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Research Article

Cytotoxic effects of synthetic alkyl-phospholipid (Erufosine) in combination with chemotherapy (5-FU) against colorectal cancer cells

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Abstract
Background: Colorectal cancer (CRC) is a commonly diagnosed malignancy. An approach used in treatment of this cancer is combinational treatment where anticancer compounds are combined for cure. Exploring the novel anticancer compounds for potential usage as combinational approach is a burning domain. In this regard, alkyl-phospholipids are novel agents and erufosine is a 3rd generation alkyl-phospholipid compound that interacts with surface membrane of cell and induces antineoplastic effects in the malignant cells.
Methods: Toxic effects of erufosine and 5-FU were identified against the three human CRC cell lines. For this purpose, the cells were exposed to the test compounds for 24-72 h and cell viability was assessed by MTT dye reduction assay. Afterwards, the cells were exposed to distinct concentrations of the test compounds (IC_{25}, IC_{50}, IC_{75}) for 48 h and expressional modulations in cell cycle regulator (P21) and stress marker (GADD45A) were identified by qRT-PCR methodology. For both MTT assay and qRT-PCR analysis, the cells were exposed to selected compounds as single agent or combination in parallel to avoid any time-lapse related differences between the experimental findings.
Results: Erufosine and 5-FU induced substantial anti-proliferative effects in SW480, SW620, HCT116 CRC cells. The inhibitory effects of erufosine were more pronounced in comparison to 5-FU. Furthermore, synergistic anti-proliferative effects were observed when erufosine was combined with 5-FU. In addition, qRT-PCR data showed noteworthy potential of the test compounds to induce expression of P21 and GADD45A genes at mRNA levels in the cells. Precisely, combination of erufosine and 5-FU induced P21 gene more prominently in the cells, especially in metastatic CRC cells (SW620).
Conclusion: Erufosine and 5-FU bear substantial cytotoxic potential against the CRC cells. Combination of erufosine and 5-FU showed synergistic anti-proliferative effects in the cells. The compounds up-regulate expression of a cell cycle inhibitor and cell stress marker (P21 and GADD45A gene) in CRC cells.
Key Words: Colorectal cancer, Erufosine, 5-FU, Proliferation, Expression

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INTRODUCTION

CRC is the most common cancer of gastrointestinal (GI) tract and a major cause of morbidity and mortality in both men and women across the globe [1]. CRC is 3rd most commonly diagnosed malignancy in males and second in females [2, 3]. Approximately 20-25% of patients with CRC have metastasis at the time of diagnosis while 50-60% of remaining proportion develop metastasis overtime [4]. Five-year survival rate is approximately 90% for the patients diagnosed with early stage CRC but 10% for the patients with advanced stage metastatic disease [3]. Currently, the basic treatment for stage I or stage II patients is surgery, standard treatment for stage III CRC is surgery followed by adjuvant chemotherapy whereas systemic chemotherapy alone/combination with targeted biological compounds is treatment of choice for mCRC. To provide the additional options for patients, treatments for primary and metastatic CRC (mCRC) have emerged including laparoscopic surgery for primary disease, more aggressive resection of metastatic disease, radiotherapy, palliative and neoadjuvant chemotherapies [5]. Most common chemotherapeutics used for adjuvant therapy in mCRC are the cytotoxic drugs like 5-FU, oxaliplatin, irinotecan, capecitabine and biological agents like bevacizumab that acts against angiogenesis, or panitumumab and cetuximab that inhibit the endothelial growth factor receptor (EGFR). Depending on patient condition, tumor histopathology and immunohistochemical properties, these drugs can be administered in different combinations [6].
5-FU was the first agent used in adjuvant treatment but when chemosensitivity to 5-FU diminished in some cases, oxaliplatin and leucovorin were added in

mCRC treatment and survival rate has been increased from 12 to 20 months [7]. Adjuvant treatment is considered to be a standard treatment for UICC stage III patients in which combination of 5-FU and oxaliplatin is used [8]. 5-FU has been an almost standard treatment modality for CRC for more than 40 years. This pyrimidine analogue enters the cells through facilitated transport and inside of the cell is converted into active metabolites that inhibit thymidylate synthase, an important enzyme in the synthesis of pyrimidine nucleotides. Although continuous infusion of 5-FU increases the response rates in advanced CRC by only 10-15% , it remains an integral constituent in CRC therapy and is also used as an adjuvant or palliative drug in breast, pancreatic, and stomach cancers, and is given in combination with other drugs [9, 10]. Administration with leucovorin increases the cytotoxicity of 5-FU, but benefits in terms of survival rate still remain around 10-15% in advanced CRC tumors [11, 12]. Despite its effectiveness, challenges concerning chemoresistance development and variable responses demand further research to establish more optimized 5-FU-based treatments.

