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Research Article

Alkyl-phospholipid mediated toxicity and regulation of transcriptomic profile of cell cycle genes in liver cancer cells

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Abstract
Background: Hepatocellular carcinoma (HCC) is the primary malignancy of the liver that accounts for about 90% of liver cancers. HCC is the sixth most prevalent cancer globally and the third driving reason for cancer associated deaths. Most used therapeutic approaches for liver cancer include surgery, chemotherapy, immunotherapy and targeted therapy. For liver disease patients, new treatment compounds are needed for a better cure for this disease. The focus of proposed study was to evaluate cytotoxic and cytostatic potential of a 3rd generation alkyl-phospholipid (erufosine) against liver cancer cells.
Methods: HepG2 liver cancer cells were exposed to different concentrations of erufosine (0.38-50μM) for 24-72 hours and effects on proliferation were determined by MTT dye reduction assay. Afterwards, the cells were exposed to three distinct concentrations of erufosine (5, 10, 25μM)) followed by total RNA extraction and cDNA synthesis. The synthesized cDNA samples were used to identify expressional alterations in our selected genes (CCNB2, CCND2, CDKN1A, and MCM4) by real-time PCR.
Results: It was clearly noticed that a substantial growth inhibition was noticed in HepG2 cells after exposure to erufosine. The genes were altered in discrete fashion where cell cycle inhibitor (CDKN1A) was induced, while cell cycle promoters (CCNB2, CCND2 and MCM4) were down-regulated especially at high concentrations of compound.
Conclusion: Erufosine showed substantial cytotoxic and anti-proliferative effects against the liver cancer cells. Expressional variations in multiple cell cycle related genes were imposed by erufosine.
Key Words: Liver cancer, HepG2, Alkyl-phospholipid, Erufosine

INTRODUCTION

Cancer is the major cause of mortality throughout the world. With expansion of population, higher average age and poor lifestyle, number of cancer diagnoses and fatalities is predicted to rise dramatically [1]. Hepatocellular carcinoma (HCC), a type of liver carcinoma, is the fifth most common carcinoma worldwide [2]. Viral infections, aflatoxins, smoking, drinking, and having a family history of cancer are all risk factors for liver cancer [3].

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For the treatment of HCC, variety of treatment interventions are available, and the choice of treatment is based on several factors, including the degree of intrinsic liver function, the presence of other chronic conditions and the availability of a particular treatment. With the fact that HCC is detected at an advanced stage, cancer symptoms and liver function impairment have limited therapy options, making treatment difficult. Surgery, chemotherapy, targeted therapy, and immunotherapy are all being used to treat liver cancer [4, 5].

Cell cycle is a closely controlled series of processes that allow cells to grow and divide. Different expressional modulations at gene levels arise during the cell cycle. Due to tightly controlled monitoring systems, cells convey the right copy of their genome to the following generation [6]. Deregulation of the cell cycle is a major characteristic of cancer cell transformation. Cancer cells manipulate regulatory

networks to circumvent regular cell cycle pathways, resulting in uncontrolled cellular proliferation. Investigating natural/synthetic compounds to counteract these alterations is a potential area for finding new cancer therapy. A number of cytostatic drugs are being developed for cancer treatment with an ultimate task to suppress cancer cell proliferation by inducing cell cycle arrest [7, 8].

Alkyl-phospholipids (ALPs) are a family of anticancer compounds that operate on cell membranes instead of DNA and have demonstrated extraordinary antineoplastic potential. They are strong sensitizers of conventional chemotherapy and radiation approach because they specifically target fast proliferating (tumor) cells, triggering growth arrest and death [9]. Based on the structural properties, ALPs have been divided into various generations. Erufosine is a third-generation ALP that interacts with lipid membranes to perform its action. Erufosine, like other ALPs, causes changes in the lipid raft of the cell membrane and exerts its anticancer effect through both cytotoxic and cytostatic mechanisms. Erufosine has been found to pass the blood-brain barrier and accumulation occurs in brain tissues, as well as having a responsive nature in brain cancers *in vitro* and *in vivo* [10]. Erufosine induces anticancer effects by apoptosis, autophagy and G2 cell cycle arrest in different cancer cell lines. Erufosine inhibited the development of rat C6 glioma tumors *in vivo* and promoted apoptosis in human astrocytoma and glioblastoma cell lines *in vitro* [11-13].

In proposed study, intentions were to investigate erufosine mediated cytotoxic effects in liver cancer cells. In addition, outcomes of the selected ALP were studied on cell cycle related genes in liver cancer cells. The data is helpful for us to understand the erufosine mediated antineoplastic effects and its subsequent utilization as anti-cancer medicine.

METHODS

Cell Culture

Human liver cancer cell line (HepG2), obtained from University of the Punjab, Lahore, was cultured in RPMI-1640 medium supplemented with L-glutamine (2 mM), 10% fetal bovine serum (FBS), streptomycin (100µg/ml) and penicillin (100IU/ml).

