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Research Article

Erufosine alters the genes associated with G2/M Phase of cell cycle in cancers: Molecular evidence from gene expression analysis

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Abstract
Background: Erufosine is a novel alkyl-phospholipid with promising anticancer activity. This study investigated the molecular mechanisms underlying the anticancer effects of erufosine on breast and colorectal cancer cells, with a focus on cell cycle regulation related genes.
Methods: Breast (MDA-MB-231, MCF-7) and colorectal cancer (SW480, SW620) cells were treated with erufosine, and cell viability was assessed by MTT assay. Afterwards, the cell lines were exposed to IC_{25}, IC_{50}, and IC_{75} concentrations of the test compound for 48 hours. qRT-PCR was used to examine expression changes in G2/M phase-related genes including CCNA2, CCNB1 and CDKN3. Fold differences were calculated by using Livak method between the groups.
Results: Erufosine exhibited potent cytotoxicity at micro-molar concentrations in the cell lines. In MDA-MB-231 cells, erufosine induced concentration-dependent downregulation of all three G2/M genes. MCF-7 cells showed minimal induction at low concentrations, followed by marked inhibition at higher concentrations. In colorectal cancer cell lines, erufosine consistently inhibited the three selected genes at all applied concentrations.
Conclusions: Erufosine induces cytotoxic effects and alterations in G2/M phase related genes in breast and colorectal cancer cells. This study provides molecular evidence for the cell cycle related effects of erufosine and supports its potential as a novel therapeutic agent for cancers, particularly for metastatic phases of disease.
Key Words: Cancer, Cell cycle, Alkyl-phospholipid, Erufosine, Cell lines

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INTRODUCTION

Breast cancer is the disease that develops from malignant cell formation in breast tissue. The breast is a mammary gland composed of lobules that produce and carry milk. Breast tissues also contain fat, lymph nodes, and blood vessels. Ductal carcinoma is the most common type of breast cancer. Other types of breast cancer include ductal carcinoma in situ, invasive breast cancer, and inflammatory breast cancer [1]. Breast cancer is the most common cancer among women and the second most common cancer worldwide. Approximately two million new cases were reported in 2018. Incidence rates vary from 19.3 to 89.7 per 100,000 women worldwide. Women diagnosed with breast cancer during 2010-14 have been found with 5-years age standardized survival rates of 70-90% in different regions of the world, with the lowest rates in Africa and Eastern Europe and the highest in North America and Oceania [2]. As of 2022, breast cancer accounts for 11.6% of all cancers worldwide. It is the second most common cancer among all sexes and the most common cancer among women. Breast cancer mortality ranks fourth (6.9%) among all cancers [3]. In addition, the age-standardized incidence rate worldwide is 46.8%, while the age-standardized mortality rate is 12.6%. Moreover, the age-standardized risk of acquiring breast cancer from birth to the age of 74 is 5.05%, which is the highest among all cancers. This age-standardized incidence rate is expected to increase by 8% by 2030 [3, 4]. Lumpectomy, mastectomy, and quadrantectomy are used to treat breast cancer in combination with chemotherapy, hormone therapy, immunotherapy, and radiotherapy. Chemotherapeutic agents target DNA to induce cytotoxicity. Different chemotherapeutic regimens, including anthracyclines, carboplatin, cyclophosphamide, fluorouracil, and taxanes are used in adjuvant and neoadjuvant therapies. Unfortunately, the available therapeutic agents also induce cytotoxicity in normal living cells [5,6].

Colorectal cancer is the third most common cancer in the world present in 13.2% and 12.7% of men and women respectively and causes 550,000 cancer deaths annually in the world [1]. There are multiple genetic and epigenetic factors as risk to colorectal

cancer development. Major risks factors involved are diet (alcohol, red and processed meat), obesity and lack of physical activity. Familial adenomatous polyposis and hereditary nonpolyposis are two autosomal dominant conditions associated with high risk of colorectal cancer. Colorectal cancer is treated in combination or individually by surgery, radiotherapy and chemotherapy depending upon stage of cancer. For most cases with stage I and II colorectal cancer, surgery alone is enough as treatment. Adjuvant chemotherapy is not supported for local colon and rectal cancers; however, lymph node metastasized cancers undergo. The strongest indication for adjuvant therapy is metastasis to regional lymph nodes. Fluorouracil is one of the effective systematic treatment options available for colorectal cancer in combination with leucovorin. Other available compounds are capecitabine, irinotecan, and oxaliplatin. Neoadjuvant chemotherapy is supported in rectal cancer and decreases risk of recurrence to ~50% in stage II and III of rectal cancer but it primarily depends upon stage of the cancer [7-9].