Alkyl-phospholipids (ALPs) are synthetic lipids and can be broadly classified into two categories, namely, alkyl-phospholipids and alkyl-phosphocholines, which include glycosylated derivatives. Due to structural similarity with the endogenous phospholipids, ALPs interfere with lipid homeostasis and impact on cellular membrane lipid rafts that influence lipid-linked signaling pathways [13]. They have advantages over the conventional chemotherapeutic agents as specific pro-apoptotic effects on tumor cells due to enhanced cellular uptake [14]. Action mechanisms of ALPs include interference with cellular processes of tumor cells, initiating cell death through several action mechanisms. One of them is through the inhibition of phosphocholine biosynthesis via the inhibition of the enzyme CTP: phosphocholine cytidylyltransferase, thereby depleting phosphocholine and inducing apoptosis through endoplasmic reticulum stress [15]. Another mechanism is through the inhibition of phosphocholine degradation into phosphatidic acid, thus breaking signaling pathways that ensure cell proliferation [16]. Additionally, ALPs affect cholesterol homeostasis by enhancing the accumulation of free cholesterol within the tumor cells, which further enhances cell death and impacts growth signaling. They can inhibit Akt activation, through disruption of specific membrane microdomains contributing to the natural activation of Akt. Finally, ALPs activate FAS/CD95 signaling, which is known to re-localize FAS to lipid rafts and promote apoptosis through activation of death-inducing signaling complexes [15]. To summarize, the above mechanisms mean that such ALPs can selectively induce apoptosis in cancerous cells while sparing normal cells, thus highlighting the utility of such ALPs as effective anticancer drugs.

Among the ALPs, erufosine is the latest generation of this class and possesses promising pharmacokinetic attributes. Erufosine has demonstrated antineoplastic effects against cell lines including leukemia, numerous myelomas, prostate, breast and squamous malignant growth cells. Erufosine forced anticancer impacts in different cell lines by apoptosis, autophagy and G2 cell cycle [17]. Erufosine also intercepted cholesterol homeostasis, generations of ATPs and proteins related to cytoskeleton. These change lead towards stress, absence of vitality and migratory activities respectively. Erufosine is metabolically stable with decreased harmfulness towards GI tract and less hemolytic action *in vivo*. These qualities empowered the intravenous implementation of erufosine to accomplish pertinent clinical fixations, which was impractical with other ALPs [18]. Structural change of erufosine made it less hemolytic than previous ALPs subsequently making its intravenous application achievable. This attribute of erufosine is because of the improvement of lamellar structures in aqueous solutions instead of micelles like other ALPs [19]. Erufosine comparatively is less lethal for bone marrow than others ALPs so empowering its higher intravenous dosages as monotherapy and mix regimens [20]. Dineva and colleagues studied erufosine for its antiproliferative movements both *in vitro* and *in vivo* against the breast cancer, while targeting PI3k/AKT pathway [21]. Erufosine found to prompted apoptosis in interminable lymphocytic leukemia. It acted by restraining caspases and initiate apoptosis by pathways that are dependent on these. It is additionally uncovered that retinoblastoma dependent signaling pathway that is vital for antineoplastic action of erufosine [22]. Human CRC cell lines have been investigated for erufosine responsiveness. Human cell line (SW480) was found sensitive to erufosine with IC50 of 3.4μM and rodent cell line CC531 was seen as sensitive to erufosine at IC50 of 25.4μM [18]. All in all, erufosine has shown substantial antineoplastic effects against cancers.

Purpose of the current study was to investigate effects of erufosine in combination with clinically used FDA approved drug (5-FU) against the CRC cells at functional and molecular levels.

METHODS

Cell Culture

Three human CRC cell lines i.e., SW480 (primary), SW620 (metastatic) and HCT116 (primary) were grown and maintained in Roswell Park Memorial Institute (RPMI)-1640, a cell culture medium which contains glutathione (a reducing agent) and high concentration of vitamins. RPMI-1640 medium lacks proteins, lipids or growth factors; therefore, the media was supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, streptomycin (100µg/ml) and penicillin (100IU/ml). To maintain the logarithmically growing cell populations, standard humidified conditions (100%) along with 5% CO_2 and 37°C temperature were provided to incubate the cells. The cell lines were cultured regularly (2-3times/week) to keep cells alive and growing efficiently under the standard conditions.

MTT Dye Reduction Assay

Cellular proliferation was measured by the MTT dye reduction assay, which is a standard cell viability assay that measures the reduction of a yellow tetrazolium dye to purple formazan crystals by mitochondrial dehydrogenases of viable cells. The assay plated the CRC cell lines; SW480 at 4000 cells/well, SW620 at 4000 cells/well, and HCT116 at 3000 cells/well for overnight in 96 well plates for adherence. The cells were then incubated with different concentrations of erufosine and 5-FU either as a single or in combination for three time points that are; 24, 48, and 72 h as explained in Table 1. Then the MTT solution was added to the treated cells and incubated for 3 h further to allow formation of crystals of formazan. The crystals were later dissolved in DMSO, and the optical density was assessed with an ELISA reader at 546/650 nm wavelength. Percent survival rates in untreated controls were determined, and GraphPad Prism 6 software was used to find inhibitory concentrations (ICs). All the assays were performed at least twice in triplicates. Comparison was made with relevant untreated control cells growing side by side.

