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Research Article

Antineoplastic effects of erufosine in combination with oxaliplatin in colorectal cancer

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Abstract

Background: Combinations of the anticancer agents is a commonly practiced strategy in cancer treatment as it provides an efficient way to control the disease burden with reduced side effects. Finding novel compounds to be combined with the available chemotherapeutic drugs for better treatment of colorectal cancer is a much-needed research domain. In this context, alkyl-phospholipids comprise an attractive class of compounds which induce anticancer effects by targeting the cell surface membranes. Erufosine is a 3rd generation of alkyl-phospholipid class and interacts with the surface lipid molecules of the cell membranes to induce antineoplastic effects in cancer cells.

Methods: Toxic effects of the selected alkyl-phospholipid (erufosine) and a commonly used chemotherapeutic agent (oxaliplatin) for colorectal cancer were identified in the three human cancer cell lines. For this purpose, the cells were exposed to various concentrations of the test compounds (single agent and combination) for 24-72 hours and cell viability was assessed. Afterwards, the cells were exposed to the distinct concentrations of the test compounds for 48 hours and expressional modulations in a cell cycle master regulator (P21) and stress marker (GADD45A) were identified by qRT-PCR methodology.

Results: The results indicated that erufosine and oxaliplatin induced substantial anti-proliferative effects in colorectal cancer cells. The inhibitory effects of erufosine and oxaliplatin alone were significantly pronounced. In addition, synergistic anti-proliferative effects were observed when erufosine was combined with oxaliplatin. In addition, qRT-PCR data showed noteworthy potential of the test compounds to induce expression of P21 and GADD45-A genes at mRNA levels in the cells. Combination of erufosine and oxaliplatin induced P21 gene more prominently in the cells, especially in metastatic colorectal cancer cells.

Conclusion: Erufosine and oxaliplatin impose substantial cytotoxic effects in the colorectal cancer cells. The combination of erufosine and oxaliplatin showed synergistic anti-proliferative effects. The compounds up regulated the expression of cell cycle inhibitor and cell stress marker (P21 and GADD45A) in colorectal cancer cells.

Key Words: Colorectal cancer, Alkyl-phospholipid, Oxaliplatin, Treatment, Combination, Synergism

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INTRODUCTION

Colorectal cancer (CRC), cancer of large intestine, is the most prevalent malignancy of gastrointestinal (GI). CRC is a significant contributor to morbidity and mortality in both genders globally [1, 2]. In the late 1990s, CRC ranked as the fourth leading cause of cancer-related deaths among both men and women under 50 but it has risen to become the leading cause of cancer death in men and the second leading cause in women [3]. In 2012, there were approximately 1.4 million new cases and 693,900 deaths attributed to CRC, while by 2018, these figures rose to over 1.8 million new cases and 881,000 deaths, making it the second leading cause of cancer mortality and third in incidence worldwide [4, 5]. The primary treatment modality for CRC is surgery followed by chemotherapy. At the time of diagnosis, around 20-25% of CRC patients exhibit metastatic stage, and an additional 50-60% develop metastasis over time [6]. The five-year survival rate is approximately 90% for early-stage CRC patients, but this drops to about 10% for those with advanced metastatic disease [5]. For patients diagnosed at stage I or II, surgery is the standard treatment. In contrast, stage III CRC treatment typically involves surgical intervention followed by adjuvant chemotherapy. For metastatic colorectal cancer (mCRC), systemic chemotherapy—either alone or combined with targeted biological therapies—is the preferred treatment approach. Emerging treatment options for both primary and metastatic CRC include laparoscopic surgery for initial disease management, more aggressive resections, radiotherapy, palliative care and neo-adjuvant chemotherapies [7, 8].

Most common chemotherapeutic agents for adjuvant therapy in mCRC include cytotoxic drugs such as 5-fluorouracil, oxaliplatin and capecitabine. Biomarker-based treatments like using bevacizumab to target angiogenesis, panitumumab and cetuximab, which inhibit the epidermal growth factor receptor (EGFR) are also employed. Selection and combination of these drugs depend on various factors including the patient's overall health, tumor histology, and specific immunohistochemical characteristics. This tailored approach allows for the optimization of treatment efficacy in individual patients [9-11].

Oxaliplatin, a platinum derivative and alkylating agent, mediates its mechanism of action by formation of DNA adducts. Oxaliplatin exhibits *in vitro* activity against cisplatin-resistant human tumor cells, including CRC cells. When used as single agent, oxaliplatin achieves a response rate of 20-25% in patients who are chemo-naive and a 10% response rate in previously treated patients. Oxaliplatin has been approved for treatment of CRC. Preclinical observations suggest that oxaliplatin has synergistic antitumor activity with 5-FU both *in vitro* and *in vivo* [12-15]. Incorporation of oxaliplatin into treatment regimens for mCRC has significantly improved survival rates, extending the average survival from 12 months to approximately 20 months [16, 17]. Adjuvant treatment in which combination of oxaliplatin and 5-FU is used considered to be a standard treatment for UICC stage III patients [18, 19]. A combination of all three agents named as FOLFOXIRI is a feasible treatment option for patient survival and increased response rate but with increased toxicity including neurotoxicity and neutropenia [20]. Further research on oxaliplatin based combination therapy is the need of the hour to ace the challenge of chemoresistance as the survival and quality life of CRC patients is significantly influenced by treatment decisions and the optimization of therapeutic strategies.

