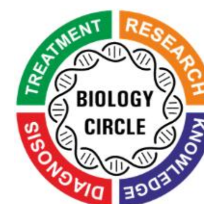
Cancer Research and Medicine
DOI: https://doi.org/10.63626/tx5xhy81
ISSN Online: XXXX-XXXX
ISSN Print: XXXX-XXXX



Research Article

Cytotoxic effects and expressional modifications induced by alkyl-phospholipid (erufosine) in lung cancer cells

Marriyam Nasim, Adeela Batool, Kinzah Kanwal
Medical Laboratory Technology, Riphah International University, Islamabad, Pakistan

Abstract

Background: Lung cancer is the leading cause of morbidity and mortality in the world. The treatment of lung cancer is a challenge because of the aggressive nature of this disease and limited therapeutic options. The situation demands to investigate novel compounds for better treatment of lung cancer with lesser side effects. Alkyl-phospholipids (ALPs) are an attractive class of antineoplastic agents which target DNA and act upon cell membrane to produce anticancer effects in tumor cell specific manners. Erufosine is the 3rd generation ALP and induces anticancer effects by causing changes in the lipid rafts of the cell membrane.

Methods: Selected lung cancer cell line was cultured in 96-well plates and exposed to various concentrations of erufosine (1.56-50µM) followed by MTT dye-based assessment of viable cell fractions. For expression modifications, the cells were exposed to erufosine followed by RNA extraction, cDNA synthesis and real-time PCR based assessment of the selected genes.

Results: Erufosine induced substantial cytotoxic effects in the lung cancer cells as shown by growth inhibition of 25 and 50% by using 24- and 33µM concentration respectively. At lower concentrations (6.25µM), there were negligible cytotoxic effects, which gradually increased with increasing concentrations. As far as expressional changes are concerned, the three selected genes (CCNB1, CDKN3, CASP3) were downregulated in response to erufosine exposure.

Conclusion Erufosine is a potential cytotoxic compound, induces notable anti-proliferative effects and modulates expressional levels of the genes in lung cancer cells.

Key Words: Lung Cancer, Alkyl-phospholipid, Erufosine, Cytotoxicity, Gene Expression

Corresponding Author: Marriyam Nasim
Email: marriyam.nasim@cemb.edu.pk

INTRODUCTION

Lung cancer is a disease in which cells in the lungs proliferate abnormally to form a tumor. Lung cancer is the second most diagnosed cancer type (11.4%) and accounts for 18% of cancer related deaths. Lung cancer in its earliest stages is asymptomatic. Signs and symptoms occur when disease is at chronic stage. Five most used therapeutic approaches for the lung cancer treatment are: surgery, therapy by radiations, drugs as a part of chemotherapy, targeted therapy and immunotherapy. Treatment of lung cancer is a challenge because of the aggressive nature of the disease and limited therapeutic option. The situation demands for a novel compound to treat lung cancer with lesser side effects [1].

ALPs are a class of antineoplastic agents which target DNA and act upon cell membranes to produce anticancer effects. Structural modifications in various members of ALPs have generated new compounds with reduced toxicity and high specificity overtime. ALPs comprise a class of antineoplastic agents such as miltefosine, perifosine, erucylphosphocholine and erufosine [2]. Erufosine is a 3rd generation ALP and has been found active against human cancer cells. In addition to the inhibition of proliferation and growth arrest of cancer cells, erufosine interferes with cholesterol homeostasis, ATP generation and cytoskeleton related proteins, which lead to stress, lack of energy and migratory activities in different cancer cell lines, respectively. The addition of 22 carbon chain structure of erufosine makes it metabolically more stable with less toxicity towards GI tract and minimally generate hemolytic activity *in vivo*. These characteristics permit the intravenous administration of erufosine to attain relevant clinical concentrations, which was not achievable with other ALPs [3]. The present study was designed to investigate the anti-proliferative effects of erufosine against lung cancer cells. Furthermore, potential modifications in expression levels of the genes in response to erufosine exposure were determined in lung cancer cells.

