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REVIEW ARTICLE

Nanoparticles and drug delivery for chemoprevention in cancers

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Abstract
Cancer is a major global issue that is responsible for morbidity and mortality all over the world. Radiation therapy, surgery, and chemotherapy are categorized into traditional cancer treatment methods that cause systemic toxicity and limited efficacy because of non-specific drug delivery. As a result, various side effects compromise a patient's quality of life. Thus, innovative approaches are required that can enhance therapeutic outcomes and result in fewer adverse effects. Nanotechnology addresses such challenges and provides a promising avenue for treating cancer. Engineered nanoparticles offer a platform for targeted drug delivery, that enhances therapeutic efficacy and shows minimum adverse effects. In this context, researchers have now developed strategies to improve cancer chemoprevention. This review demonstrates the application of various nanoparticles in cancer chemoprevention and focuses on their mechanisms of action, advantages, and novel research advancements. In cancer chemoprevention, the mechanisms of action of nanoparticles are diverse and are dependent on targeted cancer and particular nanoparticle types. Nanoparticles can inhibit tumor cell proliferation, induce apoptosis, and cause tumor cell destruction. They can promote an immune response against tumor cells, causing effective anti-tumor effects. Researchers are developing various novel nanoparticles platforms that have improved targeting efficiency, biocompatibility, and drug release kinetics. Furthermore, researchers are also trying to combine nanoparticles with other therapeutic applications such as immunotherapy, to get synergistic anti-tumor effects.
Keywords: Nanoparticles, Chemoprevention, Drug Delivery Systems, Enhanced Permeability and Retention (EPR) Effect, Biocompatibility, Tumor Targeting, Controlled Release

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INTRODUCTION

Cancer remains one of the major causes of death worldwide despite ongoing advancements in the medical field and technology because of its dynamic nature [1]. Many cancer therapies including radiation, surgery, and chemotherapy are in use today but these are facing major challenges. Chemotherapy may result in systemic toxicity by presenting its action on non-cancerous cells along with normal cells [2]. The potential of chemotherapy can further be increased by combinational strategies. Nano-oncology is now emerging as a multidisciplinary field that is expected to outperform various traditional cancer treatment methods [3]. Nanotechnology can be utilized in diagnosis and managing various types of cancers [4]. In comparison to conventional drugs, nanoparticle-based drug delivery possesses advantages, such as biocompatibility and enhanced stability, increased permeability and retention effect, as well as precise targeting [5]. The targeting mechanisms are passive and active targeting in which one relies on the EPR effect where nanoparticles accumulate in tumor tissues due to poor lymphatic drainage and their leaky vasculature [6]. The other relies on modifying nanoparticles with ligands that further specifically bind to receptors that are overexpressed on tumor cells and finally increase targeting efficiency [7]. The use of nanotechnology to achieve chemoprevention is considered an effective strategy that enhances the bioavailability of phytochemicals and ultimately their therapeutic efficacy [8].

Mechanisms of Chemoprevention

Cancer chemoprevention aims to delay, suppress, or prevent tumor occurrence by using natural or synthetic bioactive agents as shown in Table 1. Mechanistically, these chemo-preventive agents are also helpful in mitigating cancer development, either by blocking the division of premalignant cells or by impeding DNA damage [9]. The agents for chemoprevention are categorized into two principal categories: (a) "blocking" agents and (b) "suppressing" agents [10, 11]. Blocking agents are those that block the initiation stage, and the ones that affect the promotion stage are

known as suppressing agents [12]. The first group of compounds tends to neutralize RNS/ROS as well as reactive metabolites, change the metabolism of carcinogen, and cause an increase in detoxification and repair. In conditions where the initiation phase has taken place already, suppressive agents are responsible for promoting apoptosis, eliminating reactive oxygen species, reducing cell proliferation, altering gene expression and inflammation [13].

Chemoprevention approaches are classified into three categories: primary, secondary, and tertiary chemoprevention. Primary chemoprevention implies a healthy population that is highly susceptible to develop cancer. Secondary chemoprevention applies to those who are already exposed to carcinogenic agents or have developed premalignant lesions, and tertiary chemoprevention is specific for those individuals who have already developed the disease before and then recovered from the treatment. In this way, this tertiary chemo-preventive approach targets the development of new as well as second primary cancers [14, 15]. Different pharmacological characteristics or various mechanisms are vital for a potential candidate to be an effective chemo-preventive agent such as anti-inflammatory properties, anti-proliferative properties, cell cycle arrest, an inhibition of growth factor pathways, and angiogenesis [16]. Hence, various compounds show chemo-preventive characteristics at all stages of carcinogenesis such as curcumin, quercetin, and resveratrol [17-19].

Tamoxifen

It was the first FDA-approved chemo-preventive agent, that helps reduce the risk of estrogen receptor (ER)-positive breast cancer as shown in Figure 1 [26]. The mechanism of action is carried out by its binding to estrogen receptors and then blocks proliferative actions of estrogen on the mammary epithelium. For this antiproliferative action, the suggested mechanism is the synthesis of the cytokine transforming growth factor-β (TGF-β) by tamoxifen, which serves as the negative autocrine molecule [27].

Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are known as anti-inflammatory agents that have been classified as cancer chemoprevention agents [28]. Their mechanism of action is that they inhibit cyclo prostaglandin E2 and oxygenase (COX) pathways, as well as via COX-independent pathways [29].

Piperine

Piperine is an active alkaloid having various therapeutic properties such as anti-inflammatory, antioxidant, and immunomodulatory effects and has garnered attention for treating cancer [30].

Mechanisms of Nanoparticles-Based Drug Delivery

Nanotechnology has been widely studied for cancer treatment, and nanoparticles of various types are in use today, playing a significant role in a drug delivery system as shown in Table 2 and 3 [5]. In comparison to conventional drugs, nanoparticle-based drug delivery possesses advantages such as biocompatibility and improved stability, increased permeability and retention effect, and finally, precise targeting as shown in Figure 2 and Figure 3 [31, 32]. Nanoparticles possess the EPR effect to assemble in tumor sites because of their leaky vasculature that allows for targeted drug delivery [33]. The surface of nanoparticles can easily be modified with antibodies or ligands to improve their targeting capabilities. This functionalization increases binding to specific receptors on various target cells, which facilitates uptake as well as controlled release [34]. Nanoparticles can further be engineered to release therapeutic payloads. This can be established through several mechanisms such as degradation of nanoparticle matrix, diffusion, or changes in environment (temperature or pH) that enhance drug release [35]. Nanoparticles can penetrate their biological barriers and further taken up by cells in an efficient manner than the larger particles [36].

Table 1: Agents for Cancer Chemoprevention

Active Compound	Type	Mechanism of Action	Cancer targeted	References
Resveratrol	Natural	Inhibits cell proliferation and induces apoptosis	Colon, Breast	[20, 21]
Sulforaphane	Natural	Induces phase II detoxification enzymes	Colon, Lung	[22, 23]
Tamoxifen	Synthetic	Selective estrogen receptor modulator	Breast	[24]
Eflornithine	Synthetic	Irreversibly inhibits activity of ornithine decarboxylase	Colorectal cancer	[25]

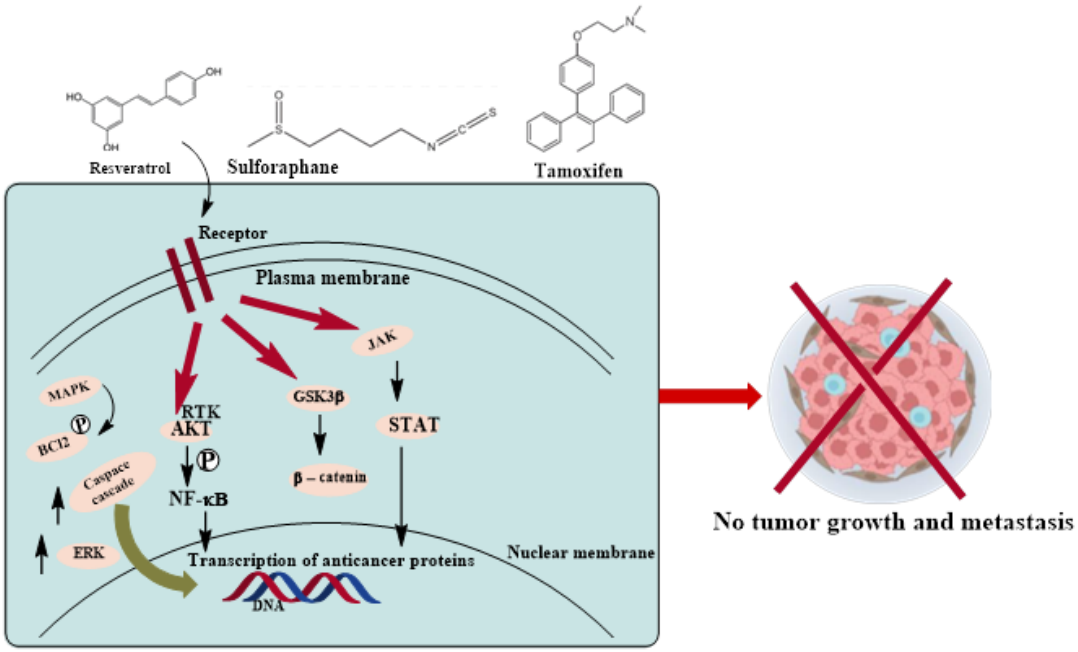


Figure 1: Chemoprevention at different stages of cancer

Table 2: Nanoparticles-based FDA-approved drugs being used in cancer treatment.

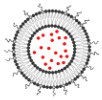
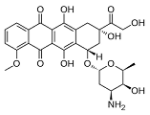
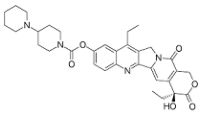
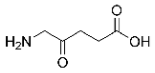
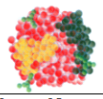
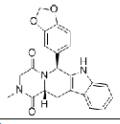
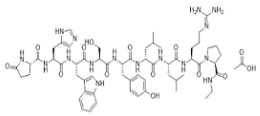
Product	Drug Nanoparticle system	Drug	Company/ Year	Mechanism of action	Indication	Nanoformulation Advantages	Reference
Doxil® (Caelyx) 	Liposome-PEG (Lipid-based nanodrugs)	Doxorubicin 	Janssen/ 1995	DNA double strand breaks. Replication arrest. Topoisomerase II activity inhibition.	Metastatic Ovarian cancer, Metastatic breast cancer	↑blood circulation time ↑tumor uptake (EPR) ↓cardiotoxicity	(37-39)
Onivyde® 	Liposome (Lipid-based nanodrugs)	Irinotecan 	Merrimack/ 2015	Serves as apoptotic death inductor. Results in inhibition of topoisomerase I activity.	Pancreatic cancer	↑blood circulation time ↑tumor uptake (EPR) ↓toxicity	(37, 39)
Ameluz® 	Lipid-based nanodrug	5-aminolevulinic acid 	Biofrontera AG/ 2016	Cytotoxic activity by production of oxygen-free radicals.	Actinic keratosis, Squamous cell carcinoma	sustained release ↓toxicity	(37, 40)
Ontak® 	Protein-drug conjugate nanoparticles	Denileukin diftitox 	Eisai Co., Ltd./ 1999	Inhibits protein synthesis. Binds to IL2R and shows cytotoxic activity.	Human CD25+ cutaneous T-cell lymphoma (CTCL)	↑blood circulation time ↑tumor uptake (EPR) ↑selectivity ↓severe toxicity	(37, 41-43)
Eligard® 	Protein-drug conjugate nanoparticles	Leuprolide acetate 	Tolmar, Inc./ 2002	Anti-tumor activity by serving as: GhRHR agonist Decreases gonadotrope secretion of FSH/ LH. Stops gonadal production of sex steroid.	Prostate cancer	↑blood circulation time ↑tumor uptake (EPR)	(37, 39, 44)

Table 3: Nanoparticles in Cancer Therapy

Type of Nanoparticle	Description	Mechanism of Action	Clinical Applications	References
Polymeric Nanoparticles	Made from biodegradable polymers. Allows controlled drug release.	It can be engineered for active and passive targeting by modification of surface properties.	Targeted delivery in ovarian and breast cancers.	[40, 41]
Liposomes	Spherical vesicles that can encapsulate drugs. Enhance stability and solubility.	Passive targeting through increased Permeability and Retention (EPR) effect.	Can be used for delivering chemotherapeutic agents.	[42]
Iron Oxide Nanoparticles	Magnetic nanoparticles can be utilized for imaging as well as for drug delivery.	Magnetic targeting permits precise localization at various tumor sites.	Employed in MRI imaging as well as drug carriers for many targeted therapies.	[43]
Nano-emulsions	Colloidal systems that increase bioavailability of poorly soluble drugs.	Improve drug stability and solubility Increased absorption at the tumor site.	Used in formulations such as fisetin for treating cancer.	[44, 45]
Dendrimers	Highly branched macromolecules have a well-defined structure for carrying out drug delivery.	Helps in targeted delivery through receptor-mediated endocytosis.	Effective in delivering small molecule drugs and nucleic acids.	[46, 47]