Alkyl-phospholipids (ALPs) are synthetic lipids that can be generally divided into two main categories: alkyl-phospholipids and alkyl-phosphocholines, which carry glycosylated derivatives. As the structure of ALPs resembles to natural phospholipids, they disrupt lipid homeostasis and affect the lipid rafts within cellular membranes, which in turn influences lipid-linked signaling pathways and biochemical routes [21, 22]. They exhibit specific pro-apoptotic effects on tumor cells compared to conventional chemotherapeutic agents, primarily due to increased cellular uptake [23]. ALPs exert their effects through different mechanisms of action including disruption of cellular processes within tumor cells leading to cell death through various pathways. One such mechanism is the inhibition of phosphocholine biosynthesis by targeting the enzyme CTP: phosphocholine cytidylyltransferase. This inhibition results in depletion of phosphocholine (PC), which in turn, triggers apoptosis by inducing endoplasmic reticulum stress [24]. Other mechanism involves the inhibition of the breakdown of PC into phosphatidic acid, which disrupts the signaling pathways regulating cell proliferation [25]. ALPs promote the accumulation of free cholesterol in tumor cells and influence cholesterol homeostasis resulting in increased cell death and affects growth signaling pathways. ALPs hinder AKT activation by disruption of specific membrane microdomains essential for natural activation of the protein. Furthermore, ALPs stimulate FAS/CD95 signaling by redistribution of FAS to lipid rafts and facilitate apoptosis through the activation of death-inducing signaling complexes [24].

Erufosine is the third and latest generation ALP with promising pharmacokinetic and pharmacodynamics properties and significant cytotoxic effects with minimal GI toxicity. It triggers apoptosis in various tumor models and specifically harm cancer cells while sparing normal bone marrow cells at the same concentration [26, 27]. Erufosine is the first intravenously applicable compound of ALP family because it exhibited significant antineoplastic activity as well as reduced bone marrow and hemolytic toxicity *in vivo* which allows its use as a monotherapy and combination regimes at higher doses. The lamellar structure of erufosine makes it potent to use intravenously as compared to other ALPs having micellar structure. Cytotoxic effects of erufosine have been documented in various cell lines related to leukemia, multiple myeloma, prostate cancer, breast cancer, and squamous cell carcinoma. It exerts its anticancer effects by modulating several signaling pathways such as apoptosis, autophagy and G2 cell cycle arrest. Erufosine induced cytotoxicity in dose dependent manner in human squamous cell carcinoma cell lines. Erufosine inhibited the colony growth, induced apoptosis and G2 arrest while down-regulated m-TOR signaling pathway [28-30]. Erufosine can cross blood-brain barrier and accumulate in brain tissue, making it a potential therapeutic option for treating brain tumors, particularly glioblastoma. Reduction in cellular growth and viability of rat and human astrocytoma cell lines was observed after treatment with erufosine [31-33]. Two prominent breast cancer cell lines (MCF-7 and MDA-MB-231) had shown sensitivity when exposed to concentrations of erufosine ranging from 6.25-100μM. Antineoplastic efficacy of different concentrations of erufosine in combination with single ionizing radiation therapy was observed in PC3, DU145 and LNCaP cells i.e. prostate cancer cell lines [34-37]. Antineoplastic effects of erufosine leukemic cells have been determined by using various concentrations of erufosine (0.001-250μM) *in vitro* where IC_{50} was 60.2, 27.2 and 22μM after 24, 48 and 72 hours respectively. More than 60μM erufosine without significant toxicity can be achieved in humans according to phase I trial

CLL patients [28]. Cytotoxic effects of erufosine were demonstrated on two bladder carcinoma cell lines IC_{50} was 4-5μM [38]. Antiproliferative and apoptotic effects of erufosine were observed in human SW480 cell line in a dose-dependent and time-dependent manner after exposure with concentration ≥12.5μM. Cell survival of rat CC531 cell line was also inhibited in concentration and time dependent manner on erufosine exposure [39].

This study was designed to explore the effects of erufosine in combination with oxaliplatin, while investigating anti-proliferative and gene modulation potential of the test compounds against CRC cell lines.

METHODS

Cell Culture

Three human CRC cell lines; SW480 (primary), SW620 (metastatic), and HCT116 (primary), were cultured and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium. This cell culture medium is enriched with glutathione, a reducing agent, and contains a high concentration of vitamins. The RPMI-1640 medium is deficient in proteins, lipids, and growth factors; thus, it was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics including streptomycin (100μg/ml) and penicillin (100IU/ml). To maintain logarithmic growth of cell populations, standard humid conditions (100% humidity), along with 5% CO2 and a temperature of 37°C, were maintained for cell incubation. The cell lines were sub-cultured regularly to maintain their viability and promote optimal growth under the standard conditions.

MTT Dye Reduction Assay

Cellular proliferation was assessed using the MTT dye reduction assay, a widely used method for evaluation of cell viability. This assay quantifies the reduction of a yellow tetrazolium dye to purple formazan crystals through the activity of mitochondrial dehydrogenases of viable cells. The assay involved seeding of the CRC cell lines in 96-well plates for overnight adherence where SW480 and SW620 cells were plated at a density of 4,000 cells/well, and HCT116 cells were plated at a density of 3,000 cells/well. The cells were subsequently incubated with different concentrations of erufosine and oxaliplatin, either as a single agent or in combination, for three different time points: 24, 48, and 72 hours, as detailed in Table 1. Following this incubation period, MTT solution was added to the treated cells and allowed to incubate for an additional three hours to facilitate the formation of formazan crystals. The tested crystals were then dissolved in DMSO, and the optical density was measured using an ELISA reader at wavelengths of 545nm and 650nm. The percent survival rate in untreated controls were assessed, and the inhibitory concentration (IC) values were calculated using GraphPad Prism 6 software. All the assays were performed at least twice in triplicates. Results were compared to those of the corresponding untreated control cells cultured alongside.

