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Research Article

Alkyl-phospholipid mediated cytotoxic and cytostatic effects in liver cancer cells

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Abstract

Background: Hepatocellular carcinoma is a prevalent cancer in the world. Due to limited therapeutic options, this malignancy is causing substantial morbidity and mortality world-wide. Alkyl-phospholipids (ALPs) are synthetic lipids and comprise a promising class of anticancer compounds. Erufosine is the latest available generation of this class and has shown anti-tumor effects against a variety of cancer cell lines. This study was designed to uncover its cytotoxic and gene expression modulations by erufosine in liver cancer cells.

Methods: Human liver cancer secondary cell line (HepG2) was cultured and exposed to erufosine. Afterwards, cytotoxic effects were monitored via MTT assay. Following the exposure with the compound, gene expression analysis of cell cycle related genes (CCNA1, CCNA2, and CCND1) was performed by using real-time PCR methodology.

Results: Erufosine demonstrated substantial cytotoxicity in liver cancer cells with increased cell death at higher concentrations and longer exposure intervals. Erufosine imposed discrete expression modifications in the selected genes, where CCNA1 and CCND1 genes were up-regulated while CCNA2 was down-regulated in response to the treatment.

Conclusion: Erufosine is a significant anticancer compound and needs further attention to translate it as a medicine.

Key Words: Alkyl-phospholipid, Erufosine, Liver cancer, Cytotoxic, Cell cycle

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INTRODUCTION

On a global scale, primary liver cancer ranks second in terms of mortality and seventh in terms of cancer incidence. Hepatocellular carcinoma (HCC) is the most common type of liver cancer, representing up to 75% of all cases. Men have an incidence rate of HCC that is two to four times higher than women [1]. HCC is a liver malignancy composed of epithelial cells with hepatocellular differentiation. Chronic inflammation followed by fibroses and cirrhosis ultimately leads to the development of liver carcinoma. HCC can be caused by a range of risk factors, such as alcohol intake, hepatitis B and C virus infection, cirrhosis, and non-alcoholic fatty liver disease. Patients with HCC may present with clinical signs and symptoms related to the tumor or underlying chronic liver disease. The symptoms include abdominal pain, weight loss, nausea, jaundice, ascites, hepatomegaly, and splenomegaly [2]. For the early diagnosis of HCC, with ultrasound every six month and apparently positive patients must be confirmed with contrast enhanced CT or MRI. Several liver function tests including clotting test, viral hepatitis test, kidney function tests or whole blood cell count are also used to assess the liver functionality and possibility of any cancerous conditions [3]. The prognosis for patients with HCC is generally poor, with about 5-10% of individuals surviving beyond 5 years following diagnosis. This pertains to the delayed detection of HCC patients, with only a mere 15% of individuals meeting the criteria for liver transplantation and surgical intervention [1]. Various treatment strategies for HCC include surgery, radiotherapy, targeted agents and chemotherapy. Despite the availability of diverse therapeutic options, 5-year survival rates are low (10-30%) especially in advanced stages of HCC. This, in turn, highlights the need to identify new therapeutic agents/compounds for HCC treatment [4].

Cell cycles comprise the sequence of events leading from DNA duplication to cell division. Several molecular pathways and molecular factors contribute to successful completion of a cell cycle. These factors include cyclins, cyclin dependent kinases (CDKs) and CDKs inhibitors, which are important players of the cell cycle. In a normal cell, a number of cell cycle checkpoints work to eliminate any errors occurring during any stage of the cell cycle. Cancer is defined as uncontrolled proliferation of the cells. Cell proliferation is under the control of a cell cycle process. Thus, cell cycle deregulation often leads to extensive proliferation of the cells and leads to cancer formation. During this transformation, the cell cycle checkpoints are compromised over their normal functions. Because of the key role of a cell cycle in cancer development, drugs are being developed to exploit cell cycle as a therapeutic target for cancer treatment [5].

Alkyl-phospholipids (ALPs) comprise a class of synthetic agents such as edelfosine (prototype), miltefosine (1st generation), perifosine (2nd generation) and erufosine (3rd generation). Unlike conventional chemotherapeutic drugs, which target DNA, ALPs act on cell membranes to induce antitumor effects [6]. Structural modifications in various members of ALPs have generated new compounds with reduced side-effects and high specificity overtime. ALPs are the effective treatment option for parasitic, bacterial and fungal infections. Perifosine and Erufosine have anti-cancer potential. Recent studies have proven erufosine to be involved in suppressing the operations of protein kinase B and triggering apoptosis in different cancerous cells (7-9]. Perifosine has been noted to be triggering apoptosis in myeloma transplants. Additionally, erufosine administration has not been observed causing haemolysis and cytotoxicity like earlier compounds from same family [10-12].