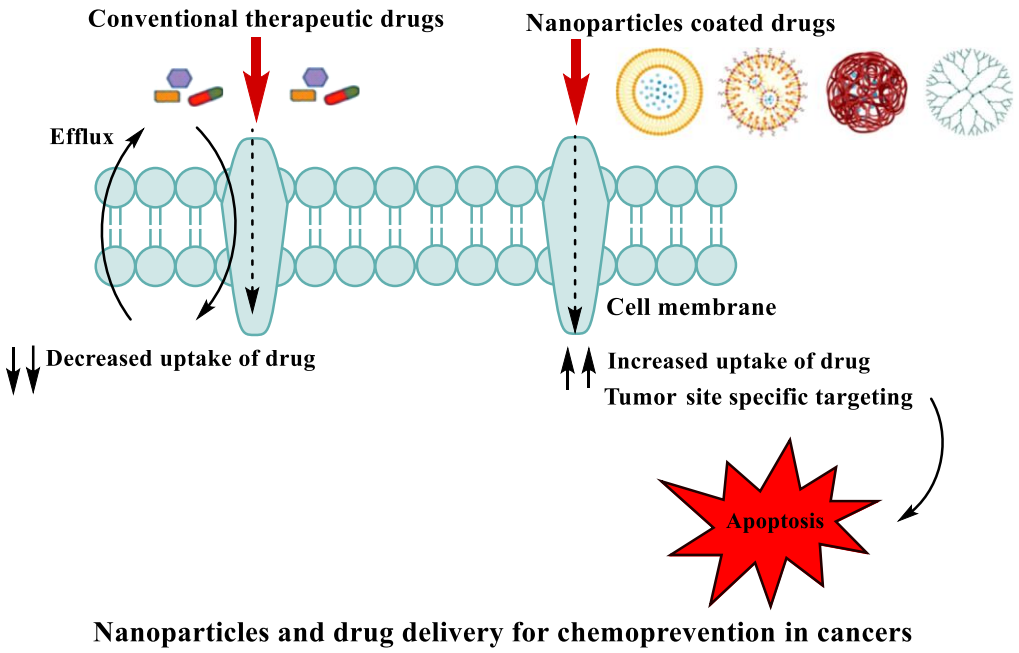


Figure 2: Nanoparticles and drug delivery for chemoprevention in cancers

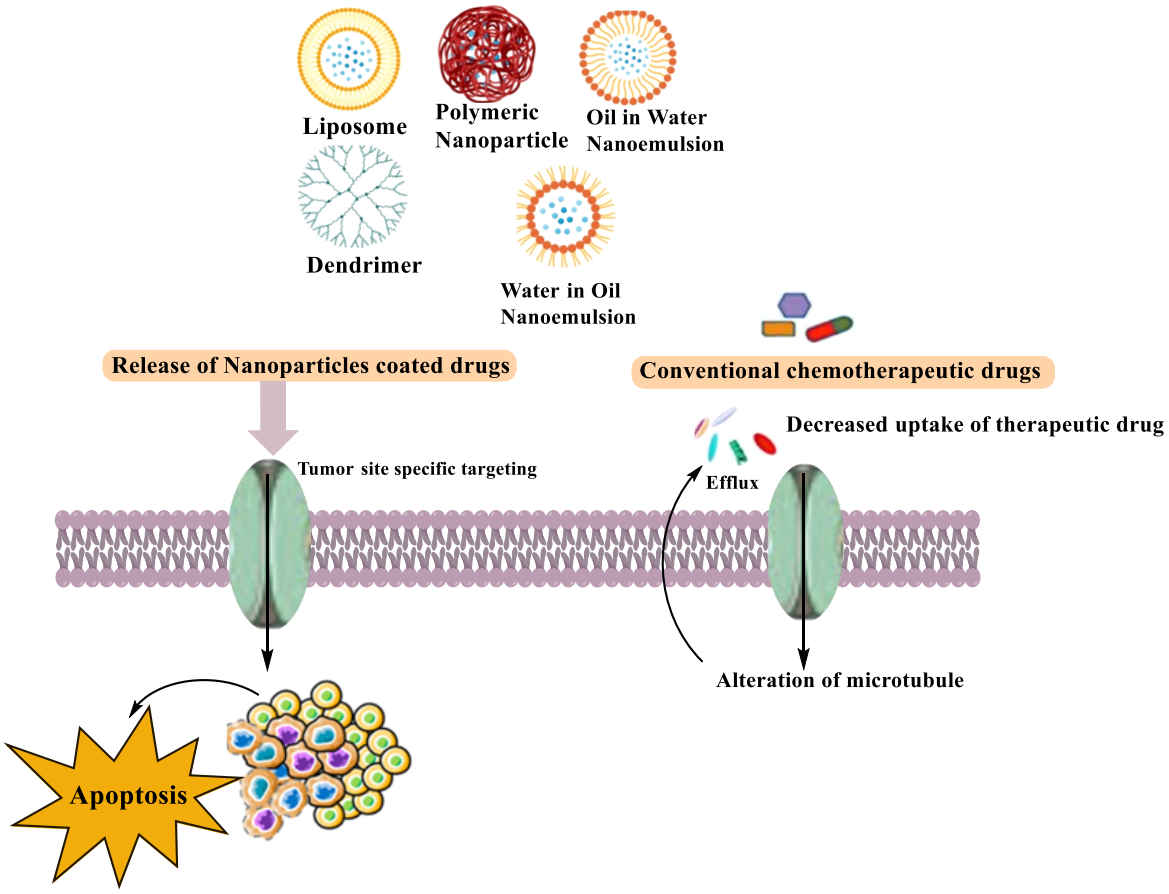


Figure 3: Mechanism of Nanoparticle-based Drug Delivery

Conclusion and Future Perspectives

Nanoparticles harbor a comparatively more effective approach to deliver the drugs for targeted cancer therapeutic purposes. Unlike conventional/traditional procedures for cancer treatment, nanoparticles provide advanced treatment options to overcome various limitations like toxicity and unrelated damage to healthy cells. Due to certain diagnostic features, such as improved cell permeability and retention effect. Nanoparticles can selectively target cancerous cells, thereby reducing toxicity. Different tailoring strategies like surface and charge modifications may assist in precise tumor treatments with better outcomes. There is a dire need to investigate innovative technologies that may enhance the nanoparticles' efficacy for targeted cancer therapy. Tailoring strategies must be developed with the assistance of artificial intelligence to boost the potential of nanoparticles and make them more biocompatible without any hint of toxicity. More mechanistic research is inevitable to locate the gaps, optimization of the procedures and establishing multidisciplinary research collaborations for a better future of nano-particles-based strategies. Finally, industrial collaboration is necessary for long-term solutions based on the global impacts of nanoparticles for cancer-related issues.

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RESEARCH ARTICLE

Effects of chemokine receptor 5 (CCR5) blockages on cell survival and expression levels of apoptosis related genes (FAS, FASL) in hepatocellular carcinoma cells

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Abstract

Background: Hepatocellular carcinoma (HCC) is a malignant tumor which arises from the liver cells (hepatocytes). HCC is the 3rd leading cause of cancer related mortalities worldwide. Various treatment strategies for HCC include surgery, radiotherapy, targeted agents and chemotherapy. Despite the availability of diverse therapeutic options, 5-year survival rates are low (20-30%) especially in advanced stages of HCC. This, in turn, highlights the need to identify new therapeutic targets/compounds for HCC treatment. The purpose of this study was to evaluate effects of blocking a chemokine receptor (CCR5) on cell proliferation and apoptosis related genes (FAS/FASL) in HCC cells (HepG2).

Methods: CCR5 receptor was blocked by using an FDA approved antagonist (Maraviroc) and effects on proliferation of HepG2 cells were identified. For this purpose, the cells were exposed to various concentrations (7.5-500µM) of the test compound and cell viability was monitored by MTT dye reduction assay for 24, 48 and 72 hours. Afterwards, HepG2 cells were exposed to three distinct concentrations of maraviroc (IC_{25}, IC_{50}, IC_{75}) in a separate experiment and expressional modulations in two apoptosis related genes (FAS and FASL) were identified by qRT-PCR methodology.

Results: The results indicated that blocking CCR5 via maraviroc induced substantial anti-proliferative effects in HepG2 cells. The effects were time and concentration dependent and were especially clearer following exposure with 100µM of maraviroc. Blockage of CCR5 induced marginal up-regulation of FAS gene in the cells. In contrast, blocking of CCR5 inhibited the expression of FASL gene in HepG2 cells in a concentration dependent format with a maximum inhibition of 5fold.

Conclusion: CCR5 blockage by maraviroc induces prominent cytotoxic effects in HCC cancer cells. Expressional modulations in apoptosis related genes are imposed in response to blockage of the chemokine receptor in the cells. Further studies are needed to understand the precise nature of growth inhibitory effects observed in response to blockage of CCR5 in HepG2 cells.

Key Words: Liver cancer, Chemokine, CCR5, Maraviroc, Cytotoxic, Genes expression

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INTRODUCTION

Uncontrolled proliferation of cells leads to the formation of tumor mass and is known as cancer. There are more than 100 types of cancer, and this division is based on either type of tissue or organ involved. Liver cancer is the sixth most frequent type of cancer and there are different types of liver cancer. HCC is the most common type of liver cancer and globally it is the well-known cause of deaths. HCC is different from "secondary" liver cancer which establishes because of spreading of tumor cells from other organs and migrating to the liver during a process called metastasis [1]. Some patients with liver cancer have no symptoms which becomes the reason for presenting at late stage of cancer. In liver cancer, the current standard staging system BCLC (Barcelona Clinical Liver Cancer) is used for treatment. There are also other treatment options to eliminate the growing tumor including microwave therapy, radiofrequency ablation, cryoablation, percutaneous injection of ethanol and electroporation treatment. Despite the availability of multiple therapeutic options, HCC is considered as a top ranked cancer and "silent killer". Major reasons are delayed diagnosis and tumor heterogeneity along with expressional modifications in genes and associated pathways.

Chemokines are small sized (8-14kDa) chemo-attractive cytokines, which are mainly responsible for the directed migration of various kinds of leukocytes, endothelial and epithelial cells [2-4]. The chemokine network is comprised of about 50 ligands and 20

receptors. Chemokine receptors are found in the lipid bilayer with their seven trans-membrane loops. These receptors are G protein coupled receptors (GPCRs) and classified according to the type of their corresponding ligands. A typical chemokine receptor contains almost 350-370 amino acids in length with a short N-terminus, located in extracellular space. C-terminus of the receptor, located in intracellular space, contains serine and threonine residues, which act as phosphorylation sites for the receptor regulation and signaling [5, 6]. Receptors that couple to C-, CC-, CXC-, and CX3C-chemokines are named XCRn, CCRn, CXCRn, and CX3CRn, respectively.

Among the chemokine network, CC-chemokine receptor 5 (CCR5) is an important member and plays vital roles in inflammatory response of the body [7]. Macrophage-tropic strains of both types of human immunodeficiency virus type 1 and 2 (HIV-1, HIV-2) use CCR5 as cofactor, making it an integral part of pathogenesis of the infection and viral transmission. Inflammatory CC-chemokines of CCR5 which act as agonists are MIP-1f, MIP-1g, MCP-2, HCC-1 and RANETS. CCR5 is expressed in both innate immunity and cellular components of acquired immunity mainly in memory T-cells, macrophages, and immature dendritic cells, and is up-regulated by pro-inflammatory cytokines. CCR5 plays an important role in inhibition of cAMP production and activates P13-MAP kinase. It stimulates Ca^{+2} release and causes activation of tyrosine kinase cascades [8]. CCR5 and CCL5 levels increased in patients with chronic liver disease. Overexpression of CCR5 and its ligands (CCL3, CCL4, CCL5) have been correlated with increased disease burden and reduced overall survival [9-11]. HIV-1 strains are transmitted through co-receptor CCR5, so different pharmaceutical companies have synthesized antagonists against CCR5 which are being used as antiviral therapies. These specific antagonists are helpful for blockage of CCR5 signal transduction [12]. This CCR5 antagonist (maraviroc) has been approved by FDA for treatment of HIV infections. Blocking CCR5 with maraviroc has shown reduction in tumor burden during HCC development *in vivo* studies [12-15]. The objectives of the current research were to block CCR5 receptors of HCC cells by maraviroc and investigating the corresponding effects on cell proliferation. Furthermore, it was aimed to measure the expressional modulations in apoptosis relevant genes (FAS, FASL) in response to blockage of CCR5 in HCC cells.

METHODS

Culturing of Cancer Cells

HCC cells (HepG2) were obtained from Centre for Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore. The cells were cultured in DMEM medium (Gibco, Cat#11965-092) supplemented with 10% FBS, 100µg/ml Penicillin/Streptomycin and 2mM L-glutamine. Standard cell culture incubation conditions (5% CO_2, 37˚C, 100% humidity) were provided for cultures throughout the experiments. The cells were passaged routinely to keep a well growing cell population.

Cytotoxicity Assay

Cytotoxic effects of blocking CCR5 receptor were investigated by MTT dye reduction assay. For this purpose, the cells were cultured in 96-well plates (4000 cells/well/100µl media) and treated next day with maraviroc (7.5-500µM, Selleck, Cat#S2003) antagonist for 24, 48 and 72 hours. Afterwards, surviving cell fractions from treated and untreated cells (control group) were examined by adding 10 µl/well MTT solutions (10 mg/mL in PBS) and dissolving newly formed formazan crystals with 50µL of DMSO. Optical density was measured by ELISA plate reader at wavelength 540nm with 690nm reference filter. Cell survival rates were calculated as the percentages of untreated control cells and inhibitory concentrations (IC) were determined by using software GraphPad Prism v5.

Gene Expression Analysis

HepG2 cells were cultured in 6-well plates at a density of 150,000 cells/well/2ml media and exposed to IC_{25} and IC_{50} and IC_{75} concentrations (identified from MTT dye reduction assay after 48 hours exposure time) of CCR5 antagonist (maraviroc). The cells were exposed to the antagonist for 48 hours, followed by collection of cell palettes and storage at -80^{0}C immediately. Total RNA content from collected cell pellets was extracted by using a commercially available kit (Thermo Fisher, Cat#K0731) following the manufacturer's protocol. A total of 1000ng extracted RNA/sample was used to synthesize cDNA (20µl) by using Revert Aid First Strand cDNA synthesis kit (Thermo Fisher, Cat#K1622).

Real Time PCR

Primers for selected FAS (*F: TCACCACTATTGCTGGAGTCA, R: GGTACTTAGCATGCCACTGC) and FASL (F: CAGGCACCGAGAATGTTGTAT, R: TGGTAGCTGCTTTTTCATGCT*) genes were designed by using Primer3 software after choosing the gene sequence from NCBI Gene bank. qRT-PCR was performed by using SybrGreen florescence dye (Thermo Fisher, Cat#K0221) for the selected genes by using prepared cDNA samples from HepG2 cells treated with different concentrations of maraviroc for 48 hours. Following amplification procedures, $2^{-\Delta\Delta CT}$ method (Livak) was used to calculate expressional changes in the selected genes. Results were compared with untreated controls while expression levels of a

reference gene (HPRT1) were used in these experiments for normalization of the data sets.

RESULTS

Blockade of CCR5 induces substantial toxic effects in liver cancer cells

HepG2 cells were exposed to for various concentrations of maraviroc (7.5-500µM) for 24, 48 and 72 hours. Following the exposure intervals, the viable fractions of the cells were identified by MTT dye reduction assay. Maraviroc exposure induced significant cytotoxic effects in HepG2 cancer cells as shown by Figure 1. Precisely, the effects were noticed for all three-time intervals. Considering the highest concentrations applied (500µM), the effects were time dependent as shown by inhibition of cell proliferation by 54, 76 and 88% after 24, 48 and 72 hours of exposure time respectively. For the three-time intervals, the effects were moderate till 250µM concentration of maraviroc, while there was a steep decline in viable cell fraction for next applied concentration (500µM). Overall, CCR5 blockage induced substantial cytotoxic effects in the liver cancer cells (Figure 1).

Expression Modulations in Genes

HepG2 cells were exposed to various concentrations of maraviroc (IC_{25}, IC_{50}, IC_{75}), total RNA was extracted followed by cDNA synthesis. Real time PCR based amplification of the two selected genes (FAS and FASL) was performed and data was normalized by amplifying a reference gene and fold changes were determined by $2^{-\Delta\Delta CT}$ method. CCR5 blockage by maraviroc induced the expression of FAS gene mildly as shown by an induction of 1.8 and 1.4fold with IC_{25} and IC_{75} concentrations of the test compound respectively (Figure 2). CCR5 blockage by using maraviroc inhibited FASL gene in HepG2 cells as shown below. The effects were concentration dependent as shown by inhibition of 1.5, 2.1 and 5.3fold in response to IC_{25}, IC_{50} and IC_{75} concentration of maraviroc. The results were almost in line with our cytotoxicity outcomes as there was also a consistent inhibition of proliferation in response to increasing concentration of maraviroc.

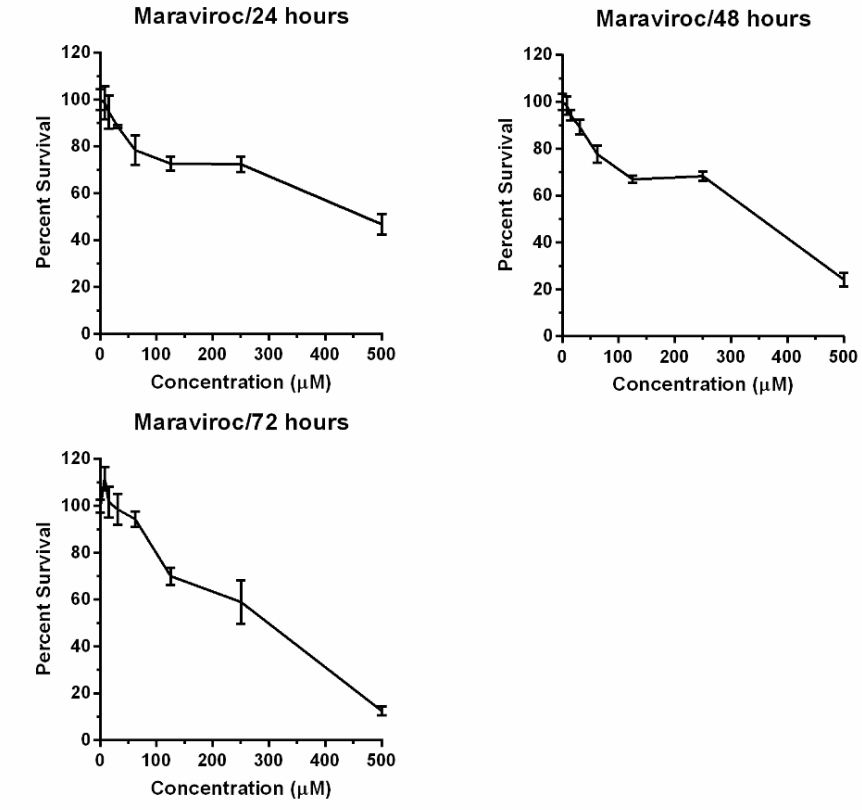

Figure 1: MTT result after CCR5 blockage for 24-72hours. Cells were exposed to different concentrations (7.5-500µM) of antagonist (maraviroc) and resulting effects on the cell proliferation were identified by MTT dye reduction assay. CCR5 blockage inhibited liver cancer cell proliferation.