The cell cycle is the sequence of events from the duplication of DNA to cell division. This results in two daughter cells from one parent cell. Cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors/facilitators and checkpoints regulate cell cycle. In carcinogenesis, these checkpoints and players are deregulated, and uncontrolled growth of cancer cells begins [10]. Currently, several cycle-targeting drugs are available for clinical use. However, these drugs have been found to induce considerable side effects, such as alopecia, bone marrow, and gastrointestinal toxicity [11]. Thus, novel therapeutic agents with minimal hemolytic activity and gastrointestinal toxicity are required. Erufosine, a 3rd generation alkyl-phospholipid, is a cytostatic compound that forms lamellar structures in water. It contains a 22-carbon chain as a modified side chain as compared to other members of alkyl-phospholipid family. The unique characteristics of this compound include less hemolytic activity, reduced bone marrow toxicity, and the ability to cross the blood-brain barrier [12]. Erufosine is found to be effective cytostatic agent against several cancers including colorectal cancer, leukemia, multiple myeloma, oral squamous cell carcinoma, prostate and breast cancer as demonstrated by *in vitro* and *in vivo* studies [13-18]. The current study aimed to determine the molecular basis of erufosine-induced arrest in breast cancer cells. For this purpose, following the antiproliferative effects of the compound in cancer cells, three representative genes related to cell cycle were investigated at mRNA levels.

METHODS

Cancer Cell Lines

Two human breast (MDA-MB-231 and MCF-7) and colorectal (SW480 and SW620) cancer cell lines were cultured in RPMI-1640 medium (21875-034, Gibco) supplemented with 10% fetal bovine serum (10270-106, Gibco), L-glutamate (A2916801, Gibco), mixture of streptomycin (100µg/ml) and penicillin (100IU/ml) (15140-122, Gibco). Among the cell lines, MDA-MB-231 and SW620 were metastatic in nature, while MCF-7 and SW480 were primary. A regular use of recommended antibiotics was maintained to keep cell culture free of contaminants. Cells were incubated under culture conditions (5% CO_2, 37˚C, humidified environment) during the experiments.

MTT Dye Reduction Assay

MTT dye reduction assay was performed to observe cell viability and proliferation following treatment with erufosine. The cell lines were seeded in 96 well plates (triplicate) with optimized cell densities (4000 cells/well/100µl media) and treated with various concentrations of erufosine dissolved in 1X PBS (0.75-50µM) for three-time points i.e., 24, 48 and 72 hours. Following treatment intervals, MTT solution (10mg/ml in PBS, 20395.01, Serva) was added (10µl/well) and cells were incubated under standard conditions for 3 hours. Formazan crystals, formed by the viable cells, were dissolved by adding 10µl of acidic solvent (0.04 N HCL in 2-propanol) in each well. Optical density was measured by ELISA reader (Krefeld, Germany) at 560 nm and 690 nm reference filter. Cell survival rates were calculated as a percentage of untreated cells. Inhibitory concentrations (ICs) were calculated using GraphPad Prism 6 software.

Treatment with Erufosine

Selected cell lines were cultured in 6-well cell culture plates (150,000 cells/well/2ml medium) and treated the next day with erufosine (IC_{25}, IC_{50}, and IC_{75}) for 48 hours. The cell pellets were collected by trypsinizing step and stored at -80˚C. Likewise, the cell pellets of untreated cells as controls were also collected and stored under similar conditions.

