

Alkyl-phospholipids and cell cycle check point inhibitor induces antineoplastic effects in breast cancer cells: Evidence from in vitro investigations

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Abstract

Background: Combination of anticancer compounds often lead to better control of tumor progression. In this context, searching for novel combinations of anticancer compounds is a continuous process. Alkyl-phospholipids are synthetic lipids and have shown anticancer effects. Combining the alkyl-phospholipids with potential other drugs is an attractive field of interest. In this study, we investigated the combined anticancer effects of an alkyl-phospholipids (erufosine and perifosine) and a cell cycle check point inhibitor (ATR) with the hypothesis that combining the two above-mentioned classes can be instrumental against breast cancer.

Methods: Human breast cancer cell lines (MDA-MB-231 and MCF-7) were exposed to the two compounds and resulting toxicity was assessed by using a mitochondrial based dye reduction assay. The effects were calculated numerically, and percentages were compared with untreated controls. To give insights at gene levels, expression profiles of cell cycle associated genes (CDKN family) were investigated by using real-time PCR methods. The effects in both assays were uncovered as single agent and combinational approaches in parallel for comparison purposes.

Results: Exposure with the compounds (erufosine, perifosine and ATR inhibitor) reduced the cell proliferation of the breast cancer cells. Overall, alkyl-phospholipids inhibited more effectively the cell proliferation as shown by the MTT assay. Combination of the two classes (alkyl-phospholipids and ATR inhibitor) showed synergistic effects while inhibiting cell proliferation. The effects were comparable in the two breast cancer cell lines. The compounds either as single agent or in combination also induced the expression of CDKN family members. However, these effects were less pronounced in ER/PR/HER2 negative cells (MDA-MB-231) as compared to ER/PR positive cells (MCF-7).

Conclusion: Alkyl-phospholipids and ATR inhibitor can be combined to achieve synergistic antineoplastic effects against breast cancer. The compounds can be applied as a combinational therapeutic approach against breast cancer.

Key Words: Breast Cancer, Alkyl-phospholipids, Cell cycle inhibitor, Combinational therapy

INTRODUCTION

Deregulation in cell cycle is a hallmark of cancer cell transformation leading to fast cell proliferation. Cell cycle regulation is governed by specific checkpoints that ensure integrity of the DNA content while going through the cell cycle stages. However, when compromised, the cell cycle machinery leads to defects in DNA duplication and damage of genome. This condition initiates DNA damage response (DDR) through multiple players including ATM and ATR serine/threonine kinases. Among these, ATR is activated by a considerably broader spectrum of genotoxic stressors and serves as a key mechanism for genome stability maintenance [1-4].

Many chemotherapeutic medicines and radiation have an anti-tumor impact by inducing DNA damage in cancer cells; hence, stimulating DDR pathways are regarded as a significant factor influencing treatment outcomes. When DDR pathways are inactivated, the inhibition of other pathways can induce cancer cell-specific lethality. For cancer treatment, ATR is a promising therapeutic target due to its functions as a facilitator in regulating responses to DNA mutilation. Many types of cancer are being addressed with chemotherapeutic medicines in combination with inhibitors of ATR [5, 6]. EPT-46464, AZ20 (AstraZeneca), AZD6738, VE-821, and VX 970 (VE-822) (Vertex) are some of the ATR inhibitors that have been identified and are currently in Phase I clinical studies [7]. VE-821 was shown to be a potent ATP-competitive inhibitor of ATR with minimal cross-reactivity against related kinases. Under replication stress, ATR and CHK1 keep cancer cells alive, and

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inhibitors of both kinases are now being tested in clinical studies. Indeed, when the CHK1 inhibitor AZD7762 is combined with ATR inhibitor VE-821 in cancer cells, replication stress is induced, resulting in replication arrest and apoptosis. *In vitro* and *in vivo*, combination of ATR and CHK1 inhibitors causes replication fork arrest, ssDNA buildup, replication collapse, and synergistic cell death in cancer cells. VE-822 was the first selective ATR inhibitor, while AZD6738 was second to enter clinical development [8-10].

Synthetic chemicals known as alkyl-phospholipids (ALPs) have been known to harbor anti-cancer and anti-proliferative properties. ALPs are glycosylated lipids and glycosylated phospholipids. Due to their resemblance to cellular phospholipids, these agents disrupt lipid homeostasis, targeting membrane lipid rafts and changing lipid-linked signaling, resulting in cell death. ALPs have an advantage over traditional anti-cancer drugs in that they target cell membrane phospholipids rather than cancer cells' DNA. These ALPs produce biophysical disruption by inserting a micelle in the lipid bilayer at clinical concentrations. Destructive cellular pathways are triggered when cell membrane metabolism is disrupted [11, 12].