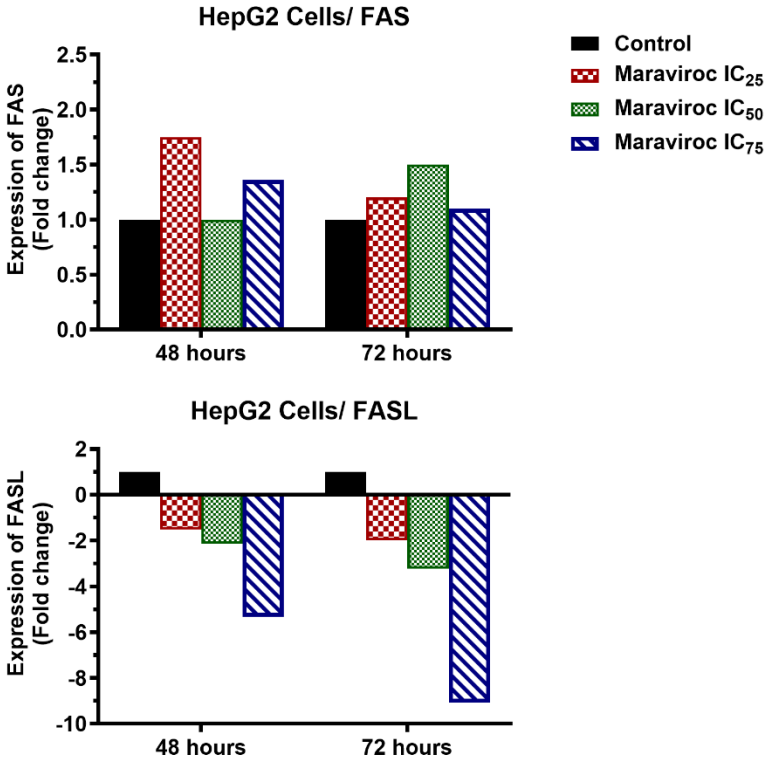

Figure 2: Expressional changes in FAS and FASL genes after CCR5 blockage. In response to exposure with maraviroc, expressional changes in the genes were identified by qRT-PCR. CCR5 blockage by maraviroc induced FAS marginally and inhibited FASL gene in HepG2 cells in a concentration dependent format.

DISCUSSION

CCR5 (CD195) chemokine receptor is mainly known for its association with HIV infections. Despite its role in HIV infections, a number of normal physiological roles are associated with this chemokine receptor. CCR5 along with its three cognate ligands (CCL3, CCL4, CCL5) is mainly responsible for recruitment of T cells, monocytes and macrophages to the site of inflammation [16]. Due to involvement of CCR5 receptor in the viral entry phase, antagonists and monoclonal antibodies are being developed to block this receptor, which, in turn, could serve as entry inhibitors for HIV infections. In this regard, a CCR5 antagonist "Maraviroc" has been approved by FDA for treatment of HIV infections. Apart from involvement in HIV infections, the CCR5 receptor is being studied for its proactive role in the pathology of various other diseases including cancers. In this respect, the inhibition of this receptor via available antagonists is being exploited as therapeutic options.

HCC is a lethal condition and imposes a significant morbidity and mortality burden. During HCC development, changes in expressional profile of various genes including chemokine receptors have been noticed. In this context, changes in CCR5 profile along with their corresponding ligands have shown significant up-regulation during HCC development. Higher expression of CCR5 has been found with poor prognosis, reduced overall survival and metastasis of HCC. Furthermore, these higher expressions of CCR5 have shown to promote the migratory and proliferative behavior of the HCC cells and abrogation of the receptors lead to the reduced tumor growth in *in vivo* conditions [12]. HCV/HBV mediated inflammation is hall mark for HCC development, where CCR5 along with its ligands play a pivotal role to attract various cell populations like macrophages, neutrophils and T-cells. Blocking CCR5 with maraviroc has shown reduction in tumor burden during HCC development in *in vivo* studies [13]. All in all, CCR5 could serve as independent prognostic markers and therapeutic targets in HCC.

In this study, we blocked the CCR5 by using corresponding antagonist (maraviroc) and studied the cytotoxic effects via MTT dye reduction assay. Furthermore, the impact of blocking the chemokine receptor on two important apoptosis related genes (FAS, FASL) was studied. As a first necessary step, it was important to validate the expression of CCR5 in selected HCC cells (HepG2) before blocking the receptors by using their corresponding antagonists. The real-time PCR analysis was performed to check expression of CCR5 in untreated HepG2 cells (data not shown). The receptor showed a good amplification as

shown by qRT-PCR with Ct value of 24.34 for CCR5 (Ct of HPRT1: 16.8).

Continuous proliferation is a major hallmark of cancer cells and drugs/compounds affecting this functional property are important entities to be exploited. Keeping in view of this phenomenon, potential cytotoxic effects of blocking CCR5 in HepG2 cells were worth investigating. To accomplish this task, HepG2 cells were exposed to various concentrations of CCR5 (maraviroc: 7.5-500µM) for 24-72 hours. Afterwards, the viable cell fractions were identified by MTT dye reduction assay. Blocking CCR5 induced substantial inhibition of cell proliferation for all time points as shown in Figure 1. The effects were induced almost in time and concentration dependent format as shown by continuous decrease in viable cell fractions with increasing concentrations of the antagonist and longer time interval. Precisely, it was not possible to attain 50% inhibition of cell viability even with highest concentration of maraviroc (500µM) after 24 hours exposure time, while more than 50% cell proliferation inhibition was noticed with 500µM concentrations of the test compound after 48- and 72-hours exposure. The results showed that selected HCC cells respond to longer exposure intervals and become sensitive over period of time in the presence of maraviroc. Our results indicated that blocking CCR5 via maraviroc induces substantial effects on cell proliferation of HepG2 cells.

Avoiding the apoptotic process is a necessary requisite for cancer cells as it gives them a chance to survive for a longer time. For this purpose, cancer cells often up-regulate anti-apoptotic and down-regulate pro-apoptotic genes. FAS and FASL are two important apoptosis related genes and alterations in their expressional levels are often observed in cancer cells. Considering this, expressional profiling of FAS and FASL genes were compared between untreated HepG2 cells with the cells treated with various concentrations maraviroc by using real-time PCR methodology. Three different concentrations (IC_{25}, IC_{50}, IC_{75}) of maraviroc were selected for the real-time PCRs.

FAS are a well-known death receptor found in cell surface membrane and play a central role in regulation of programmed cell death by activation of caspases. Furthermore, it is reported to induce cell proliferation signaling in the cells like fibroblasts and T-cells. In this study, a fractional induction of FAS gene was noticed (maximum 1.8fold) in response to CCR5 blockage by exposing the cells to IC_{25} of maraviroc (Figure 2). For the next two higher concentrations of maraviroc (IC_{50} and IC_{75}), there was no or even less induction of the gene. In contrast, there was a concentration dependent inhibition of FASL gene as shown in Figure 2. A maximum of 5.3fold inhibition of FASL gene was observed when the cells were exposed to IC_{75} of maraviroc. Observing a fractional induction of the receptor (FAS) and inhibition of its corresponding ligand (FASL) indicate a moderate connection between levels of CCR5 blockage via maraviroc and expressional modulations in FAS-FASL axis. Furthermore, the changes in FAS-FASL axis are not in line with the cytotoxic effects (Figure 1), which indicates a potential involvement of other death mechanisms being initiated after CCR5 blockage in HepG2 cells.

Overall, the study revealed substantial cytotoxic effects on HepG2 cells in response to CCR5 blockage by using maraviroc. Expressional modulations in two selected apoptosis related genes (FAS and FASL) were not uniform in response to CCR5 blockage in HepG2 cells. All in all, the cytotoxic effect on HepG2 cells by blocking CCR5 via maraviroc is an effective anti-proliferative option. Provided validation in larger cell line pool and *in vivo* investigations, CCR5 blockage can be instrumental in HCC treatment.

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

RESEARCH ARTICLE

Cytotoxic effects and expressional modifications induced by alkyl-phospholipid (erufosine) in lung cancer cells

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Abstract
Background: Lung cancer is the leading cause of morbidity and mortality in the world. The treatment of lung cancer is a challenge because of the aggressive nature of this disease and limited therapeutic options. The situation demands to investigate novel compounds for better treatment of lung cancer with lesser side effects. Alkyl-phospholipids (ALPs) are an attractive class of antineoplastic agents which target DNA and act upon cell membrane to produce anticancer effects in tumor cell specific manners. Erufosine is the 3rd generation ALP and induces anticancer effects by causing changes in the lipid rafts of the cell membrane.
Methods: Selected lung cancer cell line was cultured in 96-well plates and exposed to various concentrations of erufosine (1.56-50µM) followed by MTT dye-based assessment of viable cell fractions. For expression modifications, the cells were exposed to erufosine followed by RNA extraction, cDNA synthesis and real-time PCR based assessment of the selected genes.
Results: Erufosine induced substantial cytotoxic effects in the lung cancer cells as shown by growth inhibition of 25 and 50% by using 24- and 33µM concentration respectively. At lower concentrations (6.25µM), there were negligible cytotoxic effects, which gradually increased with increasing concentrations. As far as expressional changes are concerned, the three selected genes (CCNB1, CDKN3, CASP3) were downregulated in response to erufosine exposure.
Conclusion Erufosine is a potential cytotoxic compound, induces notable anti-proliferative effects and modulates expressional levels of the genes in lung cancer cells.
Keywords: Lung Cancer, Alkyl-phospholipid, Erufosine, Cytotoxicity, Gene Expression

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INTRODUCTION

Lung cancer is a disease in which cells in the lungs proliferate abnormally to form a tumor. Lung cancer is the second most diagnosed cancer type (11.4%) and accounts for 18% of cancer related deaths. Lung cancer in its earliest stages is asymptomatic. Signs and symptoms occur when disease is at chronic stage. Five most used therapeutic approaches for the lung cancer treatment are: surgery, therapy by radiations, drugs as a part of chemotherapy, targeted therapy and immunotherapy. Treatment of lung cancer is a challenge because of the aggressive nature of the disease and limited therapeutic option. The situation demands for a novel compound to treat lung cancer with lesser side effects [1].

ALPs are a class of antineoplastic agents which target DNA and act upon cell membranes to produce anticancer effects. Structural modifications in various members of ALPs have generated new compounds with reduced toxicity and high specificity overtime. ALPs comprise a class of antineoplastic agents such as miltefosine, perifosine, erucylphosphocholine and erufosine [2]. Erufosine is a 3rd generation ALP and has been found active against human cancer cells. In addition to the inhibition of proliferation and growth arrest of cancer cells, erufosine interferes with cholesterol homeostasis, ATP generation and cytoskeleton related proteins, which lead to stress, lack of energy and migratory activities in different cancer cell lines, respectively. The addition of 22 carbon chain structure of erufosine makes it metabolically more stable with less toxicity towards GI tract and minimally generate hemolytic activity *in vivo*. These characteristics permit the intravenous administration of erufosine to attain relevant clinical concentrations, which was not achievable with other ALPs [3]. The present study was designed to investigate the anti-proliferative effects of erufosine against lung cancer cells. Furthermore, potential modifications in expression levels of the genes in response to erufosine exposure were determined in lung cancer cells.

METHODS

Cell Culture

Human lung cancer cells (H1299) were cultured in RPMI-1640 medium augmented with L-glutamine (2mM), fetal bovine serum (10%), streptomycin (100µg/ml) and penicillin (100IU/ml). Standard cell

culture incubation conditions (at 37°C in moisturized air with 5% carbon dioxide) were maintained.

Growth Curve Analysis

A growth curve is a graphical presentation of how a specific number of viable cells increases over time. This was done by MTT assay where the cells were cultured in 96-well culture plates (1000-6000 cells/well/100µl medium,) followed by incubation for three different time points (24, 48 and 72 hours) at standard cell culture incubation conditions. MTT solution dissolved in PBS (10mg/ml) was added (10µl concentration per well). NADPH enzymes of the viable cells reduce the MTT dye to the insoluble formazan crystals. Insoluble formazan crystals were dissolved by adding DMSO (50µl/well). Optical densities were measured at absorbance wavelength of 540nm with the reference filter of 690nm by an ELISA plate reader and growth curves were generated with time intervals on X-axis and number of cells on Y-axis. The dark purple color of the solution shows a greater number of viable and metabolically active cells. This data led to the identification of optimal cell numbers to be used in subsequent experiments.

MTT Assay

MTT assay was performed to observe cell viability and proliferation following treatment of the cell line with erufosine. For this purpose, the cells were seeded in 96-well plates (100µl media/well) at pre-optimized cell densities (4000cells/well: the number with continuous growth over 24-72hour) obtained from growth curve experiments and were treated with increasing concentrations of erufosine (dissolved in PBS) for three distinct time points that are 24, 48 and 72 hours. Following the treatment intervals, MTT solution (10mg/ml in PBS) was added (10µl/well) and same procedure was repeated as described in growth curve analysis for measurement of viable cell population. Inhibitory concentration (IC) is a measure of the potency of a compound in inhibiting a specific biological or biochemical function. Data from the MTT experiments were analyzed by GraphPad Prism software (v. 6.0) to calculate ICs of erufosine while untreated cells grown in parallel were used as controls.

Expression Analysis of Genes

The cells were cultured in 6-well plates (200,000 cells/well/2ml medium) and were allowed to grow overnight. After incubation, the cells were exposed to the pre-determined erufosine IC25 (24.4µM) and IC50 (33.1µM) concentrations for 48 hours period. Following the exposure period, RNA was extracted from treated and untreated control cells palettes by using the Kit (Thermo Fisher Scientific, Cat#K0731). The quantity and quality of the extracted RNA was determined using spectrophotometric method (Nanodrop technology). The extracted RNA was stored at -80°C immediately for further use. A total of 40µl cDNA/sample was synthesized by using a specific kit (Thermo Fisher Scientific, Cat#K1622) along with the extracted RNA (1000ng) by using reverse transcriptase enzyme, Oligo dT primers and dNTPs. A PCR based amplification of a reference gene (HPRT1) was performed to verify the synthesized cDNA samples. 7µl of the amplified product was loaded on 2.5% agarose gel and visualized by electrophoresis. Primers for three selected genes (CCNB1, CDKN3 and CASP3) were designed by Primer3Plus software (Table 1). The primers were optimized by using gradient PCR methodology and amplified products were visualized on 2.5% agarose gel electrophoresis. qRT-PCR was performed by using SybrGreen florescence dye (Thermo Fisher Scientific, Cat#K0221) for selected 3 genes by using cDNA samples from the cell line treated with two concentrations of erufosine (IC25, IC50). $2^{-\Delta\Delta CT}$ method was used to find expressional changes in three selected genes in control and Erufosine treated samples. GAPDH was used as reference gene in these experiments and three replicates per sample were used.