Treatment and RNA Extraction

The CRC cell lines (SW480, SW620, HCT116) were grown in 6-well plates (150,000 cells/well/2ml media) and exposed to the test compounds alone or in combination (Table 2) for 48h. Concentrations of the compounds for these experiments were selected based on the above-mentioned MTT assay results. After the treatment intervals, the cells were collected by trypsinizing and washed with PBS. Total RNA was extracted from the control and treated cells by using commercially available extraction kit (Thermo Fisher Scientific, Cat#K0731).

cDNA Synthesis and Primer Optimization

A total of 20µl cDNA/sample was synthesized by using a commercial kit (Thermo Fisher Scientific, Cat#K1622) from the extracted RNA (1000ng) by using the reverse transcriptase enzyme and Oligo dT primers. PCR based amplification of a reference gene (HPRT1) was done to verify the synthesized cDNA samples. The amplified products were loaded on 2.0% agarose gel and visualized by electrophoresis. Primers for the selected genes (P21 and GADD45A) were designed by Primer3Plus software. The primers (*P21: Forward GCTTCATGCCAGCTACTTCC, Reverse CTGTGCTCACTTCAGGGTCA, GADD45A: Forward AACGGTGATGGCATCTGAAT, Reverse CCCTTGGCATCAGTTTCTGT*) were optimized by using gradient PCR methodology and amplified products were visualized on 2.0% 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 SybrGreen florescence dye (Thermo Fisher Scientific, Cat#K0221), prepared cDNA samples from the three CRC cell lines treated with different concentrations of the compounds alone or in combination (Table 2). All the samples were amplified in triplicate, while using a real-time PCR machine of Agilent company (AriaMx). Expression levels of reference gene (HPRT1) were used to normalize the data sets.

Analysis and Presentation of Data

Toxicity index in response to the exposure of test compounds was analyzed by using GraphPad Prism software while calculating the ICs. For this purpose, percentage survival of untreated control cells grown in parallel was used as equal to 100% survival ratios. Regarding PCR based generated data set, after the amplification procedures and normalization of data sets obtained from experimental (treated) and untreated control groups, fold changes were calculated by the $2^{-\Delta\Delta CT}$ (Livak) method. All the experiments were performed in triplicate and their averages were used for data analysis.

Table 1: Treatment concentrations of Erufosine and 5-FU for MTT assay

	Single agent treatment with compound/drug	
	Erufosine	**5-FU**
SW480	0.78, 1.56, 3.12, 6.25, 12.5, 25, 50μM	1.56, 3.12, 6.25, 12.5, 25, 50, 100μM
SW620	0.78, 1.56, 3.12, 6.25, 12.5, 25, 50μM	1.56, 3.12, 6.25, 12.5, 25, 50, 100μM
HCT116	0.78, 1.56, 3.12, 6.25, 12.5, 25, 50μM	1.56, 3.12, 6.25, 12.5, 25, 50, 100μM
	Combinational treatment with compound/drug	
SW480	1.56, 3.12, 6.25μM	6.25, 12.5, 25, 50μM
SW620	1.56, 3.12, 6.25μM	6.25, 12.5, 25, 50μM
HCT116	1.56, 3.12, 6.25μM	6.25, 12.5, 25, 50μM

Table 2: Treatment concentrations of Erufosine and 5-FU for Real-Time PCR

	Single agent treatment with compound/drug	
	Erufosine	**5-FU**
SW480	1.25, 2.5, 5, 10μM	6.25, 12.5, 25, 50μM
SW620	1.25, 2.5, 5, 10μM	6.25, 12.5, 25, 50μM
HCT116	1.25, 2.5, 5, 10μM	6.25, 12.5, 25, 50μM
	Combinational treatment with compound/drug	
SW480	2.5, 5μM	6.25, 12.5, 25μM
SW620	2.5, 5μM	6.25, 12.5, 25μM
HCT116	2.5, 5μM	6.25, 12.5, 25μM

RESULTS

Cytotoxic Effects of Erufosine and 5-FU

The anti-proliferative activity on SW480 CRC cells was significantly effective when erufosine and 5-FU were used in combination. The effects were shown to be dose- and time-dependent. Maximum growth inhibition was noted after exposure for 72 h (Figure 1). Treatment with compounds individually also reduced cell proliferation considerably. However, the cell survival reduction was more pronounced by combination treatments of erufosine with 5-FU than single-agent treatments. This synergistic effect, especially at later exposure times, displayed that the treatment might not only be more potent on the growth inhibition of cells but also extend to increase the overall efficacy of treatment in general. In short, while erufosine and 5-FU separately inhibited the proliferation of SW480 cells, their application together indicates a more significant anti-cancer effect.