Treatment and RNA Extraction

In a separate experiment, the three CRC cell lines (SW480, SW620, HCT116) were cultured in 6-well plates at a density of 150,000 cells/well/2ml of media. The cells were treated with the test compounds either individually or in combination (as detailed in Table 2) for a duration of 48 hours. The concentrations of compounds used were determined based on the results of MTT assay. After treatment intervals, the cells were harvested using trypsinization and subsequently rinsed with PBS. Total RNA was isolated from both untreated control and treated cells using a commercially available extraction kit (Thermo Fisher Scientific, Cat#K0731), adhering to manufacturer's protocol.

cDNA Synthesis and Primer Optimization

A total of 20μL of complementary DNA (cDNA) per sample was generated using a commercial kit (Thermo Fisher Scientific, Cat#K1622). This was accomplished from 1000ng of extracted RNA through the action of reverse transcriptase enzyme and Oligo dT primers. PCR-based amplification of the reference gene (HPRT1) was performed to validate synthesized cDNA samples. The amplified products were subjected to electrophoresis on a 2.5% agarose gel for visualization. Primers for the selected genes (P21 and GADD45A) were designed using Primer3Plus software. The primers (*P21: Forward GCTTCATGCCAGCTACTTCC, Reverse CTGTGCTCACTTCAGGGTCA, GADD45A: Forward AACGGTGATGGCATCTGAAT, Reverse CCCTTGGCATCAGTTTCTGT*) were optimized using gradient PCR technique and the resulting amplified products were visualized on 2.5% agarose gel electrophoresis.

Real Time PCR for P21 and GADD45A Genes

Quantitative real-time PCR (qRT-PCR) was performed for the selected genes P21 and GADD45A by using the SybrGreen fluorescence dye (Thermo Fisher Scientific, Cat#K0221). This analysis utilized cDNA samples derived from three CRC cell lines, which were treated with varying concentrations of compounds alone or in combination (Table 2). All samples were amplified in triplicate using an Agilent AriaMx real-time PCR machine. The expression levels of the reference gene (HPRT1) were utilized to normalize the data sets.

Analysis and Presentation of Data

The analysis of the toxicity index in response to the exposure to test compounds was conducted using GraphPad Prism software (Version 10) which

facilitated the calculation of the relevant inhibitory concentrations (IC). Percentage survival of untreated control cells, which were grown alongside the test samples, was established as the baseline representing 100% survival. In relation to the dataset generated by using real-time PCR, following amplification and normalization of data from experimental (treated) and untreated control groups, fold changes were determined using the 2-ΔΔCT (Livak) method. All experiments were performed in triplicate, and their average values were used for data analysis.

Table 1: Treatment concentrations of Erufosine and Oxaliplatin for MTT assay

	Single agent treatment with compound/drug	
	Erufosine	Oxaliplatin
SW480	0.78, 1.56, 3.12, 6.25, 12.5, 25, 50μM	0.78,1.56, 3.12, 6.25, 12.5, 25, 50μM
SW620	0.78, 1.56, 3.12, 6.25, 12.5, 25, 50μM	0.78,1.56, 3.12, 6.25, 12.5, 25, 50μM
HCT116	0.78, 1.56, 3.12, 6.25, 12.5, 25, 50μM	0.38,0.78,1.56, 3.12, 6.25, 12.5, 25μM
	Combinational treatment with compound/drug	
SW480	1.56, 3.12, 6.25μM	1.56, 3.12, 6.25, 12.5μM
SW620	1.56, 3.12, 6.25μM	1.56, 3.12, 6.25, 12.5μM
HCT116	1.56, 3.12, 6.25μM	1.56, 3.12, 6.25,12.5μM

Table 2: Treatment concentrations of Erufosine and Oxaliplatin for Real-Time PCR

	Single agent treatment with compound/drug	
	Erufosine	Oxaliplatin
SW480	1.25, 2.5, 5, 10μM	0.37, 0.75, 1.5, 3μM
SW620	1.25, 2.5, 5, 10μM	0.37, 0.75, 1.5, 3μM
HCT116	1.25, 2.5, 5, 10μM	0.37, 0.75, 1.5, 3μM
	Combinational treatment with compound/drug	
SW480	2.5, 5μM	0.37, 0.75, 1.5μM
SW620	2.5, 5μM	0.37, 0.75, 1.5μM
HCT116	2.5, 5μM	0.37, 0.75, 1.5μM

RESULTS

Cytotoxic Effects of Erufosine and Oxaliplatin

The combination of erufosine and oxaliplatin exhibited substantial anti-proliferative effect on SW480 cells demonstrating dosage and time-dependent effects. Maximum growth inhibition was observed after a 72-hour exposure (Figure 1). Treatment with each compound individually decreased cell proliferation significantly and reductions were noted at all points: 24, 48, and 72 hours. The reduction in cell survival was significantly greater when erufosine was combined with oxaliplatin compared to single agent treatment. This synergistic effect, particularly noticeable at later time intervals of exposure. In a nutshell, erufosine and oxaliplatin individually inhibited the growth of SW480 cells; however, their combined application demonstrated more pronounced anti-proliferative effects.