Data Analysis

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

Table 1: Primer sequences of selected genes

Gene	Sequences	Product size
CCNB1	CACTTCCTTCGGAGAGCATC	117
	AGAAGGAGGAAAGTGCACCA	
CDKN3	AGCCTGCGAGACCTAAGA	179
	GCAGCTAATTTGTCCCGAAA	
CASP3	GAGGCCGACTTCTTGTATGC	195
	AGCGTCAAAGGAAAAGGACT	
GAPDH	TGCACCACCAACTGCTTAGC	87
	GGCATGGACTGTGGTCATGAG	

RESULTS

Growth Curve of Lung Cancer Cells

Selected cell line was cultured in 96-well culture plates (1000-6000 cells/well/100µl medium) followed by incubation for three different time points (24, 48 and 72 hours) at standard cell culture incubation conditions and then treated with MTT solution. Afterwards, crystals formed by the viable cells were dissolved in DMSO and optical densities were measured by an ELISA plate reader. Growth curves were generated with time intervals on X-axis and number of cells on Y-axis as shown in Figure 1. The data identified 4000 cells/well as optimal number of cells to be used in subsequent experiments in 96-well platform as this number showed exponential growth during the selected time intervals particularly the middle period (48 hours). The number of cells/well were increased accordingly in the 6-well culture plate.

Cytotoxic Effects of Erufosine in Lung Cancer Cells

Human lung cancer cell line H1299 was cultured in RPMI-1640 medium and treated with increasing concentrations of erufosine. ICs of erufosine against H1299 cell line were determined via MTT dye reduction assay. Inhibitory effects were also calculated numerically as percentages of untreated controls. The growth inhibition was noticed after the exposure of the cells with erufosine for 3 different time points with maximum effects in later exposure time (72 hours) with highest erufosine concentration (50uM). A significant decline was also observed in cells survival rate when exposed to various concentrations of erufosine for 24, 48 hours. It has been shown in the graphs for three different time intervals (24, 48 and 72 hours) that the cells became more responsive beyond the 6.25µM concentration of erufosine as shown in Figure 2. Below this range some hyper-proliferation of cells was seen. All in all, erufosine imposed inhibition of proliferation in lung cancer cells.

RNA Extraction, cDNA Verification and Primer Optimization

Following the erufosine treatment, a good quality (260/280: 2.05) and quantity (65-440 ng/µl) of RNA was extracted from the cells. Microscopic visualization of the cells after treatment for these expression analyses is shown in Figure 3. Synthesized cDNA, when loaded on agarose gel electrophoresis, demonstrated a good quality of amplified bands (Figure 4). Designed primers were optimized well when especially focused on applying the three different annealing temperatures as shown in Figure 5.

Expressional Modifications in Genes

Real-Time PCR was performed by using SybrGreen master mix for selected 3 genes by using the synthesized cDNA from the cultured cell line treated with Erufosine at IC25 and IC50. These results highlighted the potential of Erufosine to inhibit expression of these genes in concentration dependent format as the effect was minimal with IC25 and more with IC50 concentrations as shown in figure 6. Specifically, Erufosine exposure inhibited the expression of CCNB1 in a concentration format as more intense inhibition was observed for IC50 application. Furthermore, CCNB1 was the more substantially inhibited gene (-3.1fold) among the three selected markers in this study. In CDKN3 and CASP3, there was a moderate inhibition (-1.5fold) in response to Erufosine exposure. All in all, Erufosine exposure downregulated the three selected genes in lung cancer cells.

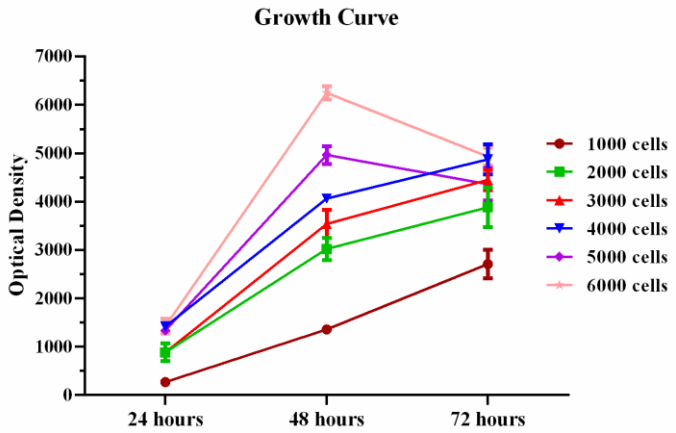

Figure 1: Growth curve generation for three different time points

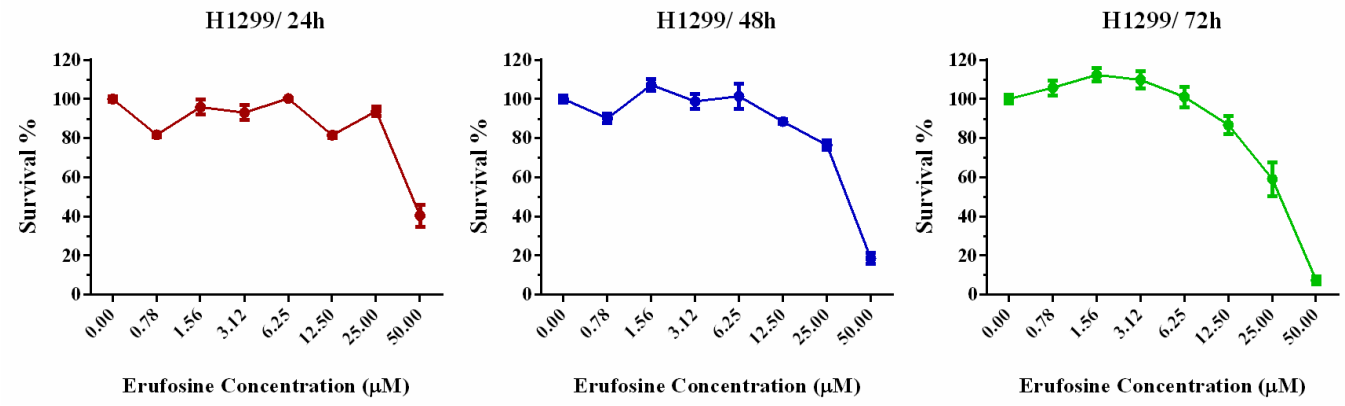

Figure 2: Inhibition of proliferation of H1299 cells after erufosine exposure determined by MTT dye assay.

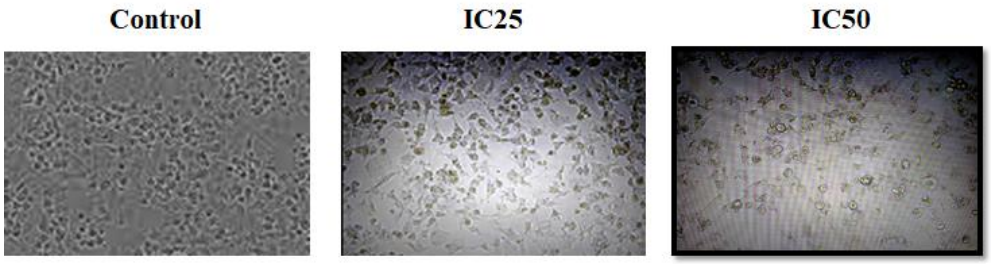

Figure 3: Microscopic images of H1299 cells after erufosine treatment for 48 hours.

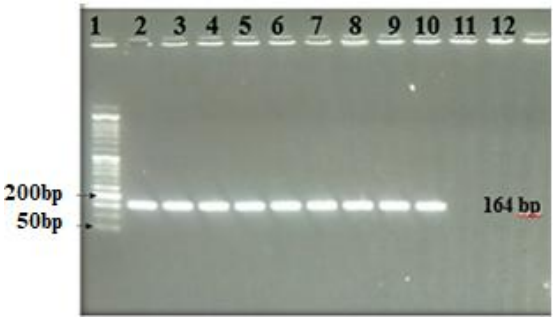

Figure 4: Gel electrophoresis of amplified cDNA of HPRT1 gene from H1299 cells. Sample distribution (left to right): Well 1: DNA marker (50bp) Well 2-4: H1299 untreated control, Well 5-7: H1299 treated with erufosine IC25, Well 8-10: H1299 treated with erufosine IC50, Well 11-12: Negative control.

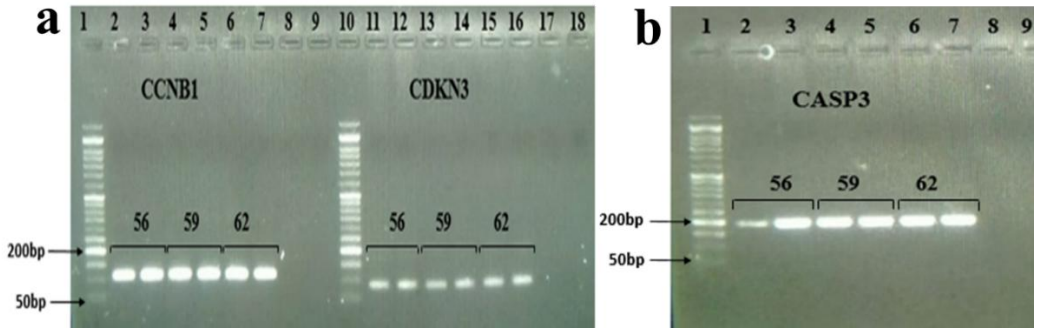

Figure 5: (a) Sample distribution (left to right) for amplification of CCNB1 and CDKN3 genes. Well 1, 10: DNA marker (50bp), Well 2-7: CCNB1 amplification at three different temperatures, Well 8-9: Negative control. Well 11-16: CDKN3 amplification at three different temperatures, Well 17-18: Negative control. **(b)** CASP3 gene Well 1: DNA marker (50bp), Well 2-7: CASP3 amplification at three different temperatures, Well 8-9: Negative control.

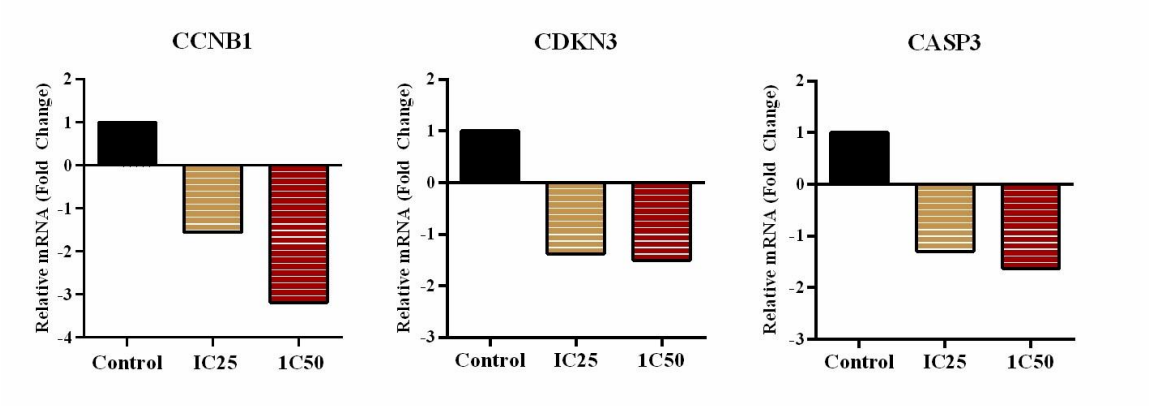

Figure 6: Verification of altered genes by Real time PCR.

DISCUSSION

Lung cancer is the most prevalent cancer while combining male and female population. It imposes a major burden of morbidity and mortality around the globe (~13%). Lung cancer, in its earliest stages, doesn't show any signs and symptoms [4]. Typical symptoms include persistent coughing, hemoptysis, breathing problems, chest cramps, hoarseness, loss of weight and pleural effusion. Treatment of lung cancer during advanced stages of the disease is challenge (5years survival <25%) [5]. Cytostatic drugs are being developed for cancer treatment with an ultimate task to suppress the multiplication of cancer cells by producing arrest in cell cycle. These cytostatic agents are particularly important in a way that they halt the cell division of fast proliferating cancer cells dominantly and spare the normal cells, thus induce minimal side effects [6].

ALPs are a class of antineoplastic agents which target DNA and act upon cell membranes to produce anticancer effects. Perifosine is a 2nd generation ALP which is being used clinically but still there are some improvements required due to its considerable side effects. Structural modifications in various members of ALPs have generated new compounds with reduced toxicity and high specificity overtime [7]. With respect to clinical applications, trials have been conducted to evaluate the efficacy of various ALPs. In this regard, clinical applications of ALPs like miltefosine and perifosine have been investigated to a larger extent, while use of recent ALPs like erucylphosphocholine and erufosine is restricted to *in vitro* and pre-clinical studies [8-10].

Erufosine is a 3rd generation ALP having significant anticancer potential against a variety of cancer cell lines. Erufosine imposes anticancer effects by autophagy, apoptosis and cell cycle arrest [11]. In addition to the inhibition of proliferation and growth arrest of cancer cells, erufosine interferes with cholesterol homeostasis, ATP generation and cytoskeleton related proteins, which lead to stress, lack of energy and migratory activities, respectively. Erufosine has lesser side effects compared to previous classes of ALPs because it is metabolically more stable than other ALPs, having lesser toxicity towards GI tract and minimally generate hemolytic activity. Structural change of erufosine with the addition of a 22-carbon change makes it less hemolytic than prior ALPs. This characteristic of erufosine is likely because of the development of lamellar structures in aqueous solutions instead of micelles like previous ALPs. These characteristics permit the intravenous administration of erufosine to attain relevant clinical concentrations, which was not achievable with other ALPs. Deregulation of cell cycles is a major hallmark of cancer. Therefore, transition of regulators of cell cycle can be target for the treatment of cancers [3].

In the present study, cytotoxic effects of erufosine on the cell line (H1299) were determined by MTT dye reduction assay. For this purpose, the selected cell line was cultured in 96-well plates and exposed to various concentrations of erufosine (1.56-50µM) followed by MTT dye-based assessment of viable cell fractions at three distinct time intervals that is 24, 48 and 72 hours. The growth inhibition was noticed after the exposure of erufosine for 3 different time points with maximum effects in later time interval that is 72 hours with 50µM erufosine concentration. This in turn indicates that erufosine is stable for longer time period and gradually alters the cellular mechanism for more effective inhibition of cell proliferation over time. One additional observation was that at three distinct time intervals, the cells became clearly responsive beyond the 6.25µM concentration of erufosine. It indicates a threshold level of the compound for H1299 cells to inhibit cellular proliferation effectively while below this range, cells tend to resist through some hyper-proliferation. A similar kind of phenomenon has been observed for other cancer cell lines mentioned in published literature [11]. This observation indicates that for clinical utilization, there must be a prior dose escalation evaluation for effective inhibition of tumor cells in clinical settings.

Cell cycles are tightly regulated and monitored via cell cycle checkpoints and in response to any stress or DNA damage, these checkpoints are often up regulated. Cyclins and CDKs are part and parcel of cell cycle and work together to assist the cell cycle progression efficiently. In this study we have selected the 3 genes (CCNB1, CDKN3, CASP3) and have checked their deregulation in lung cancer cell line H1299. The CCNB1 gene, part of the cyclin family, is found in nearly all human tissues. It interacts with a protein called p34 (cdc2) to form the maturation-promoting factor (MPF), which is essential for managing the G2/M phase transition during cell division. In normal cells, CCNB1 expression increases during the late S phase, peaks at the M phase, and plays a key role in cell cycle regulation. However, CCNB1 is often overexpressed in various cancers, such as breast, cervical, lung cancer, and melanoma, linking it to cancer progression. High levels of CCNB1, CDKN3 and CASP3 in lung cancer are associated with worse survival rates. Erufosine can slow down lung cancer cell growth by targeting important genes like CCNB1, CDKN3 and CASP3. It lowers the expression of these genes, suggesting its potential as a cell cycle inhibitor and a promising option for cancer therapy [12]. Real-Time PCR was performed for selected 3 genes (CCNB1, CDKN3, CASP3) by using the synthesized cDNA from the cultured cell lines treated with erufosine at IC25 and IC50. These results highlighted the potential of erufosine to inhibit expression of these genes in concentration dependent format as the effect was minimal with IC25 and more with IC50 concentrations. Furthermore, the concentration dependent effects of erufosine found on these three genes reflect a direct relationship between the compound's concentrations and response of the cells.