RNA Extraction

Total RNA was extracted from the control and treated cell pellets using the RNeasy Mini Kit (74104, Qiagen) according to the manufacturer's protocol. Extracted RNA was quantified using a spectrophotometer (Nanodrop ND 2000).

cDNA Synthesis

A total of 1000ng of extracted RNA/sample was used to synthesize cDNA (20µl) using olido dT and Maxima Reverse Transcriptase (EP0741, Thermo Scientific). PCR-based amplification of a reference gene (GAPDH) was performed to verify the prepared cDNA. PCR was run for 10µl of reaction including 2X master-mix (5µL), 10pmol paired primers (1µL), nuclease-free water (3µL), and synthesized cDNA (1µL). This PCR had 35 cycles (95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 45 seconds). The amplified products were visualized using agarose gel electrophoresis with 100 volts for 45 minutes on a 2.5% agarose gel prepared in TAE buffer. A total of 10µl of each amplified sample was loaded for electrophoresis along with 3µL of the DNA marker (50bp/10416014, Fermentas).

Primer Designing

Three cell cycle G2/M phase arrest genes, CCNA2, CCNB1, and CDKN3, were chosen, and β-Actin served as a housekeeping gene. Primers for these selected genes were designed by choosing gene sequences from the NCBI GenBank using Primer3 software. Primer sequences are given in Table 1.

qRT-PCR for Selected Genes

qRT-PCR was performed using SybrGreen fluorescence dye (4301955, Thermo Scientific) for selected genes using cDNA samples from cell lines treated with three concentrations of erufosine (IC_{25}, IC_{50}, or IC_{75}) for 48 hours. The $2^{-\Delta\Delta CT}$ method was used to determine expressional changes in the three selected genes in the control and erufosine-treated samples. β-Actin was used as reference housekeeping gene in these experiments.

Table 1: Primer sequences of genes

Gene	Primer Sequence
CDKN3	F: CCCTCAGAAATGATCGGAAA
	R: CAGCTTGCGATAACCAAAGG
CCNB1	F: CCTCCTTGGAAAGCAAACAG
	R: TCAAGAGGGACCAATGGTTT
CCNA2	F: CACTTCCTTCGGAGAGCATC
	R: AGAAGGAGGAAAGTGCACCA
β-Actin	F: TCCACCTTCCAGCAGATGTG
	R: GCATTTGCGGTGGACGAT
GAPDH	F: ACGGATTTGGTCGTATTGGG
	R: CGCTCCTGGAAGATGGTGAT

RESULTS

Erufosine Toxicity

The toxic effects of erufosine on the cells were studied by MTT dye reduction assay, as described in the methods section. ICs were identified using GraphPad Prism 6 software, and IC_{50} values of erufosine against the selected cancer cells are given in Table 2. Erufosine was found to be highly toxic at micro-molar concentrations in the cancer cell lines. Specifically, erufosine was more toxic to metastatic breast cancer cells (MDA-MB-231) when compared with primary cells (MCF-7) when considering the longer exposure (48 and 72 hours) and respective IC values. In colorectal cancer, the two cell lines responded in similar fashion with comparable IC values. Furthermore, one fact was cleared that erufosine loses its toxicity overtime as high IC values were observed in later time (48 and 72 hours).

Expressional Profiling of G2/M Phase Genes

Based on our understanding and known effects of erufosine to induce G2/M phase arrest, we conducted a molecular investigation of G2/M phase related genes. For molecular investigation, we extracted RNA from cell lines treated with erufosine and untreated controls. cDNA was synthesized for each RNA sample, and a confirmatory PCR of GAPDH was executed for successful RNA extraction and cDNA synthesis validation (Figure 1). The amplified products were visualized using agarose gel electrophoresis.

For the abovementioned three genes related to the G2/M phase, primers were designed and optimized by amplifying two cDNA samples. Optimized primers were used to identify expression changes in three G2/M related genes (CCNA2, CCNB1, and CDKN3) via qRT-PCR in the cells after exposure to low (IC_{25}), medium (IC_{50}), and high (IC_{75}) concentrations of erufosine for 48 hours. In a concentration-dependent manner, three selected genes were downregulated in MDA-MB-231 cells, whereas there was minimal induction of the genes at low (IC_{25}) and medium (IC_{50}) concentrations followed by marked inhibition at higher concentrations of erufosine (IC_{75}) in MCF-7 cells (Figure 2). In colorectal cancer cell lines, three genes were consistently downregulated at all applied concentrations. However, these effects were more pronounced in metastatic cells (SW620) as shown in Figure 2. Overall, erufosine demonstrated substantial potential to inhibit the three-cell cycle related genes in the primary and metastatic breast and colorectal cancer cells.