The purpose of the study was to identify the toxic effects of erufosine against liver cancer cells. To give some insights from a molecular perspective, impact of cell cycle related genes was investigated.

METHODS

Cell Culture and Erufosine

A human liver cancer cell line (HepG2), obtained from National Centre for Excellence in Molecular Biology (NCEMB), University of the Punjab, Lahore, was cultured in DMEM media (Gibco, Cat#11965-092), Standard cell culture conditions (5% CO2, 37ºC, humidified environment) were used. Erufosine was provided by Dr. Asim Pervaiz, Human Genetics and Molecular Biology Department, University of Health Sciences, Lahore, Pakistan.

Toxic Effects of Alkyl-Phospholipids

MTT dye reduction assay, a reliable method for measuring metabolically active cells, was used to assess the toxic effects of selected ALP. Briefly, the cells were cultured in 96-well plates (3000 cells/well/100µl media) and incubated overnight. These cultured cells were treated with various concentrations of the selected ALP for three-time intervals (24, 48, 72h), formazan crystals were accumulated within living cells and the proportion of surviving cells was directly proportional to the amount of the accumulated formazan crystals. Inhibitory concentrations (IC) were calculated by using GraphPad Prism 9 software.

Expressional Profiling of Cell Cycle Genes

Effects of selected ALP (Erufosine) on expressional profiling of three cell cycle relevant genes (CCNA1, CCNA2, CCND1) were assessed by qRT-PCR methodology. HepG2 cells were cultured in 6-well plates (150,000cells/well/2ml media) and exposed to IC_{25}, IC_{50} and IC_{75} concentrations of erufosine for 48h. The collected pellets were processed for total RNA extraction. RNeasy mini kit (Qiagen, Cat#74104) was used for it. RNA quantification was done using Nanodrop ND 2000 instrument. A total of 200ng extracted RNA (260/280 ratio = 1.8-2.0) was used to synthesize complementary DNA (cDNA) by using Revert Aid first strand cDNA synthesis kit (Thermo-Scientific, Cat#K1622). Confirmation of successful cDNA synthesis was done by PCR based amplification of a reference gene (GAPDH) for all samples. Following the amplification procedure, amplified products were visualized on 2.5% Agarose gel prepared in TAE buffer.

Designing and Optimization of Primers

Required primers for PCR based amplifications of the target genes were designed using the "NCBI gene" database and "Primer3plus" software. After designing, primer's specificity and self-complementarity were checked by using the online tools "NCBI-BLAST. Primers were optimized by amplification of the genes by using a gradient PCR.

Expressional Analysis of Genes

Expressions of three selected genes (CCNA1, CCNA2 and CCND1) at mRNA levels were investigated by real time PCR methodology. A house keeping gene (GAPDH) was used to normalize the data, while the amplification was done in three replicates at least for each sample. After the amplification procedures and normalization of data sets, fold changes were calculated by the $2^{-\Delta\Delta CT}$ method. Data sets from treated (experimental) and untreated (control) groups were used to compare the effects of selected ALPs on expressional modifications.

Table 1: Applied concentrations of Erufosine

ALPs	Control	Applied Concentrations (µM)						
Erufosine	UT	0.78	1.56	3.12	6.25	12.5	25	50

Table 2: Primer Sequences

Gene	Sequence 5′-3′	Sequence 3′-5′	Amplicon size (bp)
CCNA1	TAGACACCGGCACACTCAAG	CTACCAGCATAGGGGAAACTGT	312
CCNA2	CCTCCTTGGAAAGCAAACAG	TCAAGAGGGACCAATGGTTT	173
CCND1	GGGGGCGTAGCATCATAGTA	GTGGTGGCACGTAAGACACA	114
GAPDH	ACGGATTTGGTCGTATTGGG	CGCTCCTGGAAGATGGTGAT	473