To conclude, erufosine showed a substantial potential to inhibit the proliferation of lung cancer cells. Furthermore, there were prominent de-regulation of cell cycle and apoptosis related genes in lung cancer cells after erufosine exposure. Further investigations are needed to understand the molecular mechanisms affected by erufosine in lung cancer, while using additional representative cell lines.

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Competing Interests: The authors declare that they have no conflict of interest.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESEARCH ARTICLE

Anticancer effects of silkworm protein (sericin) in lung cancer cells

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Abstract
Background: Lung cancer is a wide-spread malignancy across the world. With limited availability of therapeutic options, 5-year survival is low in advanced stages of lung cancer. Sericin, a biowaste protein from silkworm cocoon has shown anticancer potential against various tumors in pre-clinical research with minimal side-effects. The study was designed to determine cytotoxic effects and expression modulations imposed by sericin in lung cancer cells.
Methods: Sericin from local cocoons was extracted by degumming process. Human lung cancer cells (H1299) were cultured and exposed to different concentrations of extracted and a commercially available sericin (0.03-1 mg/ml) for 24-72 hours. Effects on cell proliferation were determined by MTT dye reduction assay. Following the total RNA extraction and cDNA synthesis, expressional changes in cell cycle (CDKN1A, CDKN1B) and stress (GADD45A, GADD45B) related genes were determined via real-time PCR methodology.
Results: Sericin exposure induced concentration and time dependent inhibitory effects on cancer cell proliferation. Comparable results from commercially available sericin and local extracted sericin confirmed the reliable extraction process adopted in this study. CDKN family of genes (cell cycle inhibitors) was up regulated in response to sericin exposure. GADD genes (markers of cell stress) were also induced in cancer cells in response to sericin exposure.
Conclusion: Lung cancer cells were responsive towards sericin treatment as observed by inhibition of the proliferation. Sericin interfered with expression levels of cell cycle inhibitor and stress related gene families. Further studies are needed to understand the anticancer potential of sericin against lung cancer cells.
Key Words: Lung cancer, Sericin, Silk protein, Anticancer, Cell cycle, Cell stress

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INTRODUCTION

Lung cancer is a major cause of cancer associated deaths in the world [1]. Behavioral, environmental and genetic risk factors are all known to contribute towards the development of lung cancer. Overall, 5 years low survival rate with lung cancer has barely changed over the years [2, 3]. Surgery, radiotherapy, chemotherapy and targeted therapy are treatment options for lung carcinoma [4, 5]. Due to limited treatment options and associated side-effects, there is a dire need for new cost effective and safer anticancer agents to treat lung cancer. In this context, sericin, an integral part of silkworm cocoon, is a promising protein with significant potential as an anticancer agent.

An economically significant worm called silkworm (*Bombyx mori)*, feeds on mulberry leaves while in its larval stage and spins a cocoon of silk around. The cocoon of this worm is composed of spherical proteins called fibroin and sericin. When silk is produced, sericin occupies space around the two fibroin threads, thus providing protection. This also leads to the fusion of the two threads to make silk yarn. This is where the silkworm develops and completes its life cycle from larva to adulthood [6, 7]. Sericin is around 15-30% of the dry cocoon weight and connects the filaments of silk fibroin together [8]. Overall, *B. mori* cocoon is formed of 65-85% fibroin, 15-30% sericin, 1-5% non-sericin components including pigments, wax, carbohydrate and impurities. Sericin is a natural protein with multiple biological properties [9, 10]. It is a water soluble protein having molecular mass of 20-400kDa [11]. Sericin is being wasted in the silk industry during the degumming process to improve value of silk as textile fiber.

Anticancer properties of sericin have been studied in multiple cancer cell lines including colorectal, prostate and cervical cancers. Sericin was also tested *in vivo* to suppress skin tumors in the mice model induced via 7,12-dimethylbenz(α) anthracene (DMBA) and 12 o-tetradecanoyl phorbol 13-acetata. Sericin has also been showed to induce apoptosis and cell cycle arrest in cancer cells [12]. Sericin and sericin based nanoparticles have been used in experiments to see molecular changes in the human breast cancer cell lines with remarkably reduced cell proliferation [13]. Antineoplastic effects of sericin against various other cancer cells including human breast carcinoma (MCF-

7), squamous carcinoma (A431) and oral carcinoma (SAS) cells led to the suppressed cell growth [14]. Another study showed the anticancer effects of sericin in triple negative breast cancer cells (MDA-MB-468) by inhibiting P13K/AKT pathway. Different functional assays confirmed that sericin causes suppression of proliferation in breast cancer cells, induces cell cycle arrest in G_0/G_1 phase and promotes cellular apoptosis [15]. Sericin has also shown anti-proliferative effects by enhancing caspase 3 activity and suppression of BCL2 against colorectal cancer cells (SW480) [16].

This study was proposed to determine the cytotoxic effects of sericin in lung cancer cells (H1299). Additionally, expression modulations in two important gene families (CDKN and GADD) were determined to provide mechanistic reasoning. The findings led to a better understanding about antineoplastic effects of sericin in cancer cells and support its potential clinical application in the future.

METHODS

Extraction and Purification of Sericin

Sericin was extracted by using degumming process from the locally collected cocoon. Briefly, silkworm cocoons were cut into pieces, washed with deionized water along with continuous stirring followed by autoclaving at 121°C for 45-60 minutes. Afterward, the solution having soluble sericin was filtered to remove fibroin. The filtrate was processed for lyophilization to obtained sericin powder (S-Extract) and stored at 4°C until further use. Purified sericin (S-Pure) was purchased from Sigma company (S5201-5G) to compare the effects in parallel.

Cell Culture

Lung cancer cell line (H1299) was cultured in the media (RPMI-1640) along with recommended essential supplements including FBS (10%), L-glutamine (2mM), streptomycin (100ug/ml) and penicillin (100 IU/ml). The cells were maintained at standard humidified condition (5% CO2, 37^0C) for growth purposes.

MTT Dye Reduction Assay

MTT dye reduction assay was used to measure the cytotoxic effects of extracted and purified sericin in lung cancer cells. For this purpose, the cells were cultured in 96-well culture plates (3000cells/well/ 100ul medium) for overnight period. Next day, the cells were exposed to different concentrations (0.03-1.0mg/ml) of S-Extract and S-Pure dissolved in culture medium for three different time intervals (24, 48, 72 hours). Following exposure periods, cytotoxic effects were determined by adding MTT solution (10mg/ml in PBS) in each well (10ul/well). MTT solution entered cells and converted into formazan crystal in the viable cell. The crystals were dissolved by adding DMSO (50ul/well). Intensity of the color of solution reflected the number of viable cells. Untreated cells were grown in parallel as control during the experimental procedures.

Expression Analysis

Lung cancer cell line was cultured in 6-well culture plates (150,000 cells/well/2ml medium) and exposed to a relatively low, medium and high concentrations (0.25, 0.5 and 1.0mg/ml) of S-Extract and S-Pure for 48 hours. A commercially available kit (Thermo Fisher Scientific, K0731) was used for extraction of RNA from collected cell pellets according to recommended protocol. Quality and quantity of extracted RNA was estimated by Nanodrop spectrophotometer. cDNA was synthesized by using 500ng RNA per sample along with commercially available kit (Thermo Fisher Scientific, K1622). cDNA samples along with specific optimized primers (Table 1) were used for analyzing the expression profile of selected genes by using SybrGreen master mix (Thermo Fisher Scientific, K0221) and QuantStudio-3 real-time PCR machine. Samples were amplified in triplicate and expression levels of reference gene (GAPDH) were used for normalization of the data.

Presentation of Data

Cytotoxicity data generated from MTT assay was presented as percentages. For real time PCR analysis, fold changes were calculated by Livak method by comparing Ct values of treated and untreated control samples.

Table 1: Primers sequences of the selected human genes

Genes	Forward Primer	Reverse Primer
GADD45A	CGCTAGGCTTAATCGCGTT	TTCGAAGCTGGTTCGAATGC
GADD45B	TGAATGTGGACCCAGACAGC	GTCCGTGTGAGGGTTCGT
CDKN1A	GCTTCATGCCAGCTACTTCC	CTGTGCTCACTTCAGGGTCA
CDKN2A	CCCTCAGAAATGATCGGAAA	CAGCTTGCGATAACCAAAGG
GAPDH	ACGGATTTGGTCGTATTGGG	CGCTCCTGGAAGATGGTGAT

RESULTS

Cytotoxic Effects of Sericin

Lung cancer cells were grown in 96-well plates at pre-optimized cell densities in RPMI-1640 medium and were treated with the fraction of S-Pure and S-Extract (0.03-1.0mg/ml) 24-72 hours. Afterwards, MTT assay was performed to assess the growth inhibitory effects. As shown in Figure 1, inhibitory effects were calculated numerically as percentages of untreated controls. Clearly, sericin inhibited the proliferation of lung cancer cells. Cytotoxic effects were concentration dependent since the suppression of cellular proliferation became more intense as concentration was increased especially for late treatment intervals (48 and 72 hours). Furthermore, the effects of S-Extract and S-Pure were comparable in the cells. Overall, sericin inhibited the proliferation of lung cancer cells (H1299).

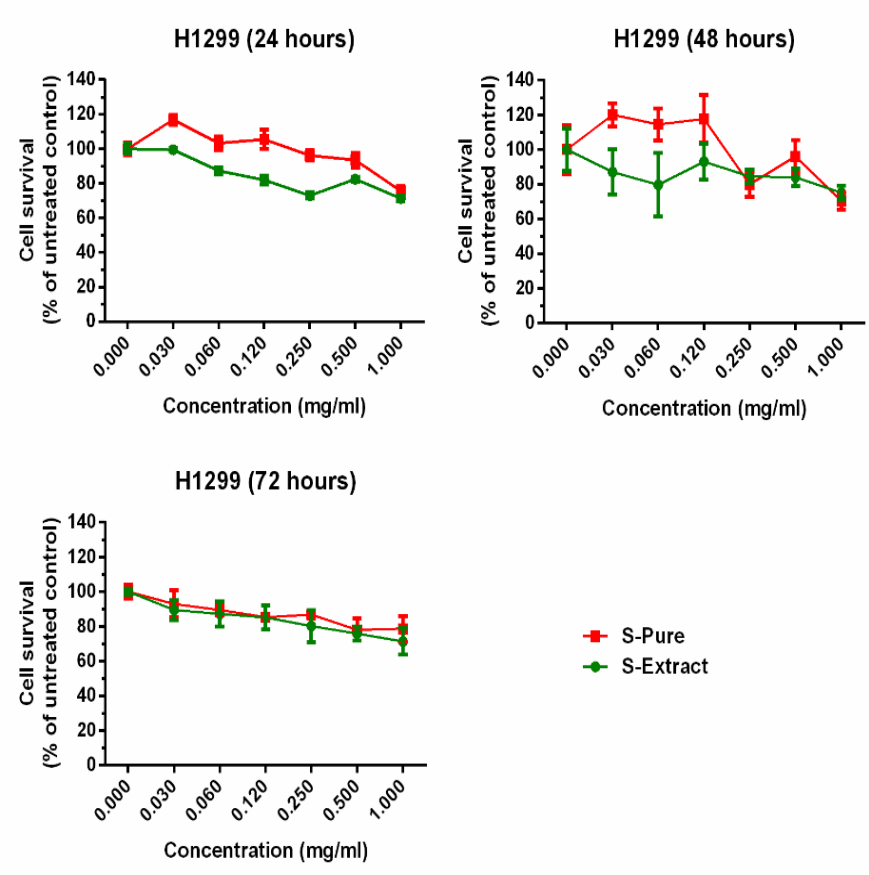

Figure 1: MTT result of H1299 cell lines after 24-, 48- and 72-hours treatment with pure and extracted sericin. The cells were exposed to different concentrations (0.03-1.0 mg/ml) of the sericin fractions and resulting effects on cell proliferation were identified by MTT dye reduction assay. Sericin exposure inhibited lung cancer cell proliferation consistently.

Expression Modulations in Cell Cycle Genes

The cDNA samples from cells treated with different concentrations of sericin fractions were used to determine expressional profile of cell cycle genes (CDKN1A and CDKN2A) by qRT-PCR methodology. For this purpose, the cells were treated with three different concentrations of S-Extract and S-Pure (0.25, 0.5 and 1.0mg/ml). Selected genes, CDKN1A and CDKN2A, were upregulated in the cells. More specifically, CDKN1A was clearly up-regulated (up to 2.7fold) in lung cancer cells (H1299) as shown in Figure 2. Both sericin fractions (pure and extracted) induced CDKN1A gene in H1299 cells. Interestingly, this induction was more pronounced at lower concentrations of sericin. CDKN2A was also consistently up regulated up to 3fold as shown in Figure 2 in the lung cancer cells. Overall, sericin induced the cell cycle inhibitor genes (CDKN1A, CDKN2A) in the lung cancer cells.

Expression Modulations in Cell Stress Genes

The modulation pattern of GADD45A and GADD45B gene in response to S-Pure and S-Extract exposure showed similar pattern as compared to CDKNIA and CDKN2A genes. There was a consistent upregulation of GADD45A gene after the sericin exposure as shown in Figure 3. Similarly, there was a uniform consistency as far as expression alterations are concerned for GADD45B gene in the cells (Figure 3). This induction was considerable in the cells (maximum of 2.9fold) in response to exposure to sericin. All in all, sericin induced the expression of GADD family of genes in the lung cancer cells.

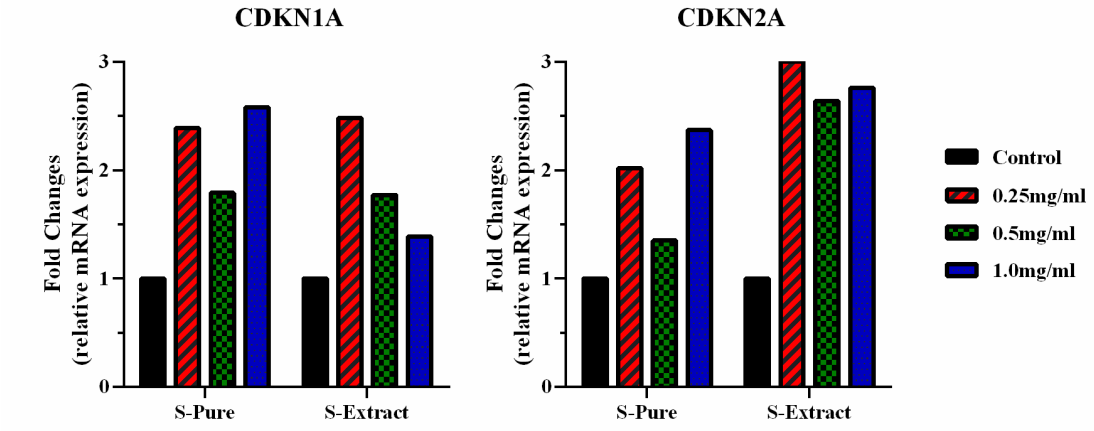

Figure 2: Expression modulation in CDKN1A and CDKN2A genes in lung cancer cells identified via real-time PCR. The cells were exposed to three different concentrations (0.25, 0.5, 1.0 mg/ml) for 48 hours and expression modulations were determined. Sericin exposure induced the levels of CDKN1A and CDKN2A transcript in lung cancer cells.