METHODS

Cell Culture

Human lung cancer cells (H1299) were cultured in RPMI-1640 medium augmented with L-glutamine (2mM), fetal bovine serum (10%), streptomycin (100µg/ml) and penicillin (100IU/ml). Standard cell culture incubation conditions (at 37°C in moisturized air with 5% carbon dioxide) were maintained for cell growth.

Growth Curve Analysis

A growth curve is a graphical presentation of how a specific number of viable cells increases over time. This was done by MTT assay where the cells were cultured in 96-well culture plates (1000-6000 cells/well/100µl medium,) followed by incubation for three different time points (24, 48 and 72 hours) at standard cell culture incubation conditions. MTT solution dissolved in PBS (10mg/ml) was added (10µl concentration per well). NADPH enzymes of the viable cells reduce the MTT dye to the insoluble formazan crystals. Insoluble formazan crystals were dissolved by adding DMSO (50µl/well). Optical densities were measured at absorbance wavelength of 540nm with the reference filter of 690nm by an ELISA plate reader and growth curves were generated with time intervals on X-axis and number of cells on Y-axis. The dark purple color of the solution shows a greater number of viable and metabolically active cells. This data led to the identification of optimal cell numbers to be used in subsequent experiments.

MTT Assay

MTT assay was performed to observe cell viability and proliferation following treatment of the cell line with erufosine. For this purpose, the cells were seeded in 96-well plates (100µl media/well) at pre-optimized cell densities (4000cells/well: the number with continuous growth over 24-72hour) obtained from growth curve experiments and were treated with increasing concentrations of erufosine (dissolved in PBS) for three distinct time points that are 24, 48 and 72 hours. Following the treatment intervals, MTT solution (10mg/ml in PBS) was added (10µl/well) and same procedure was repeated as described in growth curve analysis for measurement of viable cell population. Inhibitory concentration (IC) is a measure of the potency of a compound in inhibiting a specific biological or biochemical function. Data from the MTT experiments were analyzed by GraphPad Prism software (v. 6.0) to calculate ICs of erufosine while untreated cells grown in parallel were used as controls.

Expression Analysis of Genes

The cells were cultured in 6-well plates (200,000 cells/well/2ml medium) and were allowed to grow overnight. After incubation, the cells were exposed to the pre-determined erufosine IC25 (24.4µM) and IC50 (33.1µM) concentrations for 48 hours period. Following the exposure period, RNA was extracted from treated and untreated control cells palettes by using the Kit (Thermo Fisher Scientific, Cat#K0731). The quantity and quality of the extracted RNA was determined using spectrophotometric method (Nanodrop technology). The extracted RNA was stored at -80°C immediately for further use. A total of 40µl cDNA/sample was synthesized by using a specific kit (Thermo Fisher Scientific, Cat#K1622) along with the extracted RNA (1000ng) by using reverse transcriptase enzyme, Oligo dT primers and dNTPs. A PCR based amplification of a reference gene (HPRT1) was performed to verify the synthesized cDNA samples. 7µl of the amplified product was loaded on 2.5% agarose gel and visualized by electrophoresis. Primers for three selected genes (CCNB1, CDKN3 and CASP3) were designed by Primer3Plus software (Table 1). The primers were optimized by using gradient PCR methodology and amplified products were visualized on 2.5% agarose gel electrophoresis. qRT-PCR was performed by using SybrGreen florescence dye (Thermo Fisher Scientific, Cat#K0221) for selected 3 genes by using cDNA samples from the cell line treated with two concentrations of erufosine (IC25, IC50). $2^{-\Delta\Delta CT}$ method was used to find expressional changes in three selected genes in control and Erufosine treated samples. GAPDH was used as reference gene in these experiments and three replicates per sample were used.

Data Analysis

Categorical data generated from cytotoxicity assays were presented as frequency percentages. In case of real-time PCR analysis, fold changes were calculated by using Livak $2^{-\Delta\Delta CT}$ method by comparing Cq (quantification cycle) values of experimental (erufosine treated) and untreated control samples.