Anti-proliferative activities of erufosine and 5-FU on SW620 cells were substantial and showed maximum inhibitory activity at 72 h post-exposure (Figure 2). The maximal effect was achieved with the highest concentration of erufosine at 50μM, showing notable growth inhibition already after 24 h. By the combined treatment with erufosine and 5-FU, survival of cells was highly reduced at all time intervals (24-72 h). These combined treatments were also more potent at later time intervals to inhibit the proliferation of cells as compared to mono-treatments. Overall, erufosine and 5-FU inhibited proliferation in SW620 cells more effectively in the combinational approach.

Erufosine and 5-FU showed powerful anti-proliferative activity against HCT116 CRC cell line, with maximum inhibitory activity occurring at later intervals of exposure. Activity of the compounds was most pronounced in HCT116 cell line, exhibiting an increase over time compared with other cell lines. Low concentrations of erufosine combined with 5-FU also showed a significant inhibition on the growth of HCT116 cells. In this case, though both erufosine and 5-FU were effective, this combination proves to be highly effective in enhancing their anti-cancer activity in HCT116 cell line (Figure 3).

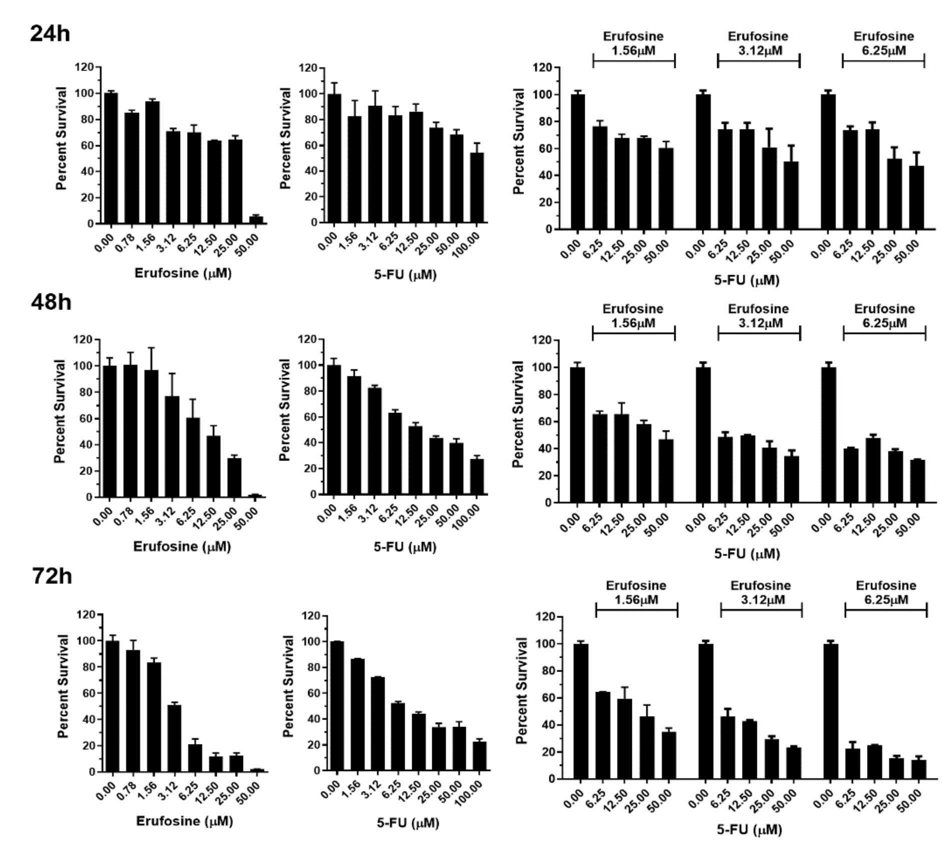

Figure 1: MTT results of SW480 after treatment with erufosine and 5-FU alone and in combinations. The effects were measured by using the dye reduction assay.