Growth Curve Analysis

Selected cell line was cultured in 96-well culture plates (1000-6000 cells/well/100µl medium) for three different time points (24, 48 and 72 hours). MTT solution (10 mg/ml in PBS) was added (10µl/well) and followed by incubation at 37^0C for 3 hours. Crystals of formazan made by the viable cells were dissolved by adding 50µl/well of DMSO. Optical densities were measured by an ELISA plate reader at 540 nm and growth curves were generated. Growth curve analysis was required to find the appropriate density of the cells for culturing during experiments.

Cell Death Assay

MTT assay was performed to observe cell viability and proliferation following treatment of the cell line with erufosine. For this purpose, the optimized cell number (4000cells/well) were seeded in 96-well plates (100µl media/well) treated with increasing concentrations of erufosine (0.38-50µM) for three-time points i.e., 24, 48 and 72 hours. Untreated cells grown in parallel were used as controls here and in all subsequent experiments of this study.

RNA Extraction

Liver cancer cells were cultured in 6-well plates and were treated with erufosine (5, 10, 25µM) for 48 hours. After exposure period, cell pellets were collected from the culture plates by trypsinization (0.25% trypsin) and total RNA was extracted by using a commercial kit (K0731, Thermofisher)

cDNA Synthesis and Verification

A total of 500ng extracted RNA per sample was used to synthesize cDNA by using the reverse transcriptase, Oligo-dT primers and dNTPs following the protocol of selected kit (K1622, Thermofisher). PCR based amplification of a reference gene (HPRT1) was performed to check the prepared cDNA. After that, 3ul of 50 base pair ladder and total of 7µl of the amplified product were loaded on 2.5% agarose gel in TAE buffer and visualized by gel electrophoresis.

Real Time PCR

Cell cycle associated genes were selected in this study as these genes play an important role in tumor cell progression. Primers for genes (CCNB2, CCND2, CDKN1A and MCM4) were designed by choosing gene specific sequence from NCBI Gene bank and using Primer3 software. Real-Time PCRs were performed by using SYBR Green master mix for selected genes by using the cDNA samples in duplicate. Expression levels of untreated controls were used for comparison purposes.

Statistical Analysis

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

Table 1: Primer Sequences of selected genes.

Gene	Primer Sequence (F.)	Primer Sequence (R.)
CCNB2	AGTTCCAGTTCAACCCACCA	ACCCTTTGGAGCCAACTTTT
CCND2	GTCTCAAAGCTTGCCAGGAG	ATATCCCGCACGTCTGTAGG
CDKN1A	GCTTCATGCCAGCTACTTCC	CTGTGCTCACTTCAGGGTCA
MCM4	GACGTAGAGGCGAGGATTCC	AGAGCAGTTTGACGTGCTTCC
HPRT1	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT

RESULTS

Optimization of cell numbers for experiments

The cells were cultivated in 96-well culture plates (1000-6000 cells/well/1000 medium) for 24, 48, and 72 hours to optimise the cell densities for further investigations. After each time point, MTT solution (10mg/ml in PBS) was applied (10µl/well), followed by the icubation period of 3 hours, formed formazan crystals were dissolved by adding 50µl/well of DMSO. Growth curves were produced after optical densities were calculated using an ELISA plate reader as shown in Figure 1. The cell number 4000/well was chosen as the apropriate number of cells for a 96-well plate experiments as this numebr exhibited the most consistent and linear growth throughout the course of three time periods.

Cell Death induced by erufosine

To measure the cytotoxic effects of erufosine in liver cancer cell line (HepG2), MTT assay was performed. HepG2 cells were seeded in 96-well plates in RPMI-1640 medium. After the incubation period of 24 hours, cells were treated with increasing concentrations of erufosine (0.38-50µM), for 24, 48, and 72 hours. Following the treatment intervals, the MTT test was carried out. Inhibitory effects were calculated numerically as percentages of untreated controls as shown in Figure 2. The cytotoxic effects were almost concentration dependent since the suppression of cellular proliferation became more pronounced as concentration was raised. The toxicity was clearly achieved at 3.12µM concentrations, which reflects that it was a threshold level, and cells responded with more sensitivity at this concentration. The effects were also time-dependent, with more cell growth inhibition being observed after 72 hours of treatment. Overall, erufosine significantly decreased cell growth and exhibited lethal effects against cells (HepG2).