Proliferation inhibitory activities of erufosine and oxaliplatin in SW620 cells were substantial where maximum inhibitory activity was observed after 72 hours treatment (Figure 2). The highest concentration of erufosine (50μM) induced drastic growth inhibition even after 24 hours of exposure time. The combined treatment with erufosine and oxaliplatin significantly reduced the cell survival at all time points (24 to 72 hours). Combined treatments presented a greater potency in inhibiting cell proliferation at later time intervals compared to the individual treatments. While both erufosine and oxaliplatin were effective in inhibiting the proliferation of SW620 cells, their combinations demonstrated a greater efficacy in suppression of cancer cell growth.

Erufosine and oxaliplatin showed anti-proliferative activity in HCT116 CRC cells with maximum inhibitory activity observed after 72 hours exposure time. Activity of these compounds was particularly notable in HCT116 cell line showing a time-dependent gradual decrease of viable cells (Figure 3). Low concentrations of erufosine when used in combination with oxaliplatin imposed a significant reduction in the growth of HCT116 cells, indicating the combination therapy is particularly effective in inhibiting cell proliferation. While both erufosine and oxaliplatin are potent as a single agent, their combinations markedly enhanced the anti-proliferative activity in the HCT116 cell line (Figure 3).

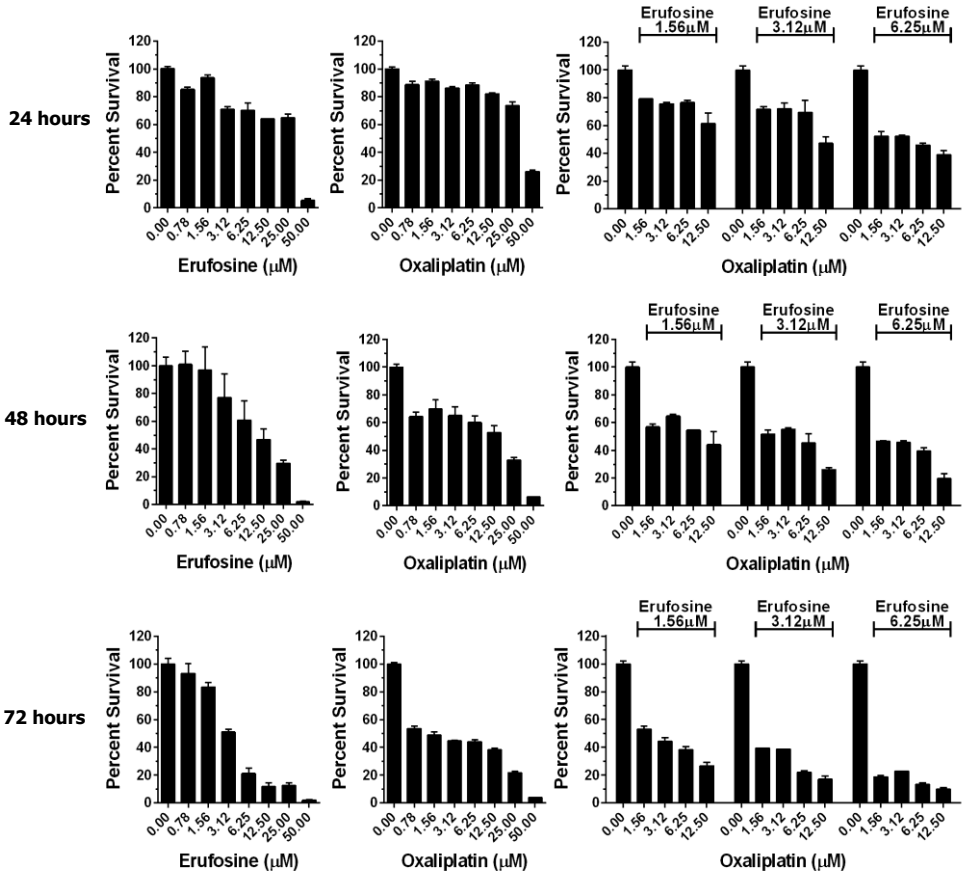

Figure 1: MTT results of SW480 after treatment with erufosine and Oxaliplatin alone and in combinations.