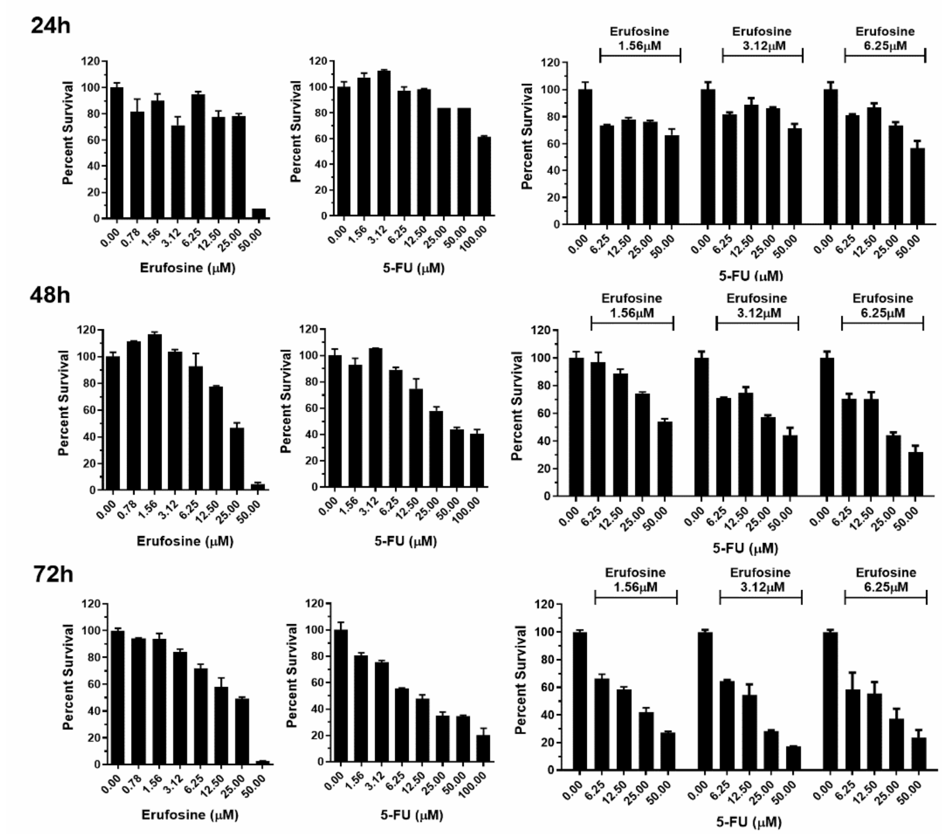

Figure 2: MTT results of SW620 after treatment with erufosine and 5-FU alone and in combinations. The effects were measured by using the dye reduction assay.

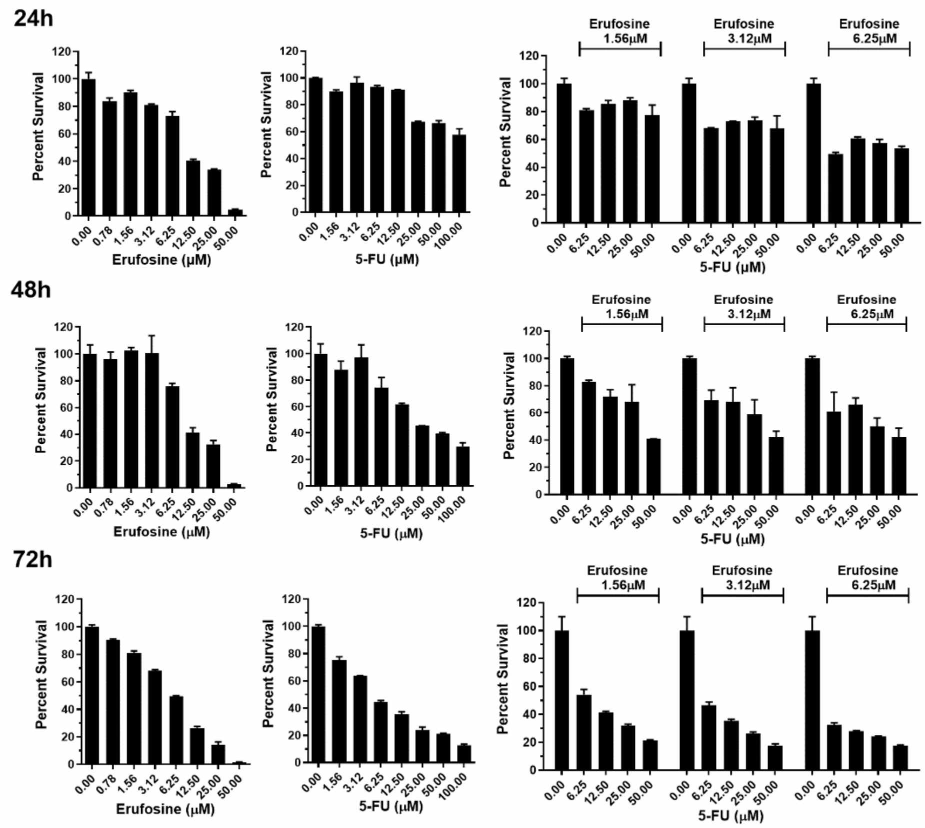

Figure 3: MTT results of HCT116 after treatment with erufosine and 5-FU alone and in combinations. The effects were measured by using the dye reduction assay.

Expression of P21 and GADD45A Genes after Single Agent Treatment

In SW480 cells, fractional inhibition of the P21 gene was observed (maximum fold -1.44) by erufosine. However, the induction of GADD45A gene was observed with a peak value of 2.39-fold. 5-FU was found to have an enhanced effect in inhibiting the P21 gene by achieving maximum inhibition at lower concentration of 25µM as -2.6fold, whereas higher concentration (100µM) had minimal effect. 5-FU had differential effects on expression of GADD45A, as it showed fractional activation at lower concentrations: 12.5 and 25µM, but minimum inhibition at higher concentrations: 50-100µM. Finally, the expressional modulations induced by erufosine and 5-FU in SW480 cells indicated that there are different ways in gene regulation; erufosine is primarily inhibiting P21 with GADD45A activated while 5-FU was successful at P21 inhibition but erratic at GADD45A based on concentration used.