Erufosine mediated expression of cell cycle genes

For expression data related experiments, HepG2 cells were treated with different selected concentrations of erufosine for 48 hours. Afterwards, the total RNA was extracted from untreated control and erufosine treated groups of HepG2 cell line by using commercially available extraction kit. cDNA was synthesized from the extracted samples and then subjected to PCR-based amplification of the HPRT1 gene. The primers for selected genes (CCND2, CDKN1A, CCNB2 and MCM4) were designed and optimized by gradient PCR methodology by using three different annealing temperatures (56, 59 and 62°C). Real-Time PCR was performed for selected genes, where three genes (CCNB2, CCND2 and MCM4) were down-regulated, while CDKN1A was up-regulated in response to erufosine (Figure 3).

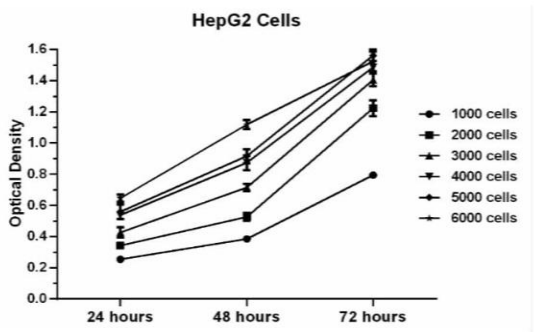

Figure 1: Growth curve analysis for three different time intervals.

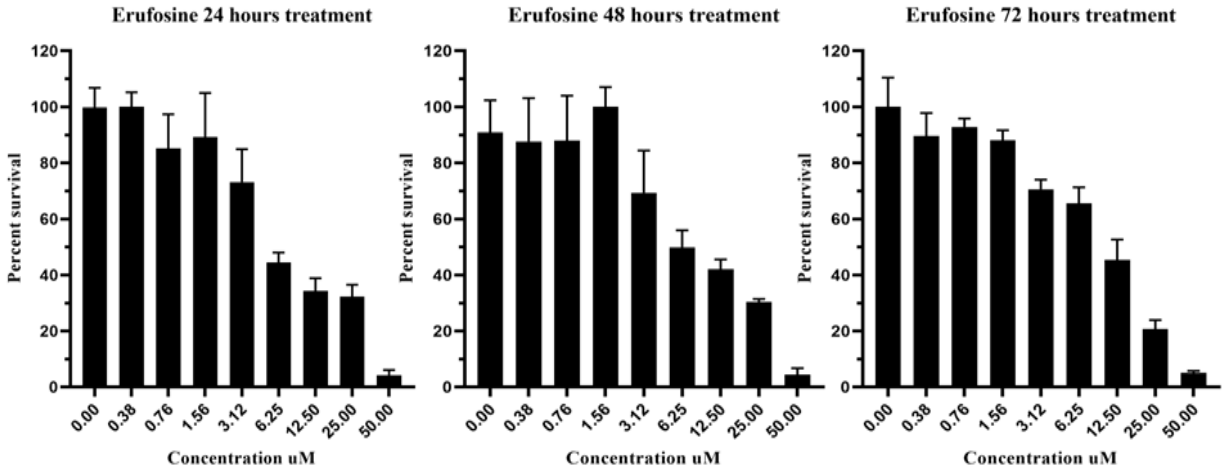

Figure 2: Inhibition of proliferation in HepG2 cells in response to erufosine exposure.