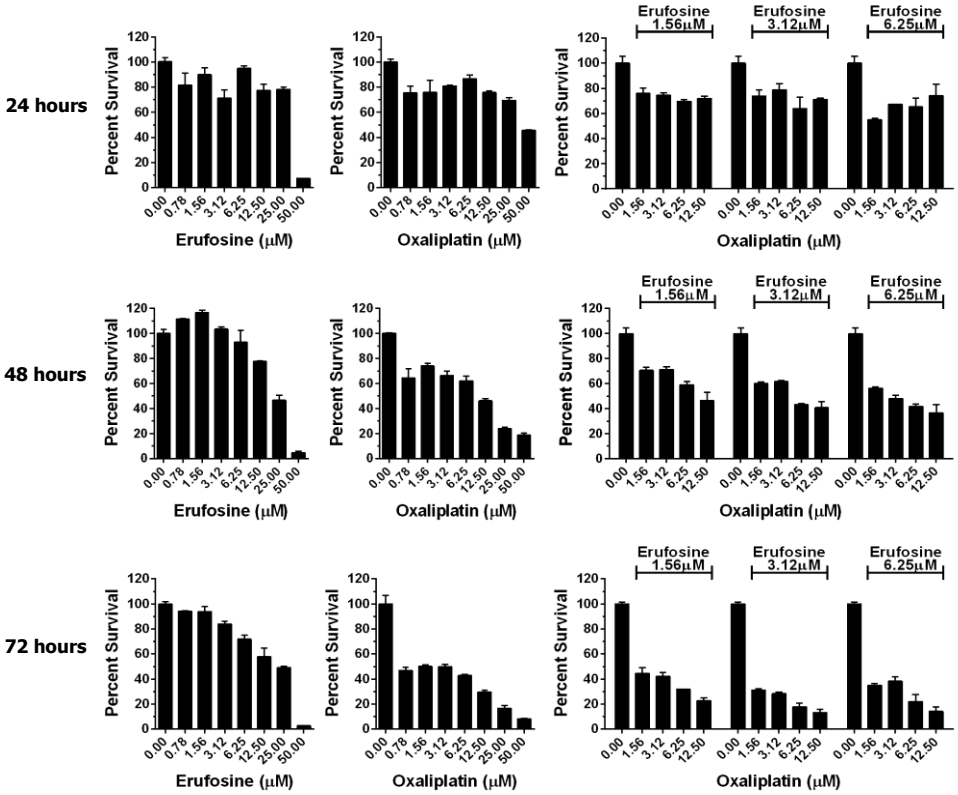

Figure 2: MTT results of SW620 after treatment with erufosine and oxaliplatin alone and in combinations.

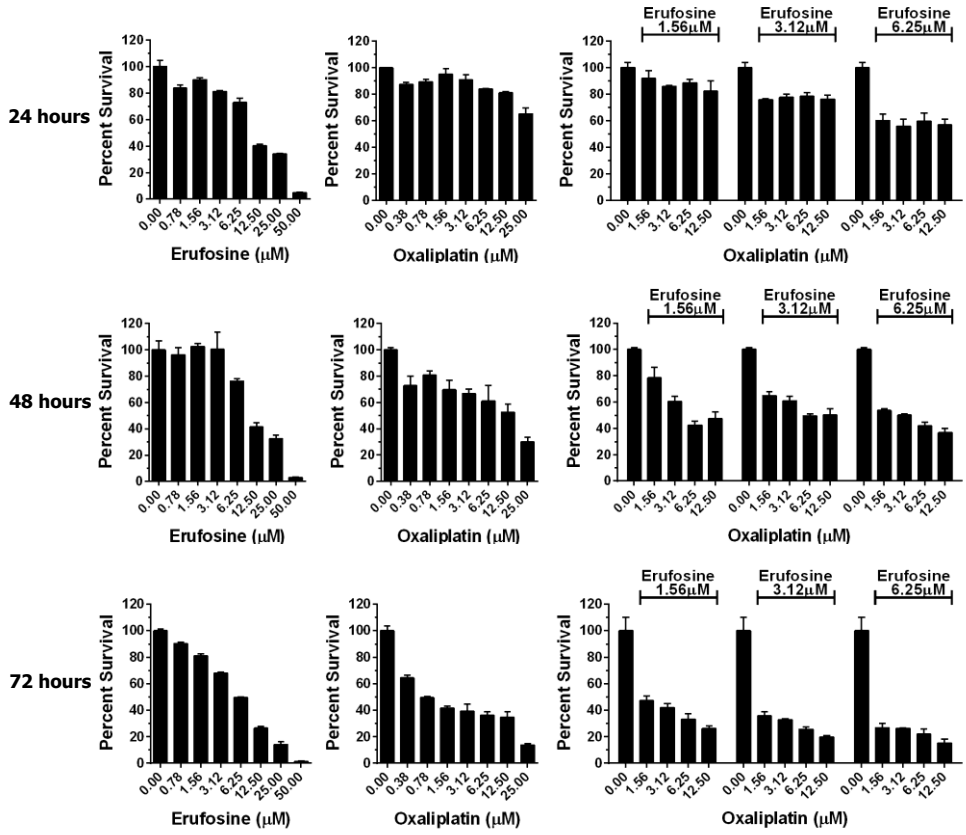

Figure 3: MTT results of HCT116 after treatment with erufosine and oxaliplatin alone and in combinations.

Expression of P21 and GADD45A Genes after Single Agent Treatment

In SW480 cells, erufosine exposure resulted in a fractional inhibition of the P21 gene, with a maximum reduction of -1.44-fold. Conversely, there was an induction of the GADD45 gene with a maximum increase of 2.39-fold in this cell line. Oxaliplatin was found to induce a significant up-regulation in expression of the P21 gene in SW480 cells. The highest concentration of oxaliplatin (3μM) resulted in a maximum activation (9.58fold) in the cells. In terms of GADD45 expression, a slight activation was observed at the lowest concentration of oxaliplatin (0.37μM), whereas moderate inhibition occurred at the higher concentrations (0.75 to 3μM).

Exposure to erufosine and oxaliplatin induced more significant expressional modulations in SW620 CRC cells in comparison with SW480 cells (Figure 4). Erufosine induced a significant increase of the P21 and GADD45 genes, with maximum change of 5.7-fold and 5.5-fold respectively. Notably, the maximum changes were observed till 5μM concentrations of erufosine but the concentrations exceeding this level exhibited the diluting effects, resulting in lower induction levels of P21 (4.06-fold) and GADD45 (3.16-fold) at further higher concentration of erufosine (10μM). Oxaliplatin was the most effective compound in inducing the expression of P21 gene in SW620 cells. Precisely, the selected drug induced 38fold up-regulation of P21 gene when the cells were exposed to 1.5μM concentration. In contrast, GADD45 gene was not altered equally as a maximum of 3.5fold was observed with 1.5μM concentration of oxaliplatin.