Exposure to erufosine and 5-FU showed significant modulations of expression in SW620 CRC cells compared to SW480 cells. Erufosine increased the levels of both P21 and GADD45A genes with the highest differences being 5.7fold and 5.5fold, respectively. The highest effects were recorded at 5µM of erufosine while greater concentrations (10µM) indicated falling levels of induction for P21 at 4.1fold and GADD45A at 3.16fold. 5-FU markedly increased the expression of the P21 gene in the SW620 cells by a maximum of 19fold at 25µM. For GADD45A, the responses were very weak with a maximum induction only of 2.3fold. Overall, it could be seen that both compounds presented quite different regulations of gene expression in SW620 cells. Erufosine showed marked up-regulation of both genes, whereas 5-FU was primarily involved in the regulation of P21 gene.

In HCT116 cells, erufosine exhibited considerable up-regulation of the P21 and GADD45A genes, with maximum changes of 1.9fold and 3.5fold, respectively. However, at 2.5µM, some level of inhibition was noted for both the genes, with P21 displaying a reduction of -1.1fold and GADD45A showing a reduction of -1.6fold. On the other hand, 5-FU also induced the P21 gene in a concentration-dependent manner, with the maximum induction of 8.9fold by 10µM. The induction of GADD45A by 5-FU was weak and maximally affected only to the extent of 1.6fold. In general, erufosine and 5-FU have different patterns of gene regulation in HCT116 cells, more ever, activation and inhibition of erufosine depends on concentration. Overall changes in P21 and GADD45A genes in response to erufosine and 5-FU exposure are shown in Figure 4.

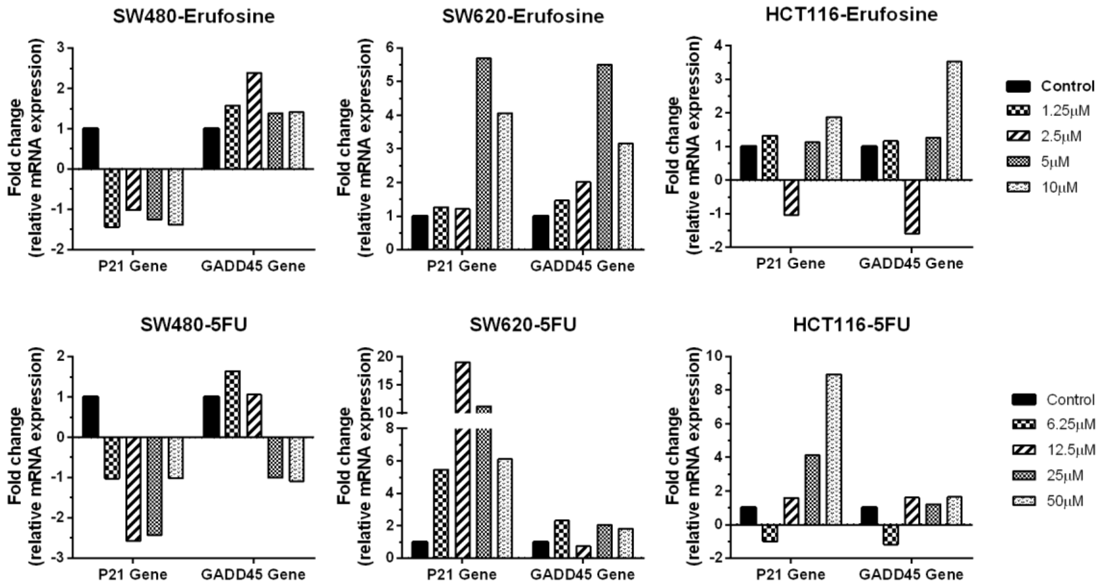

Figure 4: Expressional changes in P21 and GADD45A genes in SW480, SW680 and HCT116 cells after single agent treatment.

Expressional Modulations after Combinational Treatment

Combinational effects of erufosine and 5-FU on expressional profile of P21 and GADD45A genes in SW480 cells are shown in Figure 5. Briefly, combination of 2.5μM erufosine and 25μM 5-FU was the most effective as it induced 2.9fold induction of P21 genes. Higher erufosine concentration (5μM) did not induce any further induction as shown by a maximum of 2.1fold change in P21 gene expression. Combinational effects of erufosine and 5-FU was effective for altering the expression of GADD45A gene as shown by a maximum of 1.7fold induction when the cells were exposed to erufosine (2.5μM) and 5-FU (25μM).