Table 1: Primer sequences of selected genes

Gene	Sequences	Product size
CCNB1	CACTTCCTTCGGAGAGCATC	117
	AGAAGGAGGAAAGTGCACCA	
CDKN3	AGCCTGCGAGACCTAAGA	179
	GCAGCTAATTTGTCCCGAAA	
CASP3	GAGGCCGACTTCTTGTATGC	195
	AGCGTCAAAGGAAAAGGACT	
GAPDH	TGCACCACCAACTGCTTAGC	87
	GGCATGGACTGTGGTCATGAG	

RESULTS

Growth Curve of Lung Cancer Cells

Selected cell line was cultured in 96-well culture plates (1000-6000 cells/well/100µl medium) followed by incubation for three different time points (24, 48 and 72 hours) at standard cell culture incubation conditions and then treated with MTT solution. Afterwards, crystals formed by the viable cells were dissolved in DMSO and optical densities were measured by an ELISA plate reader. Growth curves were generated with time intervals on X-axis and number of cells on Y-axis as shown in Figure 1. The data identified 4000 cells/well as optimal number of cells to be used in subsequent experiments in 96-well platform as this number showed exponential growth during the selected time intervals particularly the middle period (48 hours). The number of cells/well were increased accordingly in the 6-well culture plate.

Cytotoxic Effects of Erufosine in Lung Cancer Cells

Human lung cancer cell line H1299 was cultured in RPMI-1640 medium and treated with increasing concentrations of erufosine. ICs of erufosine against H1299 cell line were determined via MTT dye reduction assay. Inhibitory effects were also calculated numerically as percentages of untreated controls. The growth inhibition was noticed after the exposure of the cells with erufosine for 3 different time points with maximum effects in later exposure time (72 hours) with highest erufosine concentration (50uM). A significant decline was also observed in cells survival rate when exposed to various concentrations of erufosine for 24, 48 hours. It has been shown in the graphs for three different time intervals (24, 48 and 72 hours) that the cells became more responsive beyond the 6.25µM concentration of erufosine as shown in Figure 2. Below this range some hyper-proliferation of cells was seen. All in all, erufosine imposed inhibition of proliferation in lung cancer cells.

RNA Extraction, cDNA Verification and Primer Optimization

Following the erufosine treatment, a good quality (260/280: 2.05) and quantity (65-440 ng/µl) of RNA was extracted from the cells. Microscopic visualization of the cells after treatment for these expression analyses is shown in Figure 3. Synthesized cDNA, when loaded on agarose gel electrophoresis, demonstrated a good quality of amplified bands (Figure 4). Designed primers were optimized well when especially focused on applying the three different annealing temperatures as shown in Figure 5.

Expressional Modifications in Genes

Real-Time PCR was performed by using SybrGreen master mix for selected 3 genes by using the synthesized cDNA from the cultured cell line treated with Erufosine at IC25 and IC50. These results highlighted the potential of Erufosine to inhibit expression of these genes in concentration dependent format as the effect was minimal with IC25 and more with IC50 concentrations as shown in figure 6. Specifically, Erufosine exposure inhibited the expression of CCNB1 in a concentration format as more intense inhibition was observed for IC50 application. Furthermore, CCNB1 was the more substantially inhibited gene (-3.1fold) among the three selected markers in this study. In CDKN3 and CASP3, there was a moderate inhibition (-1.5fold) in response to Erufosine exposure. All in all, Erufosine exposure downregulated the three selected genes in lung cancer cells.