Erufosine induced reasonable alterations in P21 and GADD45 genes in HCT116 cells as shown by a maximum of 1.9 and 3.5fold induction. Moreover, the trend was not uniform as some inhibition was observed in the two genes (P21/-1.1fold, GADD45/-1.6fold) with 2.5μM concentration of erufosine. Oxaliplatin was the most effective agent as far as alterations in selected genes (P21 and GADD45) are concerned. Furthermore, the compound induced the expression of the two genes in HCT116 cells in a concentration dependent format. P21 gene was maximally induced to 21fold while GADD45 gene was induced maximum to 3.7fold with the highest applied concentration of oxaliplatin (3μM). Expressional modulations in P21 and GADD45 genes induced by the selected compounds (erufosine and oxaliplatin) are shown in Figure 4.

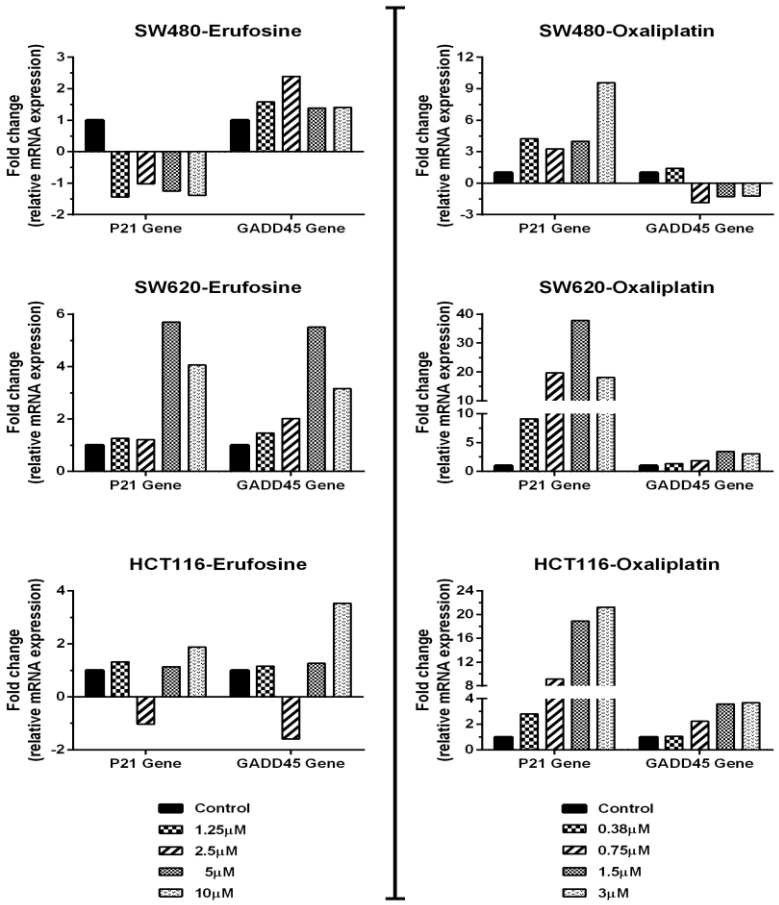

Figure 4: Expressional changes in P21 and GADD45A in SW480, SW620 and HCT116 after single agent treatment

Expressional Modulations after Combinational Treatment

Combination of erufosine and oxaliplatin induced significant alterations in P21 gene in SW480 cells. A maximum of 4.8fold induction in P21 gene was observed when the cells were exposed to 5µM erufosine and 0.75µM oxaliplatin concentrations combinedly. Interestingly, higher concentration of oxaliplatin (1.5µM) was less effective in inducing P21 gene when combined with erufosine (2.5 and 5µM). Combination of oxaliplatin and erufosine was also effective for inducing GADD45 gene. The maximum observed induction was 3.5fold while exposing the cells to 5µM erufosine along with 0.37µM oxaliplatin, however these effects were not in a concentration dependent format. Following the exposure of SW480 cells with combinational treatment approach, overall alterations in P21 and GADD45 genes were compared and shown in Figure 5.

Combining erufosine and oxaliplatin induced P21 gene effectively in SW620 cells. However, the effects were not concentration dependent. Nevertheless, highest level of induction (59fold) was observed with 5µM erufosine combined with 1.5µM oxaliplatin, while minimal induction was observed with a combination of 2.5µM erufosine and 0.37µM oxaliplatin as shown in Figure 5. Combination of erufosine and oxaliplatin inhibited the expression of GADD45 gene in SW620. Inhibitory effects were not concentration dependent, while maximum inhibition of 4.7fold was noticed with 2.5µM erufosine combined with 1.5µM oxaliplatin.

Combination of oxaliplatin and erufosine significantly induced P21 gene in HCT116 cells. A maximum up-regulation of 28fold was witnessed when the cells were exposed to 2.5µM erufosine along with 0.75µM oxaliplatin. Interestingly, regardless of erufosine concentration, maximum induction of P21 gene was observed when the cells were exposed to 0.75µM oxaliplatin. Noticeable induction in GADD45 gene was also observed when the cells were treated with erufosine combined with oxaliplatin. Maximum induction (2.2fold) was observed with 5µM erufosine and 0.75µM oxaliplatin combination. Following the exposure of HCT116 cells with combinational approach, overall alterations in P21 and GADD45 genes were compared and shown in Figure 5.