Combining erufosine and 5-FU exerted remarkable effects on expressional modulations of P21 gene in SW620 cells as shown in Figure 5. Moreover, the effects were in a concentration dependent format as shown by a more effective induction of P21 gene expression with increasing concentrations of erufosine and 5-FU combination. Precisely, minimum induction (43fold) was observed with lowest applied concentrations of erufosine (2.5μM) and 5-FU (6.25μM) in combination, while maximum induction (232fold) was observed following the exposure of SW620 cells with highest applied concentrations of erufosine (5μM) and 5-FU (25μM). In contrast to P21, GADD45A gene was inhibited by the combinational exposure of 5-FU and erufosine. Maximum inhibition (-3fold) was observed with the lowest concentrations of the two test compounds, while minimum inhibition (-1.5fold) was observed with the highest applied concentrations. This in turn reflects that inhibitory effects on GADD45A gene which dilute away with the increasing concentrations of erufosine and 5-FU (Figure 5).

In contrast to other two CRC cell lines (SW480 and SW620), combination of erufosine and 5-FU inhibited P21 gene in HCT116 cells as shown in Figure 5. Interestingly, with the increasing concentrations of erufosine and 5-FU, these inhibitory effects reduced as shown by maximum inhibition of 14fold with lowest concentrations of the two compounds (erufosine: 2.5μM, 5-FU: 6.25μM) and minimal inhibition with highest concentrations (erufosine: 5μM, 5-FU: 25μM). As far as GADD45A gene expression levels are concerned, there was a continuous induction when the cells were exposed to erufosine (2.5μM) and various concentrations of 5-FU (6.25-25μM). Surprisingly, there was moderate induction of GADD45A gene (maximum 2.2fold) when the cells were exposed to erufosine (5μM) and 5-FU (6.25-25μM).

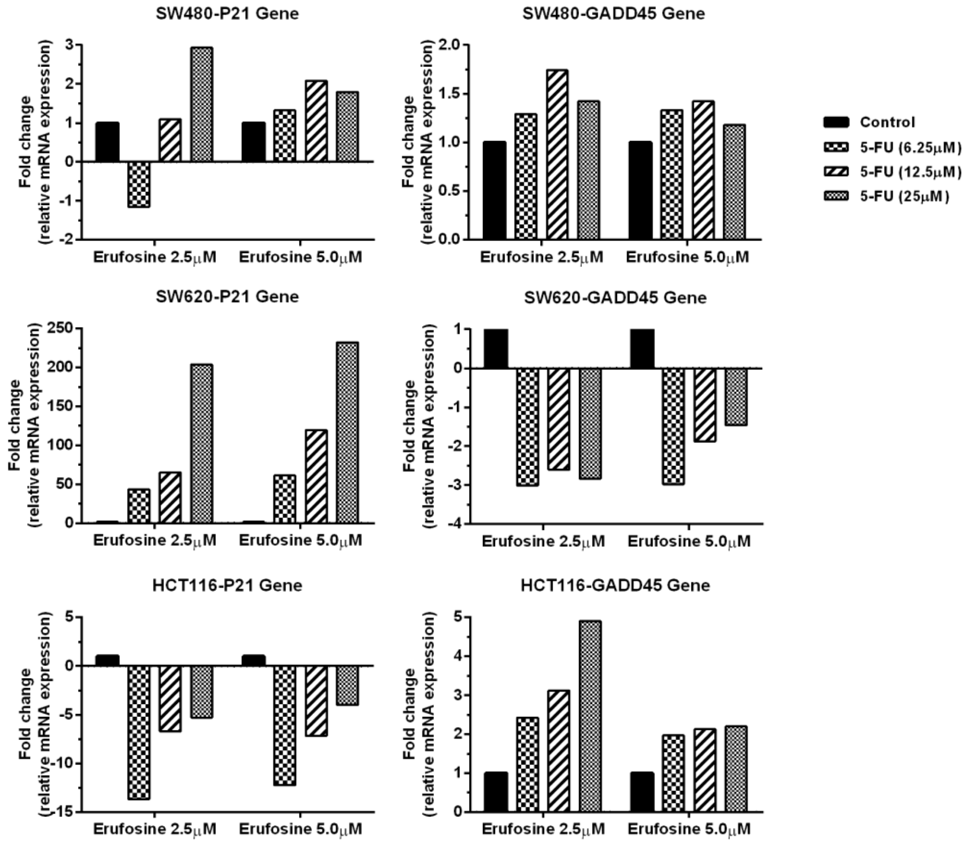

Figure 5: Expressional changes in P21 and GADD45A genes in SW480, SW680 and HCT116 cells after combinational treatments.

DISCUSSION

CRC accounts for the fourth most frequent cancer in men and third in women worldwide, and the incidence rate is sharply rising in Asian countries according to the WHO data [23]. According to the available data at diagnosis, it has been found that approximately 20-25% of CRC patients already harbor metastasis, and 50-60% of those who are seemingly free from metastasis will develop it at some time in the future [24]. The primary site of metastasis is the liver, followed by the peritoneum, lungs, bone, and brain. For non-metastatic CRC, surgery remains the treatment of choice while chemotherapy is essential for the management of inoperable metastases. The 5-year survival rate following surgical resection is still at a very low of approximately 11% for metastatic cases [18]. The cornerstone of chemotherapy is 5-FU where patients with new diagnosis are given in combination with leucovorin. The new drug is Oxaliplatin with a response rate of 10% among previously treated patients and 20-25% in cases with untreated metastasis [25]. There is an underlying problem in traditional cytotoxic chemotherapy that involves issues with more severe side effects and a high resistance rate of the drug. As an example, FOLFOXIRI combination regimen has shown higher response rates but also with the rising rate of toxicity like neurotoxicity and neutropenia. The mentioned aspects require urgent alternative therapies that may provide greater efficacy with fewer side effects [26, 27].