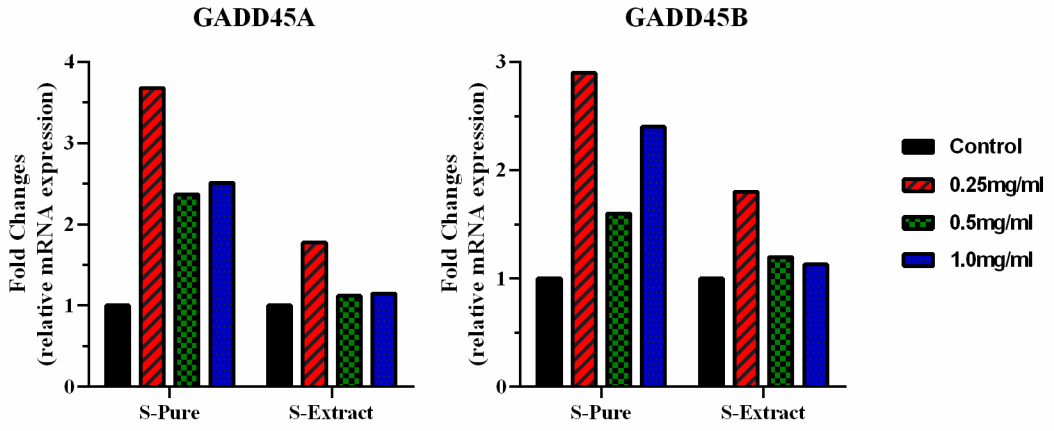

Figure 3: Expression modulation in GADD45A and GADD45B genes in lung cancer cells identified via real-time PCR. The cells were exposed to different concentrations (0.25, 0.5, 1.0 mg/ml) for 48 hours and expression modulations were determined. Sericin exposure induced the levels of GADD45A and GADD45B transcript in the lung cancer cells.

DISCUSSION

Silk is a naturally occurring macromolecular protein fiber. The bio-based substance is derived from several arthropods. Due to its ease of cultivation and high extraction yield, silk produced from *B. mori* (silkworms) cocoons is the most common form of silk. Silk sericin is an additional component of the cocoons, representing ~20–30 wt% of the total weight [17]. Sericin is a white or yellow, odorless, water-soluble protein with a pleasant flavor. Structurally, sericin is a globular protein and consist of β-sheets and random coil [18-21].The properties of the sericin may vary based on the extraction method. Thus, the application of appropriate protocols to extract and purify sericin is important and affects its molecular weight and properties. Almost ~76% of sericin consists of hydrophilic amino acids and ~24% hydrophobic amino acids. The unique amino acid combination of sericin confers the properties of oxidizing the substrates and high-water holding capacity [21, 22]. Sericin can function as a pro -oxidant B because it contain polyhydroxy amino acids like serine as well as polyphenol and flavonoids as secondary metabolites that have various biomedical properties including anticancer activity [23]. Sericin has shown anticancer effects against cancer cell lines. Sericin suppresses the proliferation related PI3K/Akt pathway, arresting the cell cycle in G_0/G_1 phase and promotes apoptosis [18]. Human oral carcinoma (SAS), breast carcinoma (MCF-7), and squamous carcinoma (A431) cells were used to study the anticancer activity of sericin. The cells were examined for anticancer activity in terms of cytotoxicity, ROS levels, cell cycle arrest, and mitochondrial membrane potential. Researchers have looked at how sericin affects the expression of p53, cytochrome C, BAX, and BCL-2 genes as well as apoptotic/anti-apoptotic proteins in cancer cells [14].

In this study, we took the human lung cancer cell line (H1299) and studied the cytotoxic effects of sericin via MTT dye reduction assay. H1299 cells were cultured under the suitable conditions and were exposed to different concentrations of the extracted sericin with various concentrations (0.03–1mg/ml for 24, 48 and 72 hours). For comparison purposes, the cancer cell line was also exposed to the commercially available purified sericin obtained from Sigma company. It was clear from the obtained result that sericin showed effective anti-proliferating effects in this cell line. Sericin does not influence the proliferation of normal cells, while cancer cells show a similar inhibition in proliferation in response to sericin exposure as reported by other researchers [13, 15, 16]. The comparable results from commercially available sericin and the local extracts confirmed the reliable extraction process adopted in this study. Overall, the cytotoxic effects were mild and concentration dependent on the cancer cells.

Identifying the molecular basis of any functional outcome is an interesting and much needed area of investigation. To figure out potential reason behind the anti-proliferative effects of sericin, expression analysis of the important cell cycle regulator genes (CDKN family) and cell stress related genes (GADD45 family) were selected in this study. CDKN family of genes was induced in the cells in response to sericin exposure, which may have contributed to slowing down the cell cycle machinery and cellular proliferation rates. As far as the GADD family of genes are concerned, the genes were unanimously induced in lung cancer cell line. GADD genes are well-known marker of cell stress and death mechanisms, while CDKN family is a well-known category of cytostatic effectors. Induction of these genes may have led to the imposed cytotoxicity and cytostatic effects via sericin exposure, respectively. If so, provided with further confirmation, sericin turned out to be a cytostatic and cytotoxic agent for lung cancer cells and may have substantial clinical applications. Findings from the current study warrant further detailed investigations to explore antineoplastic effects of sericin against lung cancer cells, while including additional representative cell lines.

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Authors contributions: Umm-E-Ammarah Mehak perfomed the experiments. Sana Iqbal helped in experiments, data analysis and manuscript drafting.
Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESEARCH ARTICLE

Riproximin mediated effects on transcriptomic profile of PI3K-AKT-mTOR pathway genes in breast cancer cell lines

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Abstract

Background: Riproximin, a type II ribosome inactivating protein, is a vital antineoplastic agent and can be used as alternative to chemotherapy specifically to kill tumor cells. Since discovery, this protein has been tested against various cancer cell lines and animal models to explore its anti-neoplastic activities. In this study, riproximin has been tested to unfold its impact on multiple genes related to PI3K-AKT-mTOR signaling pathway. This pathway has its significant importance in regulation of cellular functions which include metabolism, proliferation, survival and growth of cancerous cells. Due to the valuable importance in carcinogenesis, this pathway is also being targeted for therapeutic purposes.

Methods: Breast cancer cell lines were exposed to different concentrations of riproximin (MDA-MB-231: 1-50ng/ml, MCF-7: 1-20ng/ml) followed by total RNA extraction, cDNA synthesis and expressional profiling of 10 genes related to PI3K-AKT-mTOR signaling by using real-time PCR. Fold changes were identified via Livak method while comparing the data with untreated cells grown in parallel.

Results: Real-time PCR demonstrated a substantial potential of riproximin to alter expression of the genes. Among these targets, the most effective de-regulations in MDA-MB-231 cells were found in FOS (16fold) followed by JUN (6fold) and NFKB1 (4fold). In MCF-7 cells, most substantial modifications were observed in NFKB1 (14fold), CD14 (9fold) and PDK1 (6fold).

Conclusion: Riproximin bears significant cytotoxic potential against primary and metastatic breast cancer cell lines. Substantial expressional modulations in PI3K-AKT-mTOR signaling pathway related genes are imposed by riproximin in the cancer cells. Further detailed *in vitro* and *in vivo* studies are required to understand the precise impact of riproximin exposure on PI3K-AKT-mTOR signaling, which ultimately will pave the way for its clinical utilization.

Key Words: Cancer, Plant Protein, PI3K-AKT-mTOR, Genes, Target

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INTRODUCTION

Breast cancer develops from malignant cells in breast tissue. It is the most prevalent cancer in females and affecting a huge proportion of population. Treatment strategies being utilized to control breast cancer include surgical removal of the tumor mass, chemotherapy, radiations, hormonal therapy and immune modulation [1-4]. Despite the advancements in treatment, options are largely palliative in nature and are only used to mend the worth of life. Challenges in breast tumors treatment are still there especially for advanced stages where 5-years survival rate is not more than 30% [5-8]. A huge burden of side-effects and expensive nature of treatment modalities impose further obstacles to control the disease. Tumor heterogeneity along with complex intracellular pathways in breast cancer cells further adds to the speed breakers. Among the many cancerous signaling cascades, PI3K-AKT-mTOR signaling shows a significant role in cellular transformation and proliferation of breast tumors [9]. In cancerous conditions, often this pathway is found to be hyperactivated and leads to uncontrolled and continuous proliferation of breast cancer cells. Keeping in view the crucial involvement of PI3K-AKT-mTOR signaling in cancer progression, synthetic inhibitors are being developed against this signaling axis for therapeutic purposes. At the same time, investigating naturally occurring modulators of PI3K-AKT-mTOR signaling for therapeutic purposes is a promising area of research [10,11].

For many years, plants proved to be a rich source of medicinal compounds and are being explored

continuously for their therapeutic efficacy against diseases including cancers [12]. *Ximenia americana* is one of such plant which has been used by the local healers (Hakeem) in African countries to treat cancers. Almost 15 years ago, an active fraction from the crude extracts of this plant were purified and was named as riproximin [13]. This purified protein has been tested against various cancer cell lines for its anticancer effects [14]. Reticence of colony formation, migration, and proliferation of cancerous cells by riproximin is being reported in a previously conducted study [15]. Furthermore, riproximin was shown to induce apoptotic and cytostatic effects in this study. The data compelled us to explore the consequences of this protein on PI3K-AKT-mTOR cascade, the vital signaling pathway for tumor cells.

To validate the sensitivity of breast cancer cells towards riproximin in our experimental settings, MTT assay was performed. Afterwards, riproximin mediated expression modifications in PI3K-AKT-mTOR signaling related genes were identified via real-time PCR methodology.

METHODOLOGY

Cell Culture

Two cell lines for breast cancer, MCF-7 and MDA-MB-231, were cultured in Roswell Park Memorial Institute (RPMI)-1640. The media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 IU/ml penicillin. Breast cancer cell lines were incubated and maintained at standard incubation conditions (5% CO2, 37˚C temperature, 100% standard humidified atmosphere). Both cell lines were passaged an average of two to three times per week to maintain the cells' viability and proper development under the specified conditions.

Riproximin Treatment

The cells were seeded in 6-well plates (2000µl media/well) at optimized densities (200000cells/well) and were treated with the protein dissolved in PBS (MDA-MB-231: 1, 10, 50ng/ml, MCF-7: 1, 5, 20ng/ml) for 48 hours. Following treatment intervals, the cell palettes were collected and stored at -80C till further use. Untreated cells grown in parallel were used as controls and experiments were conducted in triplicate.

RNA Extraction and Quantification

Total RNA extraction was performed by using spin column technology and a commercially available RNA extraction kit (Thermo Fisher Scientific, Cat#K0731). A spectrophotometer-based instrument known as the Nanodrop ND2000 was used to quantify the extracted RNA.

cDNA Synthesis and Verification

Following RNA extraction and measurement, a total of 1500ng of extracted RNA/sample was utilized to synthesize 40µl of cDNA using dNTPs, OligodT, and reverse transcriptase (Thermo Fisher Scientific, Cat#K1622). To validate cDNA synthesis procedure, PCR based strategy was used to amplify the reference gene GAPDH. Agarose gel electrophoresis was used to view the amplicon.

Primer Designing and Optimization

Primers of the chosen genes—FOS, PIK3R2, CASP9, JUN, PDK1, CD14, NFKB1, FASLG, CCND1, and CDKN1B—were created by using Primer 3 and selecting gene-specific sequences from the NCBI Gene Bank (Table 1). The gradient PCR technique was used to optimize the chosen primers for the ten genes, testing a range of annealing temperatures, 56–62^{0}C. Utilizing an electrophoresis and 2.5% agarose gel, the amplified products were examined. By evaluating the gel's band quality, optimized annealing temperature was determined.

Expression Analysis

The study used cDNA samples from two cell lines that were treated with three different riproximin doses to perform qRT-PCR for ten genes. 2-ΔΔCT approach was used to determine gene expression changes in control and riproximin-treated samples. HPRT1 was used as a reference gene. 2-ΔΔCT (Livak) method was used to calculate fold changes from data of treated and untreated groups.

Table 1: Primer sequences of genes.

Genes	Primer Sequence (F)	Primer Sequence (R)
CASP9	GGAAGAGCTGCAGGTGGAC	CCTGCCCGCTGGATGTC
CCND1	GGGGGCGTAGCATCATAGTA	GTGGTGGCACGTAAGACACA
CD14	GAAGACTTATCGACCATGGAGC	AGACGCAGCGGAAATCTTCA
CDKN1B	CCGGCTAACTCTGAGGACAC	TGCAGGTCGCTTCCTTATTC
FASLG	CTGGGGATGTTTCAGCTCTC	CTTCACTCCAGAAAGCAGGAC
FOS	GGGGCAAGGTGGAACAGTTA	AGGTTGGCAATCTCGGTCTG
JUN	ACGGCGGTAAAGACCAGAAG	CTCGCCCAAGTTCAACAACC
NFKB1	CCTACGATGGAACCACACCC	ATCTGCTCCTGCTGCTTTGA
PDK1	TCCTGGACTTCGGATCAGTG	TGCAACCATGTTCTTCTAGGC
PIK3R2	CAGAGAGATCGACAAGCGCA	GTGCGTACTGGTCCTCAGTC
GAPDH	ACGGATTTGGTCGTATTGGG	CGCTCCTGGAAGATGGTGAT
HPRT1	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT

RESULTS

RNA and cDNA Verification

Total RNA was extracted from untreated and treated breast cancer cell lines using a commercial extraction kit, revealing high purity (260/280 ratio: 1.8-2.0) and quantity (>50ng/µl/sample). cDNA was synthesized using extracted total RNA and verified by using PCR-based amplification of a reference gene (GAPDH). Agarose gel electrophoresis was used to visualize the amplified product, revealing an intact, high-quality synthesis of cDNA from the extracted RNA samples, shown in Figure 1.

Primer Optimization

Primers for ten selected genes were designed and optimized by using gradient PCR methodology and visualized on agarose gel electrophoresis, indicating good quality in design and optimization protocols. Based on intensity of bands, appropriate amplification conditions were selected for subsequent experiments. Gel electrophoresis pictures of the optimized genes are shown in Figures 2-4.

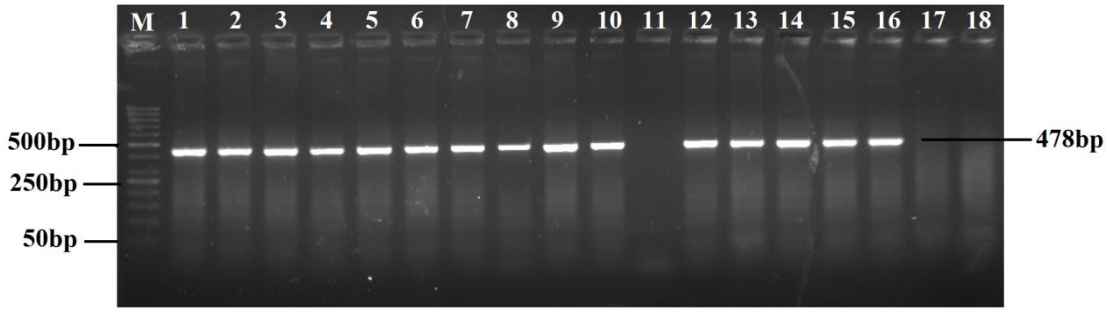

Figure 1: Electrophoresed cDNA of MDA-MB-231 and MCF-7; Sample distribution (left to right): Well 1: DNA marker (50bp) Well 2-9: MDA-MB-231 (untreated and treated samples in duplicate), Well 10-16: MCF-7 (untreated and treated samples in duplicate), Well 17-18: PCR negative controls.