Table 2: IC50 concentrations of erufosine after 24-, 48- and 72-hours exposure

	Breast Cancer Cells		Colorectal Cancer Cells	
	MDA-MB-231	**MCF-7**	**SW480**	**SW620**
24 h	8 µM	8.2 µM	5.8 µM	5.5 µM
48 h	12.5 µM	17 µM	10.1 µM	10 µM
72 h	20 µM	40 µM	15.8 µM	20.1 µM

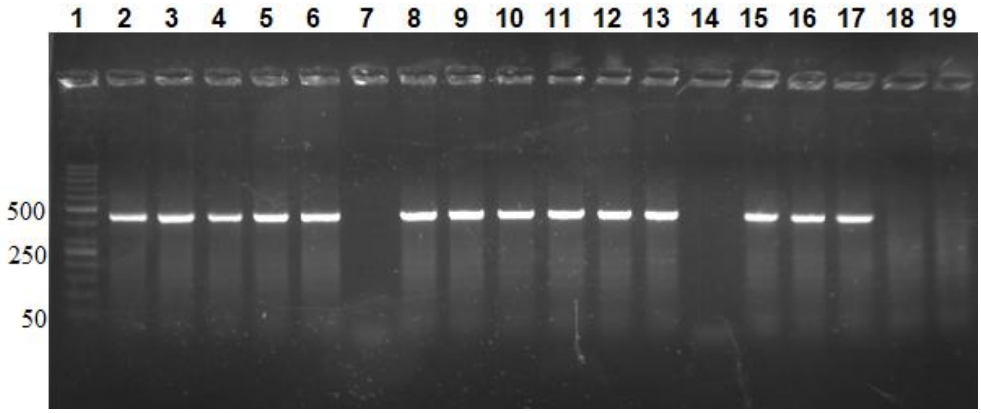

Figure 1: Electrophoresed cDNA of cells. Amplified cDNA samples for GAPDH gene were visualized by using agarose gel electrophoresis.