ALPs attach to the surface membranes of cells and exert selectively antineoplastic effects only on tumor cells. The newest generation (3rd) ALP is erufosine, and it exhibited remarkable cytotoxic and cytostatic impact toward a number of cancer cell lines. The present study examines the cytotoxic effects of the third-generation alkyl phosphocholine erufosine combined with 5-FU on three CRC cell lines, namely SW480, SW620, and HCT116. The cells were treated with different concentrations of erufosine (0.78-50 μM), 5-FU (1.56-100 μM), and viability was determined by a MTT assay afterwards. Results showed that single-agent therapies significantly decreased the viability of cells, although this effect was stronger for combination therapies with erufosine and 5-FU. The anti-proliferative effects were time- and dose-dependent, meaning greater doses over a longer period enhanced cytotoxicity. The combinations were found to have synergistic anti-proliferative effects, and these combinations could potentially provide significant inhibition of growth for

CRC cells and may lead to reduced side effects because lower doses are required. The findings point toward the therapeutic potential of erufosine in combination with established chemotherapeutics like 5-FU for treatment of CRC. Further research into the signaling pathways is therefore required to further elucidate these interactions.

Understanding the molecular mechanisms that result in changes in cell function is a very relevant consideration, especially concerning how cancer cells proliferate. Several agents meaningfully inhibited proliferation, especially for CRC cells. The cytotoxic effects of these agents, erufosine and 5-FU were further studied. Two significant regulators had been selected: GADD45A that senses stress in the cell and facilitates apoptosis, and P21 known for inhibiting the cell cycle. 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 drugs (5-FU) alone or in combination for 48 h. Following the total RNA extraction and cDNA synthesis from treated and untreated control groups, expressional alterations in two selected genes was identified via real-time PCR methodology. Expressional modifications in P21 gene in response to single agent exposure were compound and cell line dependent. Precisely, in SW480 cells, erufosine and 5-FU inhibited the expression of P21 gene moderately. In contrast, in the other two CRC cell lines (SW620 and HCT116) both compounds almost persistently induced the expression of P21 gene. Furthermore, induction of P21 gene was more prominent in SW620 and HCT116 cells. The results indicated 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 in nature while inducing the 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 in response to the combinational approach. It is important to mention that this synergistic induction of P21 gene with the combination of erufosine and 5-FU was more effective in metastatic CRC cells (SW620, Figure 5), which in turn indicates that combining 5-FU with erufosine can inhibit the proliferation of metastatic CRC cells more effectively.

GADD (Growth Arrest and DNA Damage) gene family, with low abundance in normal cells, are essential players in oncogenesis and involve in regulation of many cellular functions including DNA repair, cell cycle control, senescence and genotoxic stress. Initiation and progression of malignancies related to defects in GADD genes pathway are reported. These genes serve as tumor suppressors and stress sensors in response to diverse stimuli. An essential step to mediate anticancer activity of multiple chemotherapeutic drugs is the induction of GADD45A expression and the effects of drugs might be revoked by absence of GADD45A. So, chemotherapeutic agents often rely for their anticancer activity on GADD45A up-regulation for induction of cell cycle arrest and apoptosis in tumor cells [28]. In this study, an up-regulation of GADD45A gene was observed in response to exposure of the CRC cells with erufosine and 5-FU as single agent treatment (Figure 4). However, the induction of GADD45A gene in response to exposure with selected compounds was not as effective as observed in case of P21 gene. These observations indicate that erufosine and 5-FU rely more on signaling cascades which interfere with P21 expressional modifications 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 between erufosine and 5-FU for inducing the expression, almost negligible synergistic effects were found for GADD45A gene when the CRC cells were exposed to the compounds in combination (Figures 5). Infect, even there was a marginal inhibition of GADD45A gene in SW620 cells, when the cells were exposed to erufosine in combination with 5-FU (Figure 5). These observations indicate the possibility of negative feedback loop(s) for inhibiting the up-regulation of GADD45A gene via erufosine and 5-FU combinations.

To conclude, erufosine and 5-FU induces substantial cytotoxic effects in CRC cells. Combination of erufosine and chemotherapeutic drug (5-FU) leads 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 with the compounds. The up-regulation of P21 gene was more prominent when the cells were exposed to combination of erufosine and 5-FU. All in all, the two compounds showed ample cytotoxicity against the CRC cells and their combinations are quite effective for inhibiting proliferation of the cells. Further *in vitro and in vivo* investigations are needed to support evaluation of erufosine in combination with 5-FU against CRC in clinical settings.

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