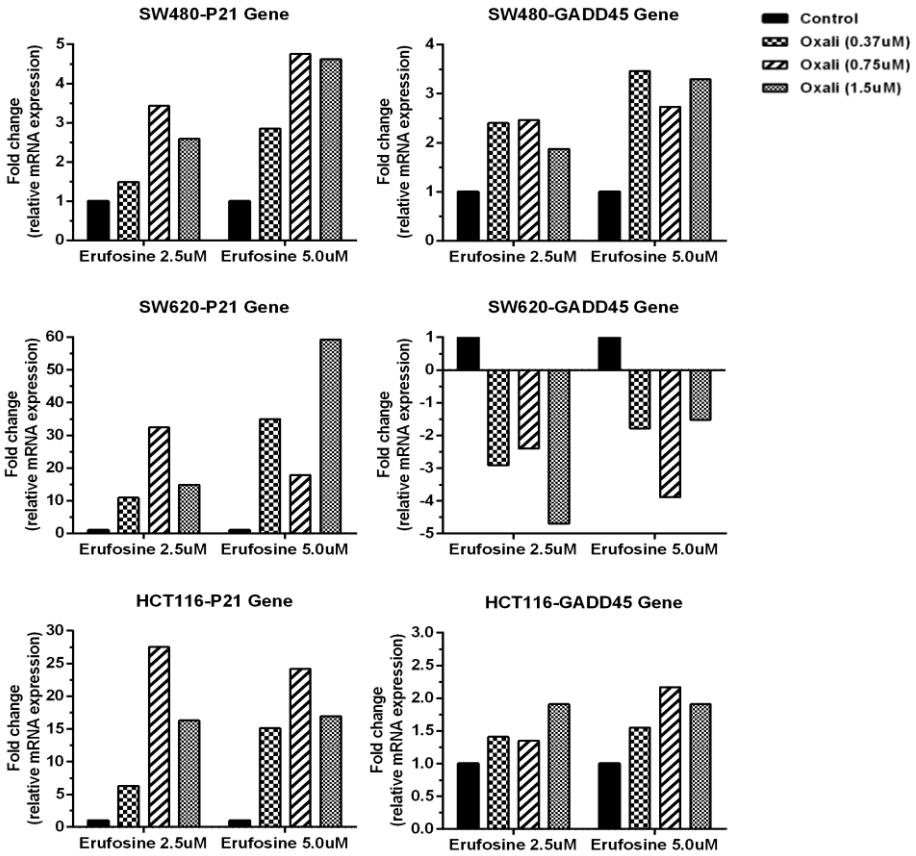

Figure 5: Expressional changes in P21 and GADD45A genes in SW480, SW620 and HCT116 cells after combinational treatment

DISCUSSION

CRC is a prevalent cancer among men and women globally. Recent data from World Health Organization (WHO) indicates a significant rise in CRC incidence and high mortality rate particularly in Asian countries [40, 41]. Initial diagnosis of CRC patients indicate almost 20-25% individuals already have metastatic and 50-60% will develop metastasis over time [42]. Most common organ involved in mCRC is liver and other metastatic sites are peritoneum, lungs, bone and brain [43, 44]. The mainstay curative treatment for non-metastatic CRC is surgery but chemotherapy is the treatment of choice to deal with the in-operable metastasis [45]. Metastatic CRC is often harder to treat and tends to have a poor treatment outcome as there is a low 5-year survival rate of about 11% of the patients with metastatic CRC. Platinum derivative, oxaliplatin, achieved a response rate of 10% for the patients already treated with 5-FU and 20-25% in untreated patients of mCRC in phase II studies. Oxaliplatin is a platinum-based compound which can induce apoptosis by linking the two adjacent guanine residues in DNA strands. A significant synergistic antitumor activity of 5-FU and oxaliplatin was observed in preclinical studies in both *in vivo* and *in vitro* where the objective response rate was not higher than 20% [46]. Despite of rapid advancement in diagnosis and treatment, conventional cytotoxic chemotherapy for the management of mCRC is still a main challenge as significant survival has not achieved yet due to severe side effects coupled with high incidence of drug resistance and increased toxicity i.e., FOLFOXIRI [45, 47].

ALPs bind to the surface membranes of cells and induce antineoplastic effects selectively in tumor cells. Erufosine is the latest generation (3rd) of ALPs and has shown significant cytotoxic and cytostatic impact against various cancer cell lines [48]. Combining the synthetic ALPs with oxaliplatin considered to be an attractive area of research. In this study, cytotoxic effects of erufosine were investigated in combination with oxaliplatin. For this purpose, three CRC cells lines (SW480, SW620 and HCT116) were selected and grown in 96-well plates in cell culture settings and exposed to various concentrations of erufosine (0.78-50μM) and Oxaliplatin (0.38-50μM) alone or in combinations followed by assessment of proliferation by MTT dye reduction assay as shown in Table 1. The CRC cell lines showed a substantial decrease in cell viability after exposure to single agents. In addition, the decline was more prominently observed in the cell lines when treated with combination of erufosine and oxaliplatin. These anti-proliferative effects were almost time and dose-dependent (Figures 1-3). In addition to this, the selected CRC cells were more responsive towards erufosine and oxaliplatin exposure as higher doses of the later compound were required to induce equal inhibition of the cellular proliferation. This observation highlights that erufosine and oxaliplatin are more efficient in inducing the cytotoxic/apoptotic routes in the CRC cells indicate more effective anticancer results can be achieved with erufosine and oxaliplatin in clinical scenarios while using lesser quantities of the compounds. As far as combinations of erufosine with oxaliplatin are concerned, synergistic anti-proliferative effects were observed. Combining the drug with erufosine induces more effective inhibitory effects on proliferation of the CRC cells as compared to single agent treatment. The results are promising as it indicates the possibility of achieving substantial growth inhibition of CRC cells in clinical settings in future while using lesser quantities of the compounds. Furthermore, it will reduce the burden of the side effects due to lower doses of chemotherapeutic drugs usage, thus improving the overall quality of life of the patients. It will be of worth to investigate the anti-proliferative signaling cascades being operated by erufosine and oxaliplatin together. Nevertheless, cytotoxic data indicates substantial inhibition of cell viability in the three CRC cell lines in response to exposure with erufosine and oxaliplatin, while there was synergism observed when erufosine was combined with FDA approved chemotherapeutic drug (oxaliplatin).