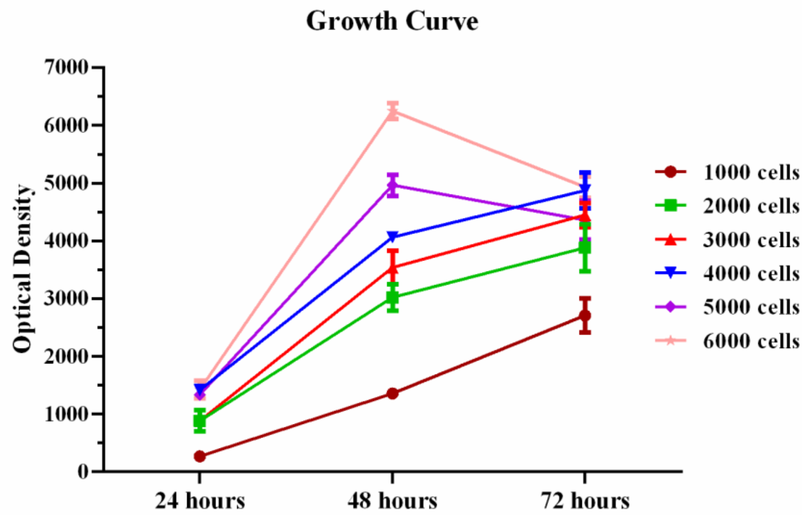


Figure 1: Growth curve generation for three different time points

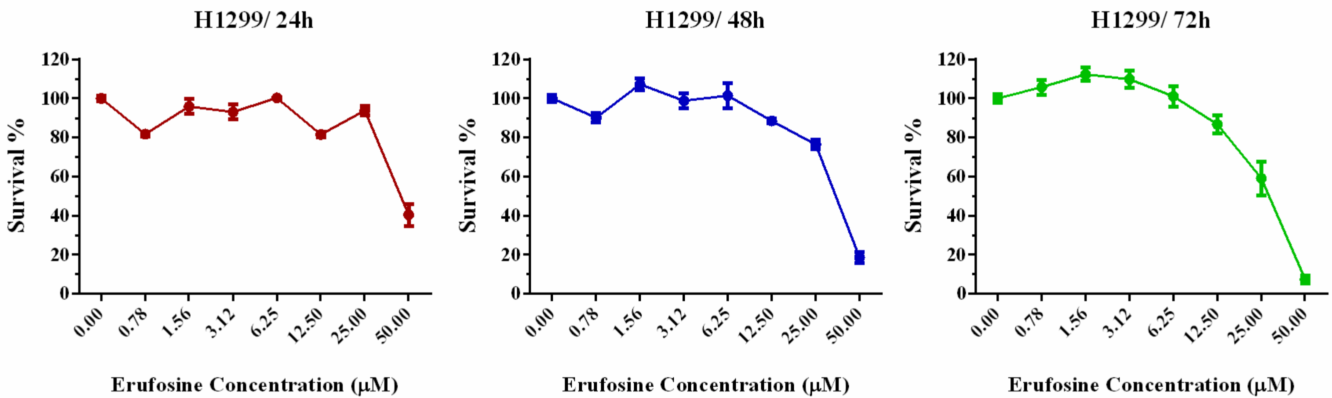


Figure 2: Inhibition of proliferation of H1299 cells after erufosine exposure determined by MTT dye assay.

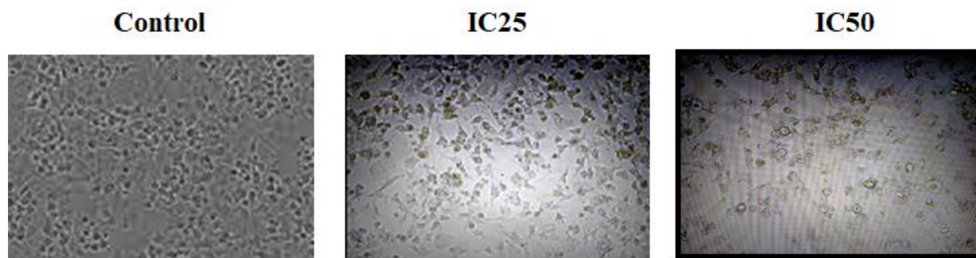


Figure 3: Microscopic images of H1299 cells after erufosine treatment for 48 hours.