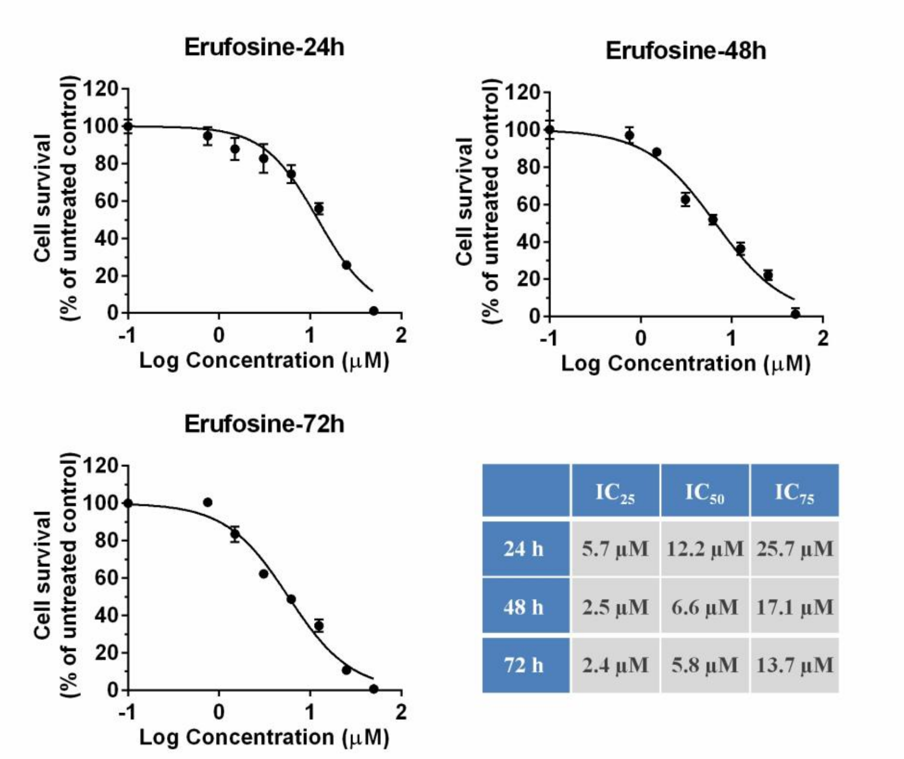
RESULTS

Toxic Effects of Erufosine

Erufosine induced substantial cytotoxic effects in HepG2 cancer cells. The effects were almost concentration dependent for the selected compound as with increasing concentrations, more cytotoxicity was observed. Furthermore, the effects were time dependent as shown by continuous lowering of IC values over the period of exposure time. Clearly, erufosine exhibited substantial anti-proliferative effects against the liver cancer cells (Figure 1).

Expressional Analysis of CCNA1, CCNA2, CCND1

Expression levels of the three selected genes i.e., CCNA1, CCNA2 and CCND1 were identified in HepG2 cells by real-time PCR methodology. In response to erufosine exposure, a concentration dependent induction of CCNA1 gene was observed. Erufosine exposure inhibited the expression of CCNA2 by 1.9 and 3.6fold at IC_{50} and IC_{75} concentrations. Higher concentrations of the tested compounds substantially induced CCND1 gene expression in the target cells as shown by a maximum 2.5fold induction in response to exposure with erufosine (Figure 2).



	IC_{25}	IC_{50}	IC_{75}
24 h	5.7 µM	12.2 µM	25.7 µM
48 h	2.5 µM	6.6 µM	17.1 µM
72 h	2.4 µM	5.8 µM	13.7 µM

Figure 1: Cytotoxic effects of erufosine were investigated by MTT dye reduction assay. The compound inhibited the proliferation at all three-time intervals as determined by corresponding ELISA readings.