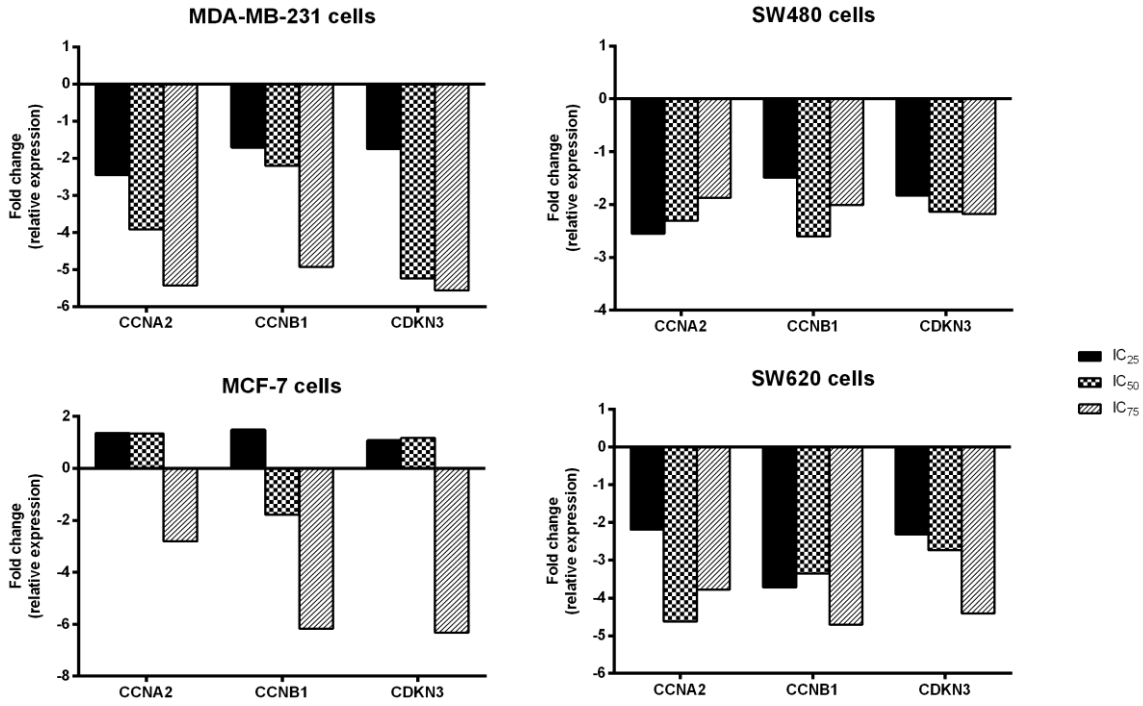

Figure 2: Expressional changes in G2/M phase related genes in breast and colorectal cancer cells treated with different inhibitory concentrations of erufosine. The expression of the genes was identified by using real-time PCR method and fold changes were determined via Livak method while comparing the data sets from treated and untreated control samples. Fold value of untreated samples was considered as equal to one.

DISCUSSION

Functional properties of cells are influenced by genetic changes. Keeping in view this fact, cell cycle is an important functional property of cancer cells and factors influencing this need to be investigated. In this study, we aimed to explore the mechanistic basis of known cytostatic effects imposed by erufosine in cancer cells. For this purpose, metastatic and primary breast and colorectal cancer cells were exposed to the test compound and qRT-PCR was used to profile the changes in G2/M phase cell cycle-related genes induced. These three genes were CCNA2, CCNB1, and CDKN3, respectively. Erufosine-mediated alterations in these genes are shown in Figure 2, while cellular viability affected by the compound is shown in Table 2. The viability results showed that metastatic breast cancer cells (MDA-MB-231) were more sensitive to erufosine than the primary breast cancer cells (MCF-7). Erufosine was found to induce concentration-dependent effects on the three selected genes in cancer cell lines, which in turn indicated its potential to affect the target cells (at the gene level) regardless of their different molecular subtypes or origin. These findings also indicate that erufosine induces its effects at gene levels on target cells by targeting components/molecular pathways that are common in primary and metastatic cancer cells. Similar trends of genetic de-regulations by erufosine were observed for colorectal cancer cells. Considering this, erufosine can be exploited as a therapeutic compound against the primary and metastatic phases of cancer cells with similar expected outcomes. As we know, metastatic cancers are difficult to treat, and available treatment strategies enable us to achieve very low five years survival rates in the advanced stages of these cancers (<22%). This dilemma requires novel therapeutic compounds with better efficacy and minimal side effects. Alterations in molecular pathways help the cancer cells for better survival and fast proliferation. Deregulated cell cycle is one of the key features of cancer cells, where canonical pathways are tuned for fast cellular proliferation [19]. Considering this, a number of chemotherapeutic and targeted agents have been introduced as therapeutic drugs to halt the rapid cell cycle of tumor cells.

Alkyl-phospholipids comprise a class which also induce cytostatic effects in target tumor cells by altering the AKT/mTOR machinery and cyclin/CDK cascades [20]. Erufosine, the latest generation of alkyl-phospholipids, has emerged as a new synthetic compound with reduced gastrointestinal toxicity and hemolytic activity [21-23]. Although the compound was synthesized almost two decades ago, significant attention has been given to evaluating its potential as an anticancer agent in preclinical settings in recent years. Erufosine is a metabolically stable compound with the potential for intravenous administration and crossing the blood-brain barrier. Pathways have been investigated and proposed as the basis for the anticancer effects of erufosine against various cancer cell lines. The key molecular pathways regulated or altered by erufosine to induce its anticancer effects include induction of apoptosis through caspases, inhibition of proliferation by targeting the AKT/mTOR pathway, and cell cycle arrest by altering the key regulatory genes (cyclins/CDKs). More importantly, these effects are tumor cells specific as erufosine affects the vital molecular pathways needed for effective tumor cell proliferation [24, 25].

In this study, erufosine was also found to be a highly toxic compound and induces cytotoxic effects at low concentrations (IC_{50}: 5.5-8.2μM) in primary and metastatic breast and colorectal cancer cells (24 hours exposure). At the same time, the compound inhibited the three-cell cycle supportive genes (CCNA2, CCNB1, CDKN3) consistently in the cells. This data shows that erufosine can effectively slow down the proliferation of cancer cells while imposing the required genetic expressional changes. In conclusion, erufosine is a potential therapeutic agent for cancer treatment further in vitro and in vivo experiments are needed to understand the effects of erufosine at molecular levels to support its clinical utilization.

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