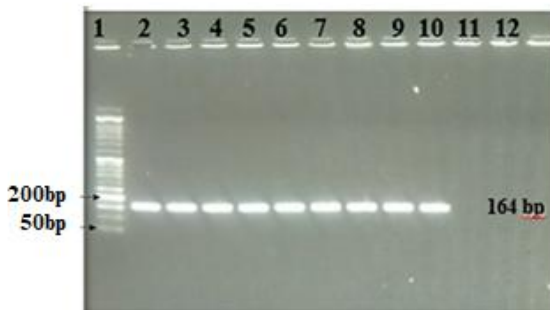


Figure 4: Gel electrophoresis of amplified cDNA of HPRT1 gene from H1299 cells. Sample distribution (left to right): Well 1: DNA marker (50bp) Well 2-4: H1299 untreated control, Well 5-7: H1299 treated with erufosine IC25, Well 8-10: H1299 treated with erufosine IC50, Well 11-12: Negative control.

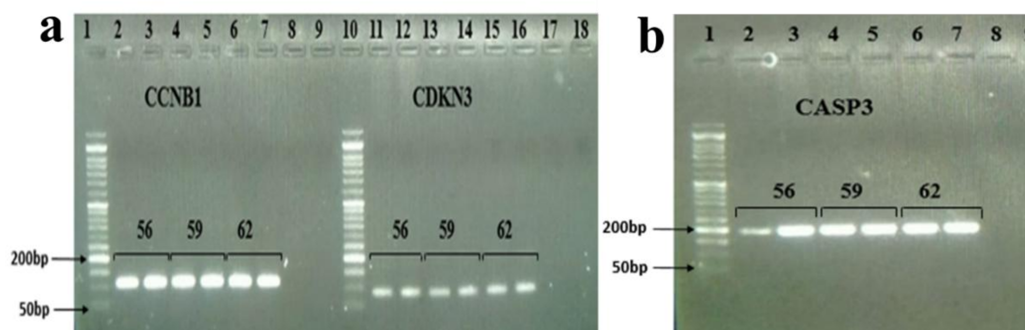


Figure 5: (a) Sample distribution (left to right) for amplification of CCNB1 and CDKN3 genes. Well 1, 10: DNA marker (50bp), Well 2-7: CCNB1 amplification at three different temperatures, Well 8-9: Negative control. Well 11-16: CDKN3 amplification at three different temperatures, Well 17-18: Negative control. **(b)** CASP3 gene Well 1: DNA marker (50bp), Well 2-7: CASP3 amplification at three different temperatures, Well 8-9: Negative control.

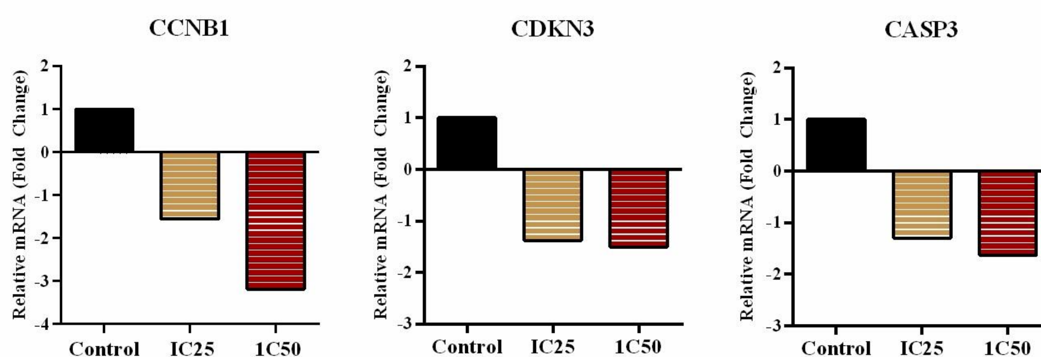


Figure 6: Verification of altered genes by Real time PCR.

DISCUSSION

Lung cancer is the most prevalent cancer while combining male and female population. It imposes a major burden of morbidity and mortality around the globe (~13%). Lung cancer, in its earliest stages, doesn't show any signs and symptoms [4]. Typical symptoms include persistent coughing, hemoptysis, breathing problems, chest cramps, hoarseness, loss of weight and pleural effusion. Treatment of lung cancer during advanced stages of the disease is challenge (5years survival <25%) [5]. Cytostatic drugs are being developed for cancer treatment with an ultimate task to suppress the multiplication of cancer cells by producing arrest in cell cycle. These cytostatic agents are particularly important in a way that they halt the cell division of fast proliferating cancer cells dominantly and spare the normal cells, thus induce minimal side effects [6].

