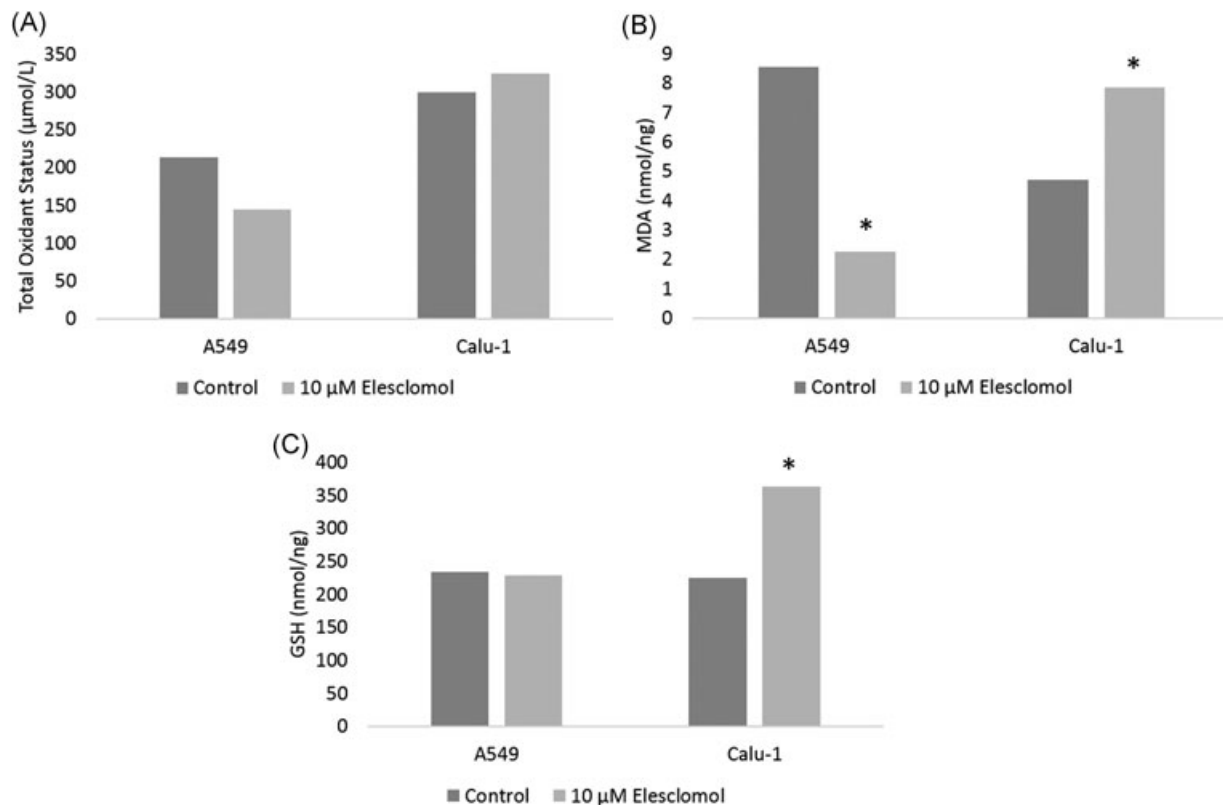


FIGURE 4 The consequences of Elesclomol treatment on TOS (A), MDA (B), and GSH (C) levels for A549 and Calu-1 cells at 24 hours (* $P < 0.05$ when compared to control group). GSH, glutathione; MDA, malondialdehyde; TOS, total oxidant status

within previous studies might be stemmed from different cellular and molecular characteristics of cell lines that were used.

Our findings revealed that 10 μM Elesclomol exposure to A549 and Calu-1 cells decreased Bcl-2 and Bcl-XL protein expressions. Bcl-2 and Bcl-XL function as antiapoptotic molecules,¹⁹ thus decreased expressions of these proteins trigger apoptosis. Elesclomol also decreased total caspase-3 and caspase-9 levels, which means the pro forms are cleaved and increased the cleaved forms.²⁰ Although we did not show the cleaved forms of caspase-3 and caspase-9, it can be interpreted by checking total levels that Elesclomol increases cleaved forms of these mentioned caspases. We also found that Akt survival signaling was induced by Elesclomol, which may reflect a cellular feedback mechanism for survival. Also, this could be the reason that 48-hour Elesclomol treatment has modest effects on cell proliferation. In consistency with our findings, Qu et al⁵ showed increased cleaved caspase-3 and Akt levels in breast cancer cells. In contrary, p-Erk activation was both diminished with Elesclomol treatment in A549 and Calu-1 cells, which demonstrates its antiproliferative effects.