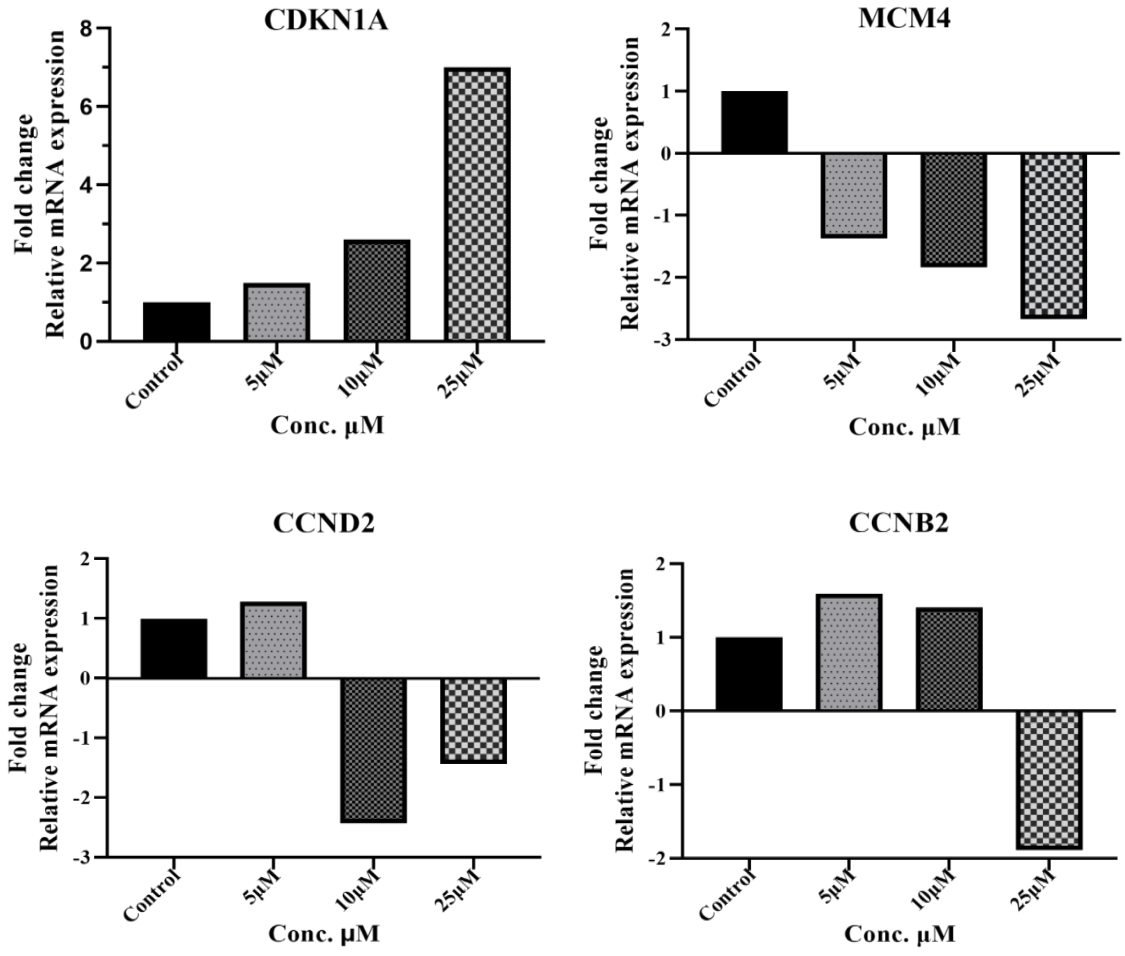

Figure 3: Expression analysis of the genes by Real time PCR.

DISCUSSION

HCC is one of the most prevalent cancers in the world. The third most frequent cause of cancer-related fatalities globally is HCC. With a life expectancy of less than 5 years in industrialized and even lower in underdeveloped countries, it is recognized as one of the fastest-spreading cancers in the United States. HCC can be treated in a variety of ways, including partial liver removal, liver transplantation, ablation, embolization, and chemotherapy [14-16].

ALPs comprise a class of antineoplastic substances that operate on cell membranes and finally target DNA to have an anticancer impact. Over time, structural changes in different ALPs have led to the creation of novel compounds with lower toxicity and higher selectivity [17]. The use of more contemporary ALPs, such as erufosine, is only permitted *in vitro* and pre-clinical investigations so far [18, 19]. Erufosine is a 3rd generation ALP having significant anticancer potential against a variety of cancer cell lines. It has lesser side effects as compared to previous classes of ALPs because it is metabolically more stable and less hemolytic than prior ALPs. Deregulation of cell cycle is a major hallmark of cancer. Therefore, transition of regulators of cell cycle can be target for the treatment of cancers.

In the present study, cytotoxic effects of erufosine on the liver cancer cells were determined by MTT dye reduction assay. For this purpose, the selected cell line (HepG2) was cultured in 96-well plates and exposed to various concentrations of erufosine (0.38-50μM) followed by MTT dye-based assessment of viable cell fractions at three distinct time intervals that are 24, 48 and 72 hours. The results showed that erufosine inhibited the proliferation of liver cancer cells effectively at all three exposure periods. Furthermore, the cytotoxic effects followed a concentration dependent format across the whole experimental period, which means more intense inhibition was observed with increasing concentrations of erufosine. With the highest applied concentration (50μM), ~95% of the cells were dead. This data shows that systemic infusion of erufosine with bearable concentrations can be instrumental in controlling HCC clinically.

Exploring the mechanistic reasoning behind the observed anti-proliferative effects was the second aim of this study. For this purpose, the cells were exposed to erufosine for 48 hours followed by RNA extraction, cDNA synthesis and expression profiling four cell cycle related genes via real-time PCR methodology. Among the down-regulated genes after erufosine exposure, CCNB1, CCND2 and MCM4 are involved in normal cell cycle progression and chromosomal complex maintenance respectively [20, 21]. This indicates that erufosine can hamper the expression of genes required for cell cycle positive progression while inducing cytostatic effects. Among the up-regulated genes, CDKN1A is a strong cell cycle inhibitor which indicates erufosine as negative regulator of cell cycle progression [22]. Overall, erufosine imposed distinct expression changes in the cell cycle related selected genes in liver cancer.

Data from current study clearly established the antineoplastic effects of erufosine against liver cancer cells. The compound induced toxic effects and showed potential to alter important regulators of cancer cell progression. Further studies are required to understand the anticancer potential of this compound and its subsequent quest as cancer medicine.

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