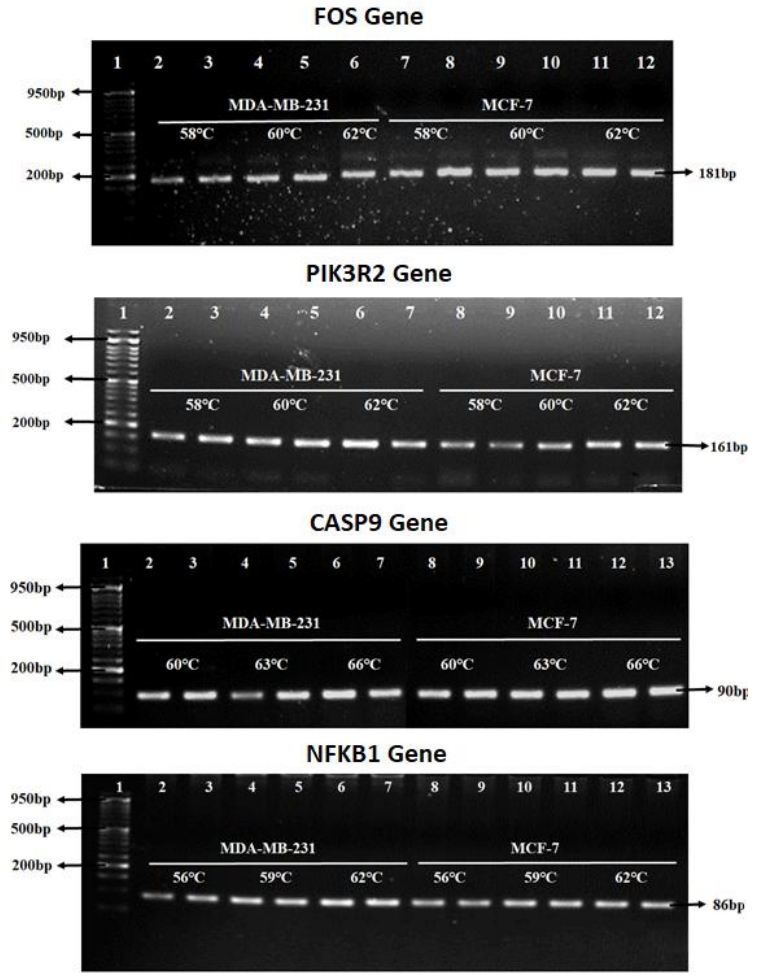

Figure 2: Primers showed specific amplification at different annealing temperatures for FOS, PIK3R2, CASP9 and NFKB1 genes. Sample distribution (left to right), well 1: DNA marker (50bp), well 2-13: MDA-MB-231 and MCF-7 untreated cell lines. Amplified products were visualized on 2.5% agarose gel electrophoresis.

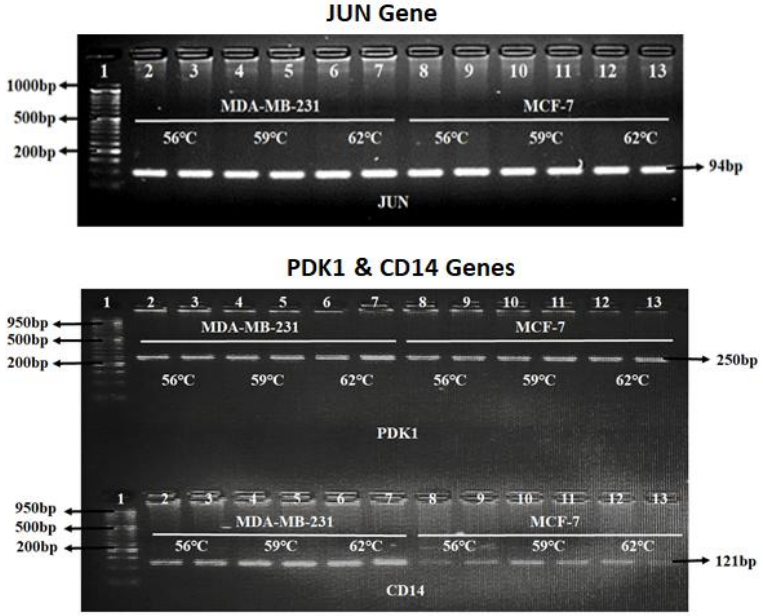

Figure 3: Primers showed specific amplification at different annealing temperatures for JUN, PDK1 and CD14 genes. Sample distribution (left to right), well 1: DNA marker (50bp), well 2-13: MDA-MB-231 and MCF-7 untreated cell lines. Amplified products were visualized on 2.5% agarose gel electrophoresis.

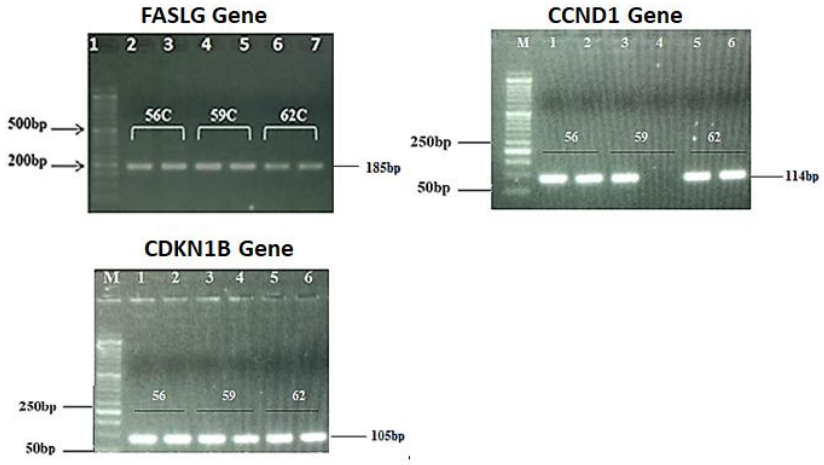

Figure 4: Primers showed specific amplification at different annealing temperatures for FASLG, CCND1 and CDKN1B genes. Sample distribution (left to right), well 1: DNA marker (50bp), well 2-13: MDA-MB-231 and MCF-7 untreated cell lines. Amplified products were visualized on 2.5% agarose gel electrophoresis.

Expressional Analysis

In this study, we evaluated the effects of riproximin on expression levels of the selected ten genes in breast cancer cell lines after exposure to three different concentrations. Expressional analysis was performed using qRT-PCR, identifying expressional modulations in MDA-MB-231 and MCF-7 cell lines, shown in Figure 5 (MDA-MB-231) and Figure 6 (MCF-7). Only three genes (CD14, CDKN1B, and JUN) in MDA-MB-231 cell line and two genes (CASP9 and JUN) in MCF-7 cells showed concentration-dependent effects. As far as the maximum de-regulation is concerned, FOS gene was maximally up-regulated (36fold) followed by NFKB1 (20fold) and Jun (18fold) in MDA-MB-231 cells. In MCF-7 cells, the most effective de-regulation was observed in CD14 (19fold) followed by NFKB1 (12fold). More interestingly, these maximum de-regulations in MCF-7 cells were observed in response to lowest concentrations of riproximin (1ng/ml). All in all, riproximin showed a prominent potential to alter the expression of PIK3-AKT-mTOR pathway genes in cell specific manner.

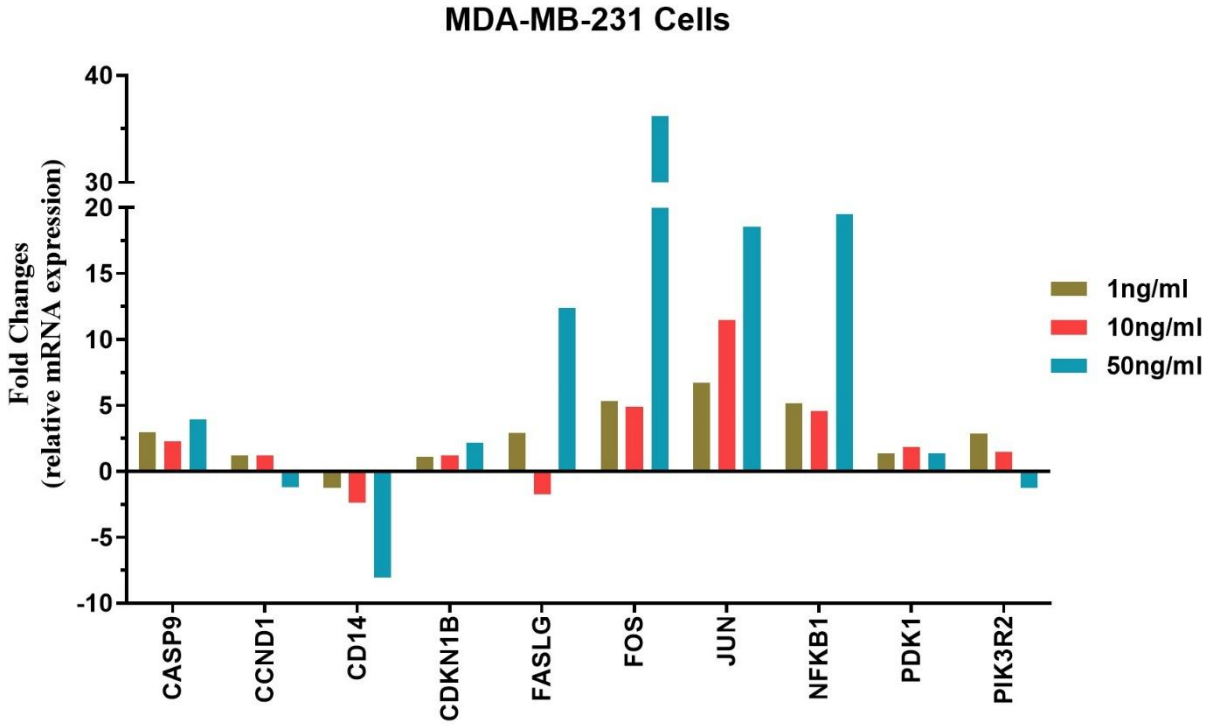

Figure 5: Fold changes in selected genes in MDA-MB-231 cell line. The cells were exposed with three different concentrations of riproximin followed by qRT-PCR based analysis. Fold changes were determined by using Livak method.

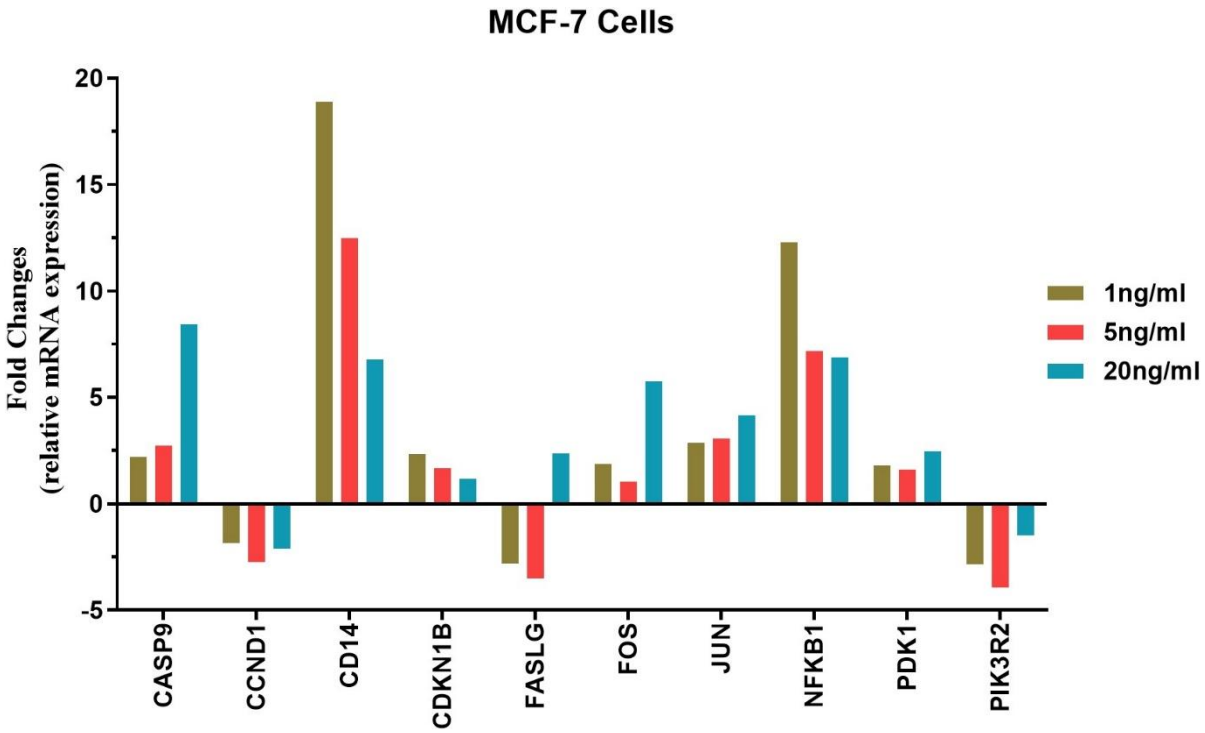

Figure 6: Fold changes in selected genes in MCF-7 cell line. The cells were exposed with three different concentrations of riproximin followed by qRT-PCR based analysis. Fold changes were determined by using Livak method.

DISCUSSION

Indeed, the leading cause of morbidity and death worldwide is breast cancer. When combining the cases, the incidence rate is rising quickly and overtaking lung cancer [16,17]. A major hurdle in controlling breast cancer is the heterogeneous nature of this disease, aggressive behavior and lack of effective therapeutic options [18]. In this context, it is of paramount importance for the scientific community to search for new safer but effective treatment options. Plants can be an attractive source of medical entities in this scenario. A huge variety of plants have been studied so far to explore anticancer components from their different parts including roots, leaves, and fruits [19]. *X. americana* is one kind of such plant, which has been used for its medicinal benefits in African countries. Particularly, seeds of this plant (in grounded form) have been used by local people to treat various diseases including cancers [20].

Several fractions from *X. americana* seeds were isolated in 2006, and their corresponding anticancer properties were examined [21]. Riproximin, a highly effective antineoplastic protein from this plant, has been investigated over time for its ability to combat a variety of malignant cell lines and animal models [22]. There have been several demonstrated genetic changes brought about by riproximin in these cell lines; nevertheless, information regarding the precise effects of this protein on different pathways is scarce. It is necessary to clearly emphasize riproximin's effects at the molecular level to comprehend the changes it is imposing on different signaling pathways. With that in mind, the PI3K-AKT-mTOR signaling cascade one of the most important and dysregulated cascades in malignant cells was the subject of our discussion.

The core part of this study was to identify the impact of riproximin exposure on PI3K-AKT-mTOR signaling related genes in the breast cancer cell lines. For this purpose, the cells were exposed to different concentrations (MCF-7: 1-20ng/ml, MDA-MB-231: 1-50ng/ml) for 48 hours followed by RNA extraction, cDNA synthesis, and real-time PCR based amplification. The treatment concentrations were selected based on a previous MTT toxicity data sets, where the aim was to use higher concentrations for having sufficient effects while avoiding too much cellular death to have enough cells for extraction of RNA at the same time [15]. Overall, the data generated from these results showed that MCF-7 cells were more responsive towards riproximin as compared to MDA-MB-231 cancerous cells. Based on known literature and a reference from Qiagen RT^2 PCR panels, a total of 10 genes related to PIK3-AKT-mTOR pathway were selected to be examined in this study. Real-time PCR data showed a discrete pattern of alterations in the expressions in response to riproximin exposure. Most effectively up-regulated genes in MDA-MB-231 cells were FOS (36fold) followed by NFKB1 (20fold) and JUN (18fold). Interestingly, all these maximum up-regulations were

observed in response to the highest applied concentrations (50ng/ml) in MDA-MB-231 cells, which shows that the cells responded in a linear fashion towards the tested compound. In contrast, a different set of de-regulation was observed in MCF-7 cells, where CD14 (19fold) was the most effectively de-regulated gene followed by NFKB1 (12fold). More importantly, these extreme de-regulations were observed in response to the lowest applied concentration (1ng/ml) in MCF-7 cells, which in turn shows the development of resistance or irresponsiveness of the cells towards higher concentrations of riproximin in MCF-7 cells. Another interesting fact is the existence of a differential response in de-regulations: for instance, CD14 was effectively inhibited in MDA-MB-231 cells (-7fold) while it was induced in MCF-7 cells (19fold). This reflects that cancer cell types from the same malignancy can respond differently towards riproximin exposure, like MDA-MB-231 triple negative cells ($ER^-/PR^-/HER2^-$) did as compared to MCF-7 double positive cells (ER^+/PR^+). Nevertheless, riproximin exhibited a substantial potential to de-regulate PIK3-AKT-mTOR pathway related genes in breast cancer cells and needs further investigations to target this pathway for therapeutic purposes. Provided with further *in vitro* and *in vivo* investigations, the compound can be an affective regulator of this vital proliferation related pathway in cancer cells.

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Ethics Approval: Not Applicable in this work
Contributions: AK and KU perfomed experiments. OS analyzed data. AP planned the work and drafted the manuscript.
Competing Interests: No competing interests
Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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).