ALPs are a class of antineoplastic agents which target DNA and act upon cell membranes to produce anticancer effects. Perifosine is a 2nd generation ALP which is being used clinically but still there are some improvements required due to its considerable side effects. Structural modifications in various members of ALPs have generated new compounds with reduced toxicity and high specificity overtime [7]. With respect to clinical applications, trials have been conducted to evaluate the efficacy of various ALPs. In this regard, clinical applications of ALPs like miltefosine and perifosine have been investigated to a larger extent, while use of recent ALPs like erucylphosphocholine and erufosine is restricted to *in vitro* and pre-clinical studies [8-10].

Erufosine is a 3rd generation ALP having significant anticancer potential against a variety of cancer cell lines. Erufosine imposes anticancer effects by autophagy, apoptosis and cell cycle arrest [11]. In addition to the inhibition of proliferation and growth arrest of cancer cells, erufosine interferes with cholesterol homeostasis, ATP generation and cytoskeleton related proteins, which lead to stress, lack of energy and migratory activities, respectively. Erufosine has lesser side effects compared to previous classes of ALPs because it is metabolically more stable than other ALPs, having lesser toxicity towards GI tract and minimally generate hemolytic activity. Structural change of erufosine with the addition of a 22-carbon change makes it less hemolytic than prior ALPs. This characteristic of erufosine is likely because

of the development of lamellar structures in aqueous solutions instead of micelles like previous ALPs. These characteristics permit the intravenous administration of erufosine to attain relevant clinical concentrations, which was not achievable with other ALPs. Deregulation of cell cycles is a major hallmark of cancer. Therefore, transition of regulators of cell cycle can be target for the treatment of cancers [3].

In the present study, cytotoxic effects of erufosine on the cell line (H1299) were determined by MTT dye reduction assay. For this purpose, the selected cell line was cultured in 96-well plates and exposed to various concentrations of erufosine (1.56-50µM) followed by MTT dye-based assessment of viable cell fractions at three distinct time intervals that is 24, 48 and 72 hours. The growth inhibition was noticed after the exposure of erufosine for 3 different time points with maximum effects in later time interval that is 72 hours with 50µM erufosine concentration. This in turn indicates that erufosine is stable for longer time period and gradually alters the cellular mechanism for more effective inhibition of cell proliferation over time. One additional observation was that at three distinct time intervals, the cells became clearly responsive beyond the 6.25µM concentration of erufosine. It indicates a threshold level of the compound for H1299 cells to inhibit cellular proliferation effectively while below this range, cells tend to resist through some hyper-proliferation. A similar kind of phenomenon has been observed for other cancer cell lines mentioned in published literature [11]. This observation indicates that for clinical utilization, there must be a prior dose escalation evaluation for effective inhibition of tumor cells in clinical settings.

Cell cycles are tightly regulated and monitored via cell cycle checkpoints and in response to any stress or DNA damage, these checkpoints are often up regulated. Cyclins and CDKs are part and parcel of cell cycle and work together to assist the cell cycle progression efficiently. In this study we have selected the 3 genes (CCNB1, CDKN3, CASP3) and have checked their deregulation in lung cancer cell line H1299. The CCNB1 gene, part of the cyclin family, is found in nearly all human tissues. It interacts with a protein called p34 (cdc2) to form the maturation-promoting factor (MPF), which is essential for managing the G2/M phase transition during cell division. In normal cells, CCNB1 expression increases during the late S phase, peaks at the M phase, and plays a key role in cell cycle regulation. However, CCNB1 is often overexpressed in various cancers, such as breast, cervical, lung cancer, and melanoma, linking it to cancer progression. High levels of CCNB1, CDKN3 and CASP3 in lung cancer are associated with worse survival rates. Erufosine can slow down lung cancer cell growth by targeting important genes like CCNB1, CDKN3 and CASP3. It lowers the expression of these genes, suggesting its potential as a cell cycle inhibitor and a promising option for cancer therapy [12]. Real-Time PCR was performed for selected 3 genes (CCNB1, CDKN3, CASP3) by using the synthesized cDNA from the cultured cell lines treated with erufosine at IC25 and IC50. These results highlighted the potential of erufosine to inhibit expression of these genes in concentration dependent format as the effect was minimal with IC25 and more with IC50 concentrations. Furthermore, the concentration dependent effects of erufosine found on these three genes reflect a direct relationship between the compound’s concentrations and response of the cells.