Bax-dependent mitochondrial apoptosis and mitochondrial permeability are regulated by several factors, including the phosphorylation by the protein kinase Akt. Simonyan

et al²¹ identified the regulation of Bax/mitochondria interaction by Akt. Elesclomol increased p-Akt and Bax protein expression levels in A549 KRAS mutant human lung cancer cells.²¹ We concluded that Elesclomol's mechanism of action over cancer cells is not only limited with the induction of ROS whereas it also regulates the Bcl-2, Bcl-XL, and Bax proteins in control of apoptosis.

The intrinsic apoptosis-mitochondrial pathway is coordinated by Bcl-2 family proteins, these proteins control mitochondrial outer membrane permeabilization (MOMP), that accounts for no return point for apoptosis.²² Bcl-2 inhibitors is now being used as anticancer therapeutics and offer treatment possibilities in small-cell lung cancers with high BCL2 expression.²³ To the best of our knowledge, this study concludes for the first time that Elesclomol might be a plausible alternative in Bcl-2 inhibition in KRAS mutant lung adenocarcinomas, however, our results are limited as being only in vitro.

The loss of epithelial and the gain of mesenchymal characteristics are attributed with the assignment of metastasis. There are several markers such as E-cadherin, Vimentin, MMPs associated with this epithelial to mesenchymal transition phenomena. In this study, we have shown for the first time that Elesclomol

has antimetastatic activity in NSCLC cells. We found that Elesclomol inhibited wound healing on A549 cells more effectively than Calu-1 cells. In accordance with that finding, we revealed that Elesclomol decreased the MMP-9 protein expression level in A549 cell line. Also, MMP-2 expression was decreased with Elesclomol in A549 cells. In line with these findings, E-cadherin was increased in A549 cells. Interestingly, Vimentin was only decreased in Calu-1 cells. It is known that the typical proliferation-associated transcription factors such as FoxM1 and β -catenin also have roles in metastasis. Although FoxM1 and β -catenin were not changed after Elesclomol treatment within groups, it is effective in the metastasis process in NSCLC cell lines as shown in our study.

Elesclomol was used as an oxidative stress inducer for melanoma cells.²⁴ Also, it was found to selectively trigger apoptosis in cisplatin-resistant lung cancer cells through ROS.¹⁸ In our study, we could not observe an increase at oxidative stress parameters such as TOS, MDA, or antioxidant molecule GSH levels in Elesclomol treated A549 cells. However, there was an increase both in TOS, MDA, and GSH levels in Elesclomol treated Calu-1 cells compared with the control group. This difference between cell lines may depend on the mutation status variance of the cell lines used in this study. Besides, it is questionable whether A549 cells trigger antioxidant systems other than GSH and revive a survival feedback mechanism to prevent apoptosis just as seen in p-Akt activation with Elesclomol treatment. Also, we collected the oxidant molecules from the whole cell content and assayed TOS, MDA, and GSH levels. It might be reasonable to isolate mitochondria and check ROS status to observe Elesclomol's direct effects on mitochondria and calculate the oxidant status of the cells.

Lastly, cancer cells can switch their metabolism to glycolysis and suppress mitochondrial respiration which drives its antiapoptotic and metastatic ability. This survival mechanism decreases dependence on oxidative phosphorylation and reduces ROS production. So, it is important to know the energy metabolism of the cancer cell and the status of tumorigenesis grade. Such as, metastatic or hypoxic tumors may not benefit from Elesclomol treatment owing to the fact that mitochondrial phosphorylation impairment for the lack of oxygen.

The antiproliferative, apoptotic, and antimetastatic understanding of Elesclomol's activity provided by this study has important implications for its therapeutic application in cancer. The findings and future studies will highlight the potential use of mitochondrial agents for a biomarker-based prioritization of patients who have KRAS mutation.

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