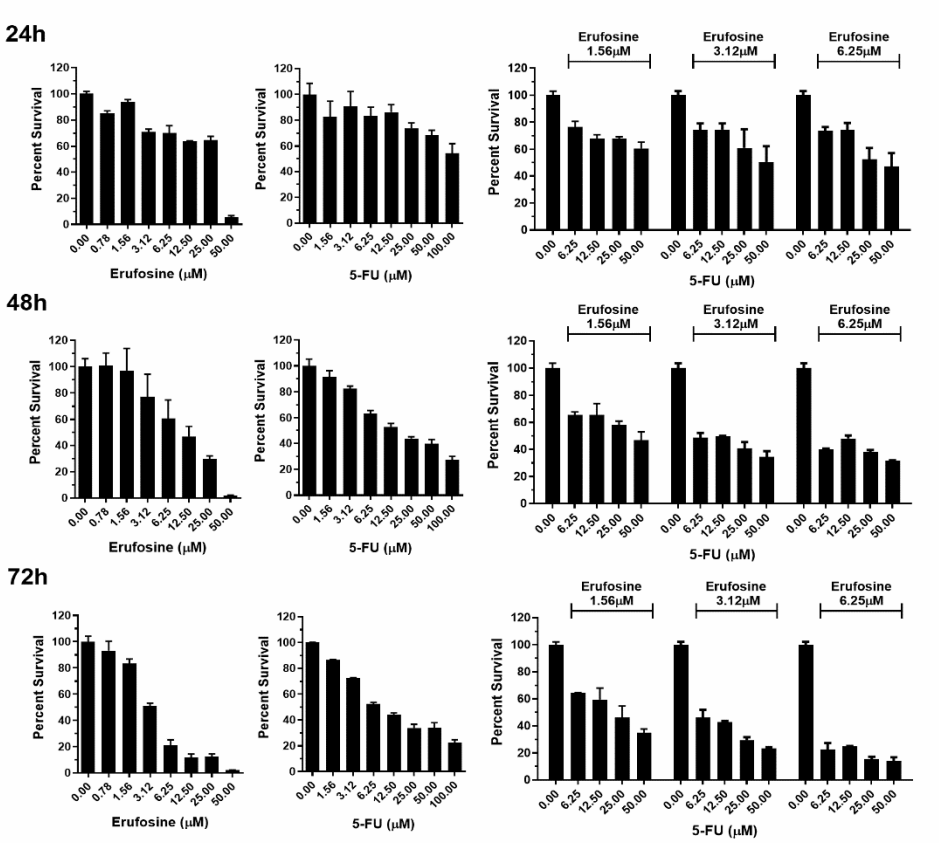

Figure 1: MTT results of SW480 after treatment with erufosine and 5-FU alone and in combinations. The effects were measured by using the dye reduction assay.

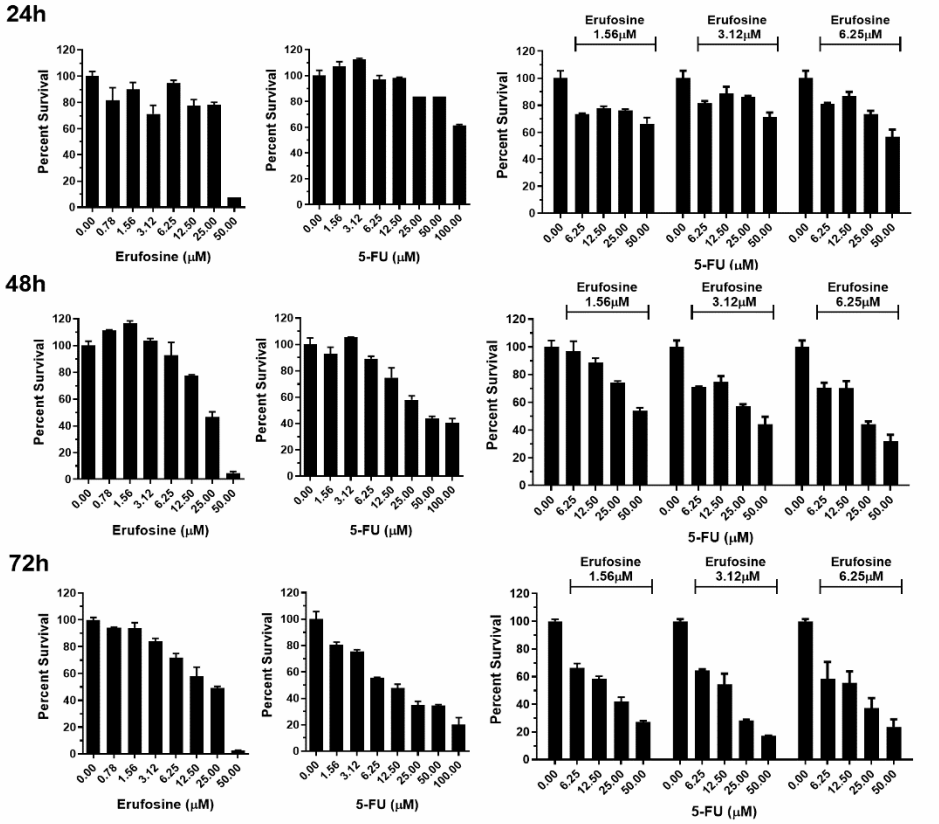

Figure 2: MTT results of SW620 after treatment with erufosine and 5-FU alone and in combinations. The effects were measured by using the dye reduction assay.

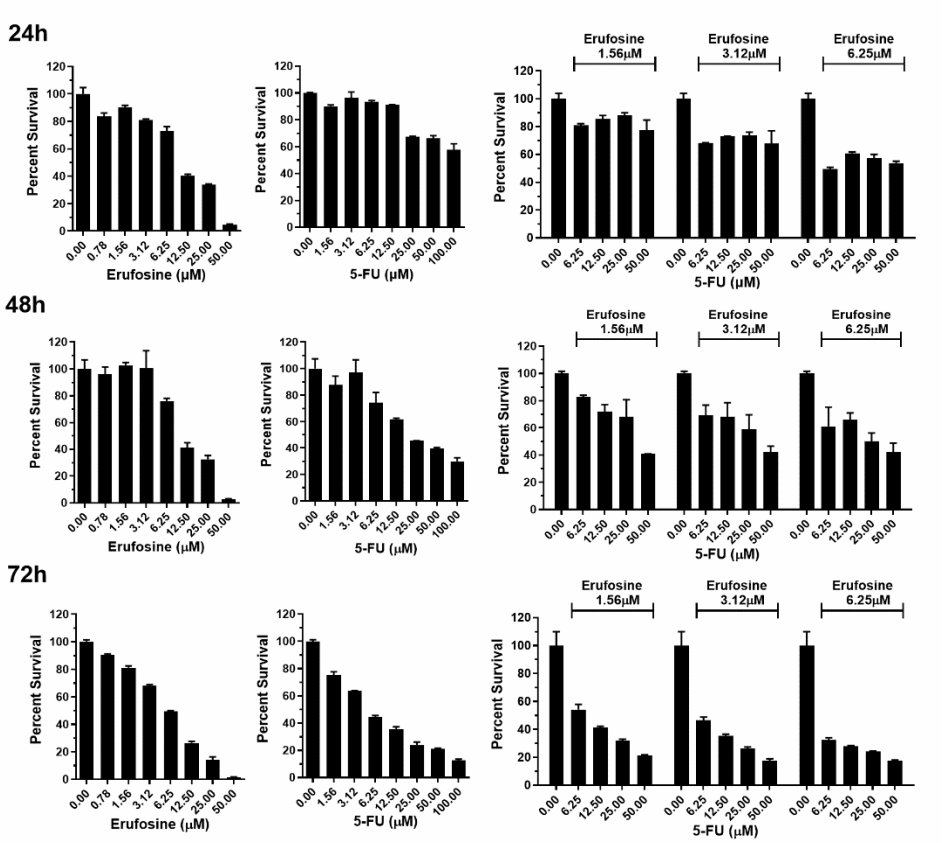

Figure 3: MTT results of HCT116 after treatment with erufosine and 5-FU alone and in combinations. The effects were measured by using the dye reduction assay.

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.

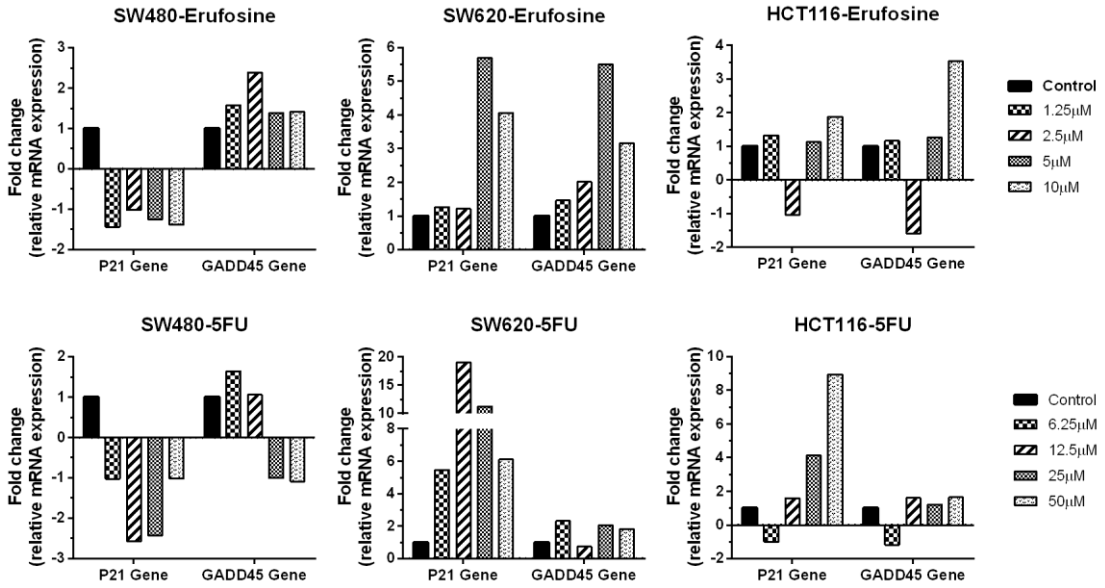

Figure 4: Expressional changes in P21 and GADD45A genes in SW480, SW680 and HCT116 cells after single agent treatment.

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).

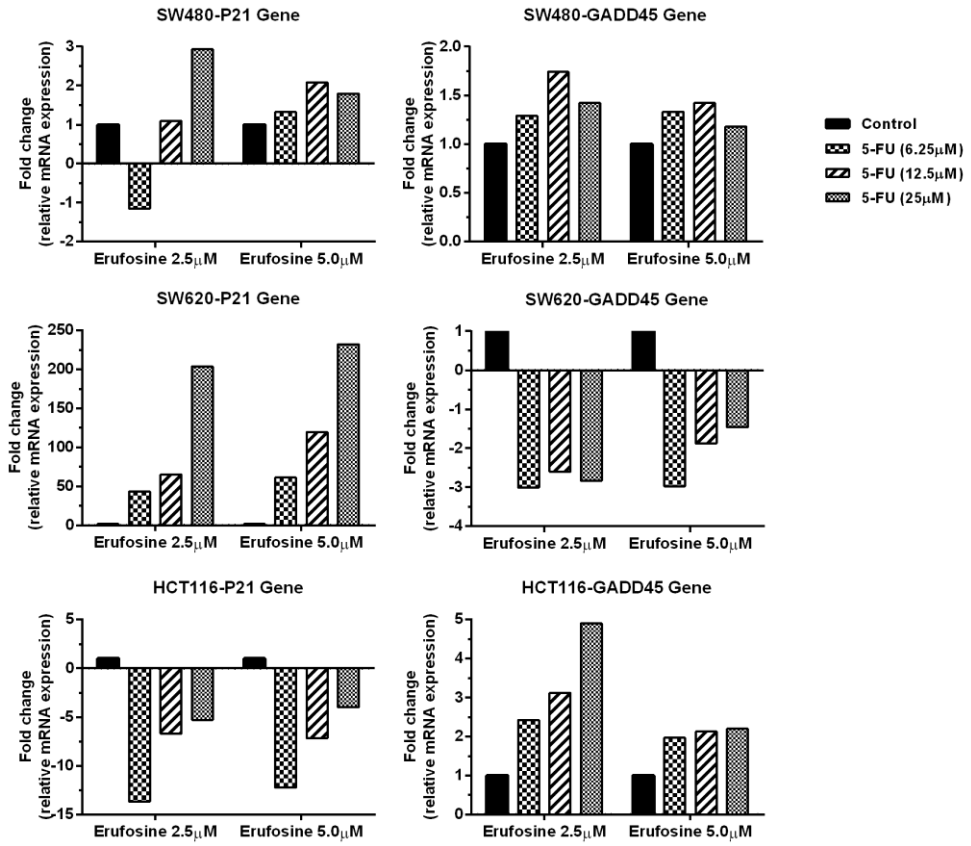

Figure 5: Expressional changes in P21 and GADD45A genes in SW480, SW680 and HCT116 cells after combinational treatments.

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 were 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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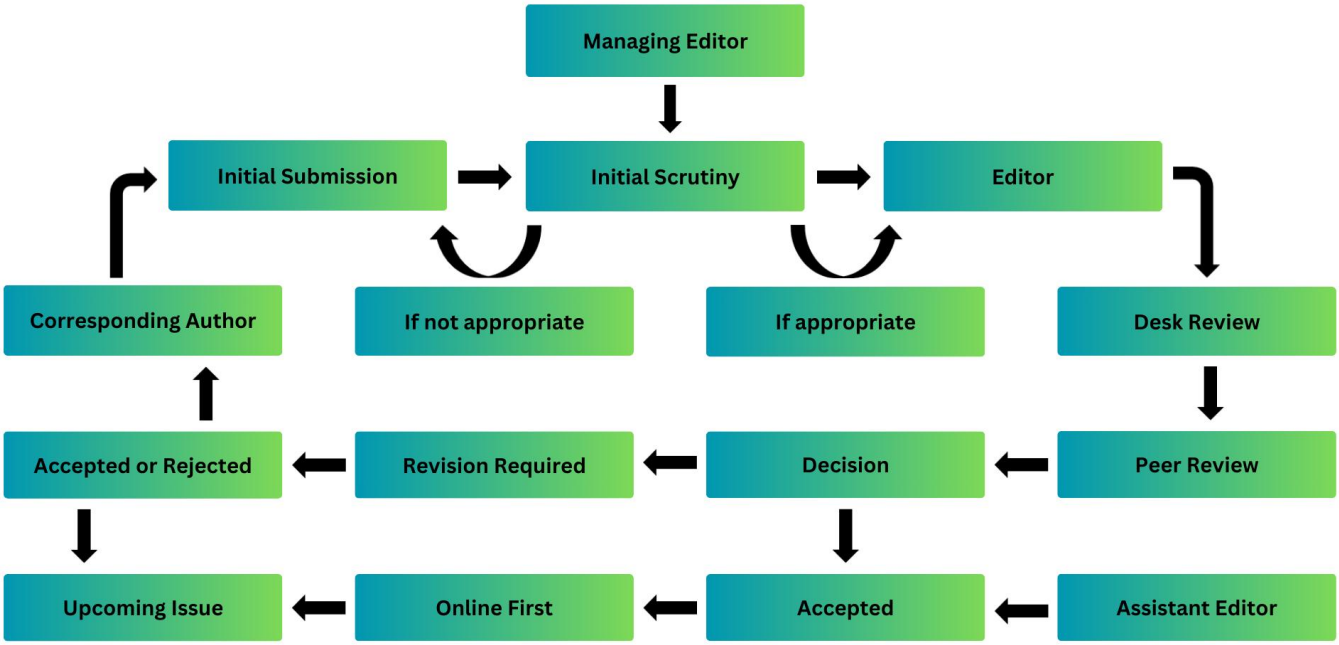

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