To conclude, erufosine showed a substantial potential to inhibit the proliferation of lung cancer cells. Furthermore, there were prominent de-regulation of cell cycle and apoptosis related genes in lung cancer cells after erufosine exposure. Further investigations are needed to understand the molecular mechanisms affected by erufosine in lung cancer, while using additional representative cell lines.

REFERENCES

1. Abdik H. Antineoplastic effects of erufosine on small cell and non-small cell lung cancer cells through induction of apoptosis and cell cycle arrest. Mol Biol Rep. 2022; 49: 2963–2971.
2. Nitulescu GM Margina D Juzenas P Peng Q Olaru OT Saloustros E Fenga C Spandidos DA Libra M Tsatsakis AM. Akt inhibitors in cancer treatment: The long journey from drug discovery to clinical use. Int. J. Cancer. 2016; 48: 869-885.
3. Van Blitterswijk WJ Verheij M. Anticancer mechanisms and clinical application of alkylphospholipids. Biochim Biophys Acta. 2013; 1831: 663-674.
4. Walter F Rubin G Bankhead C Morris H Hall N Mills K Dobson C Rintoul R Hamilton W Emery J. Symptoms and other factors associated with time to diagnosis and stage of lung cancer: a prospective cohort study. Br. J. Cancer. 2015; 112: S6-S13.
5. Spiro SG Tanner NT Silvestri GA Janes SM Lim E Vansteenkiste JF Pirker R. Lung cancer: progress in diagnosis, staging and therapy. Respirology. 2010;
6. Kim KW Roh JK Wee HJ Kim C. Cancer Discov. 2016; Springer.
7. Nitulescu GM Margina D Juzenas P Peng Q Olaru OT Saloustros E Fenga C Spandidos DA Libra M Tsatsakis AM.. Akt inhibitors in cancer treatment: The long journey from drug discovery to clinical use. Int. J. Cancer. 2016; 48: 869-885.
8. Bagley RG Kurtzberg L Rouleau C Yao M Teicher BA. Erufosine, an alkylphosphocholine, with differential toxicity to human cancer cells and bone marrow cells. Cancer Chemother Pharmacol. 2011; 68**:** 1537-1546.
9. Königs SK Pallasch CP Lindner LH Schwamb J Schulz A Brinker R Claasen J Veldurthy A Eibl H Hallek M. Erufosine, a novel alkylphosphocholine, induces apoptosis in CLL through a caspase-dependent pathway. Leuk. Res. 2010; 34: 1064-.
10. Zaharieva M Konstantinov S Pilicheva B Karaivanova M Berger M. Erufosine: a membrane targeting antineoplastic agent with signal transduction modulating effects. Ann. N. Y. Acad. Sci. 2007; 1095: 182-192.
11. Kaleağasıoğlu F, Berger MR. Differential effects of Erufosine on proliferation, wound healing and apoptosis in colorectal cancer cell lines. Oncol. Rep. 2014; 31: 1407-1416.
12. Li Y Leng Y Dong Y Song Y Wu Q Jiang N Dong H Chen F Luo Q Cheng C. Cyclin B1 expression as an independent prognostic factor for lung adenocarcinoma and its potential pathways. Oncol Let. 2022; 24(6):1-3.

Ethics Approval: Not Applicable

Competing Interests: The authors declare that they have no conflict of interest.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Open Access

This article is licensed under a Creative Commons Attribution 4.0 International License. The license permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. View the copy of this license at http://creat iveco mmons .org/licen ses/by/4.0/.