Exploring the reasoning at molecular levels is a needed fact to understand any change in functional properties of the cells. Proliferation is one of the hallmarks of cancer cells and our selected compounds (erufosine/oxaliplatin) induced substantial inhibitory effects on this functional property of the CRC cells. Thus, it was worth investigating potential reasons lying behind the cytotoxic effects of the selected compounds. For this purpose, two important players of apoptosis and cell cycle (GADD45A and P21) were selected. GADD45 is a gene involved in sensing the cell stress and leading the way for apoptotic mechanism, while P21 gene is a well-known cell cycle inhibitor. It was decided to investigate the potential expressional changes in these two important regulators in response to exposure of the CRC cells with the selected compounds. For this purpose, the CRC cell lines were allowed to grow in 6-well plates (150,000 cells/well/2ml media) and exposed to the test compound (erufosine) and chemotherapeutic drug (oxaliplatin) alone or in combination for 48 hours. Following the total RNA extraction and cDNA synthesis from treated and untreated control groups, expressional alterations in two selected genes were identified via real-time PCR methodology. Expressional modifications in P21 gene in response to single agent exposure were compound and cell line dependent as shown in Figures 4 and 5. Precisely, in SW480 cells, erufosine induced P21 gene effectively but there was a substantial induction in response to oxaliplatin. In contrast, in other two CRC cell lines (SW620 and

HCT116), the two compounds almost persistently induced the expression of P21 gene. Furthermore, induction of P21 gene was more prominent in SW620 and HCT116 cells. The results indicate that molecular differences at the level of cells play a vital role as far as expressional changes in P21 gene are concerned in response to exposure to the test compounds. Nevertheless, as a general trend, the compounds induced the expression of P21 gene in CRC cells and considering the importance of this gene as master cell cycle inhibitor, the compounds can be exploited as cytostatic agents in clinical settings. More importantly, like cytotoxic data, the effects of combinational approach were synergistic while inducing expression of P21 gene in the CRC cells. Precisely, either the inhibition of P21 gene was reverted to induction or up-regulation was more prominent (Figure 5). It is important to mention that this synergistic induction of P21 gene with the combination of erufosine and oxaliplatin was more effective in metastatic CRC cells (SW620, Figure5), which in turn, indicates that combining the compound (oxaliplatin) with erufosine can inhibit the proliferation of metastatic CRC cells more effectively.

An essential step to mediate anticancer activity of multiple chemotherapeutic drugs is the induction of GADD45 expression and the effects of drugs might be revoked by the absence of GADD45 genes. Chemotherapeutic agents often rely for their anticancer activity on GADD45 up-regulation for induction of cell cycle arrest and apoptosis in tumor cells [49]. In this study, an up-regulation of GADD45A gene was observed in response to exposure of the CRC cells with erufosine and oxaliplatin as single agent treatment (Figures 4). However, the induction of GADD45A, in response to exposure with selected compounds, was not as effective as observed in case of P21 gene. These observations indicate that erufosine and oxaliplatin rely more on signaling cascades which interfere with P21 expressional modifications more effectively as compared to pathways converging at GADD45A expression levels. Additionally, SW620 cells were found to be more prone when talking about induction of GADD45A gene by exposing the cells with the selected compounds. In contrast to P21 expressional data, where a synergism was observed with erufosine and chemotherapeutic drug (oxaliplatin) for inducing the expression, almost negligible synergistic effects were found when the CRC cells were exposed to the compounds in combination. Infect, even there was a marginal inhibition of GADD45A gene in SW620 cells, when the cells were exposed to erufosine in combination with oxaliplatin (Figures 5). These observations indicate the possibility of negative feedback loop(s) for inhibiting the up regulation of GADD45A gene via erufosine or oxaliplatin combinations.

To conclude, erufosine and oxaliplatin induced substantial cytotoxic effects in the CRC cells. Combination of erufosine and oxaliplatin lead to synergistic anti-proliferative effects in the CRC cells. Substantial induction of a master cell cycle inhibitor (P21) was observed in response to exposure of CRC cells. The up regulation of P21 gene was more prominent when the cells were exposed to combination of erufosine and oxaliplatin. All in all, these compounds showed ample cytotoxicity against the CRC cells and their combinations are considerably effective for inhibiting proliferation of the cells. Further *in vitro and in vivo* investigations are needed to support evaluation of erufosine in combination oxaliplatin against colorectal cancer in clinical settings.

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