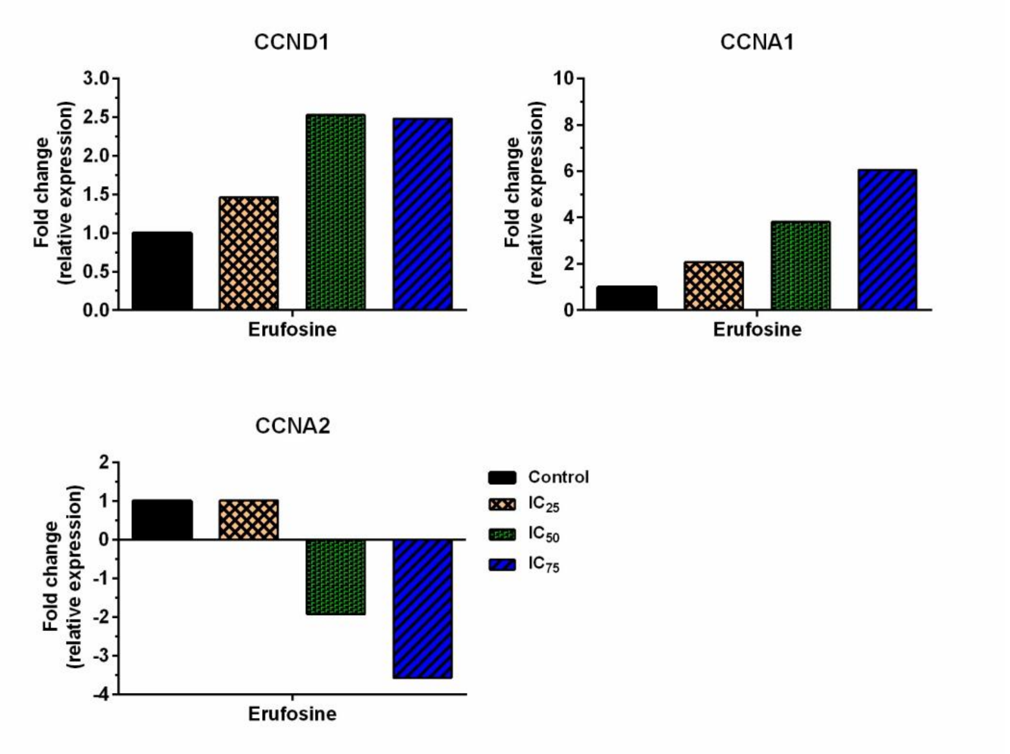


Figure 2: Expression of CCNA1, CCNA2 and CCND1 genes in HepG2 cells. Exposure with erufosine de-regulated the expression of selected cell cycle related genes in HepG2 liver cancer cells.

DISCUSSION

Synthetic ALPs have been used as therapeutic agents since the second half of twentieth century. Modifications in their structures for functional improvisation have never stopped since then. In this study, we have determined cytostatic and cytotoxic effects of erufosine by treating HepG2 cells with the compound (Erufosine). Anti-proliferative effects of erufosine on cancerous cells were estimated using MTT dye reduction assay. Moreover, effects of these treatments were directly seen on three cell cycle related genes i.e., CCNA1, CCNA2 and CCND1 by finding their expressional change in treated HepG2 cells. ALPs are known for blocking action of phosphocholine in multiplying cells. Phosphocholine is most abundantly found phospholipid in cell membrane, also this blocking action of ALP's has not been observed in non-multiplying cells [6, 8].

Erufosine, is the most advanced form of ALPs. Having lengthened hydrocarbon chain and an extra methyl group in its choline part, is known to be suppressor of protein kinase C and phospholipase C. From the results section it could be seen, the ICs of the compound was higher for 24h than 48h and 72h incubations. HepG2 cells treated with IC_{25}, IC_{50} and IC_{75} of erufosine have been found to possess the altered expression of three genes mentioned above. The altered expression of genes could be used to depict the cell cycle conditions in HepG2 cell after treatment. The up-regulated expression of CCNA1 gave us an image that cell cycle in HepG2 after treatment would have been halted in G1 phase or G2 phase. While in a much-conserved protein CCNA2, the clear down-regulated expression for all three concentrations of the compound further confirmed these halts. Protein of CCND1 gene interacts with Rb protein and clearly causes halt in just G1 phase of cell cycle. The substantial increase in expression of this gene up to 2.5 times in IC_{50} and IC_{75} of cells treated with the compound hints the halt in G1 phase of cell cycle. All in all, the study revealed anticancer potential of selected synthetic ALP against HCC cells, however further investigations are required to understand the complex mechanisms governed by erufosine against liver cancer cells. Given the fact that Perifosine (a sister compound) is already in clinical use with its limited utilization, erufosine seems to be a better and attractive option and needs due attention for further investigations as anticancer medicines against liver cancer.

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Ethics Approval: Not Applicable
Contributions: MM perfomed experiments. SI analyzed the data and drafted the manuscript.
Competing Interests: None
Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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