Perifosine is a variation of edelfosine (prototype ALP) in which choline component of alkyl phosphocholines is replaced with a heterocyclic piperidine group. This modification was an advantage over miltefosine (1st generation ALP) as stability and half-life by approximately 140 hours increased, thus halting rapid metabolic degradation [11]. Phosphocholine, that is the main metabolic product of miltefosine, is responsible for severe GI toxicity after oral therapy. Perifosine, on the other hand, is unable to produce phosphocholine and allowing for a more thorough investigation *in vitro* and *in vivo* [13, 14]. Perifosine is a 2nd generation ALP that may be given to people orally and has a low toxicity profile, as well as a high rate of tumor uptake and accumulation. In phase II clinical trials, perifosine exhibited toxicity against a variety of advanced and metastatic cancers such as breast cancer, head and neck cancer, melanoma, and hepatocellular carcinoma, but it had a toxicological profile like miltefosine and caused GI side effects (nausea, vomiting, diarrhea). It inhibits many important signal transduction pathways in human malignancies, including PI3K/AKT, one of the most important pathways for cancer cell proliferation. Perifosine has shown significant clinical effectiveness as an Akt inhibitor in combination treatments with other potent antineoplastic medicines, despite its limitations as single oral drug for treating common solid tumors [14-16].

Erufosine is a 3rd generation ALP molecule that has shown itself to be effective against leukemia, multiple myeloma, breast, prostate, colorectal and squamous carcinoma cell lines. When evaluated for anticancer

potential, erufosine has demonstrated considerably reduced off target effects due to structural alteration. *In vivo*, erufosine is more metabolically stable, with lower hemolytic activity and less bone marrow damage. Because of these properties, erufosine can be administered intravenously to obtain clinically meaningful doses that are not achievable with other ALPs. In different cancer cell lines, erufosine induces anticancer effects through autophagy, apoptosis, and G2 cell cycle arrest. Erufosine has demonstrated the ability to penetrate blood-brain barrier and accumulate brain tissue, as well as having a responsive nature in brain cancers *in vitro* and *in vivo*. As far as anticancer activities are concerned, it is limited to pre-clinical studies so far and increased understanding about its anti-cancer properties will foster the evaluations in phase I trials. So far, *in vitro* investigations are successfully conducted against acute myeloid leukemia, astrocytoma, glioblastoma, multiple myeloma, breast, colorectal and oral squamous cell carcinoma [17-25].

The objectives of current research were to investigate the cytotoxic effects imposed by ATR inhibitor and erufosine/perifosine alone and in combinations in breast cancer cells. Furthermore, the aim was to determine the effects of single and combinational exposures of the test compounds on CDKN family of genes in breast cancer cells by real-time PCR methodology.

METHODS

Toxicity Assay

Two breast cancer cell lines (MDA-MB-231 and MCF-7) were cultured in the cell culture medium (21875-034) along with 10% fetal bovine serum (10270-106), 2mM L-glutamate (A2916801), penicillin (100IU/ml) and streptomycin (100µg/ml) (15140-122) in 96-well plates. To assess toxicity response towards compounds (erufosine, perifosine and ATR), the cells were cultured and incubated at 5% CO₂, 37°C, humidified culture conditions. By using the MTT dye reduction assay methodology, a colorimetric assay, cell viability was investigated after exposure with the compounds. Cells (4000cells/well) were propagated in 96-well plates (100µl medium/well) and treated with variable concentrations (1.56-50µM) of erufosine, perifosine and ATR inhibitor (Ab219506). The treatment was carried out for three intervals i.e., 24-72 hours. The treatment was carried out as a single agent and combinations of the selected compounds as shown in Table 1 and 2. Following treatment intervals, MTT solution (20395.01) was added to dissolve formazan formed by viable cells. Optical densities were measured to calculate cell survival rates as percentage of untreated control. Inhibitory concentrations (IC) required for subsequent real-time

PCR experiments were assessed using GraphPad Prism software.

Expressional Analysis Treatments

Breast cancer cells were cultured in 6-well plates and allowed to grow overnight. Afterwards, the cells were exposed to various ICs of erufosine, perifosine and ATR alone and in combinations (Table 3). After 48 hours of incubation, cells were washed, trypsinized and the palettes were collected. Total RNA, extracted from treated and control cells by using spin column extraction kit (K0731), was quantified and used to synthesize cDNA via reverse transcriptase kit (K1622).

Prepared cDNA samples were confirmed by amplifying reference gene (HPRT1) and gel electrophoresis.

Real Time PCR

Primers of cell cycle related genes (CDKN1A, CDKN1B) were designed (Table 4) and optimized by gradient PCR followed by gel electrophoresis to visualize the products. Real time PCRs were performed by using SybrGreen dye (K0221), cDNA samples and gene specific primers. The samples were amplified in triplicate. Quantstudio-3 real-time machine was used for expression profiling. Expression level of HPRT1 reference gene was used to normalize the data. Fold changes were calculated by Livak ($2^{-\Delta\Delta CT}$) method.

Table 1: Treatment of breast cancer cells against different concentrations of selected compounds for MTT assay

Cell Lines	Erufosine (μM)	Perifosine (μM)	ATR (μM)
MDA-MB-231	1.56, 3.12, 6.25, 12.5, 25, 50	1.56, 3.12, 6.25, 12.5, 25, 50	1.56, 3.12, 6.25, 12.5, 25, 50
MCF-7	1.56, 3.12, 6.25, 12.5, 25, 50	1.56, 3.12, 6.25, 12.5, 25, 50	1.56, 3.12, 6.25, 12.5, 25, 50

Table 2: Exposure with inhibitors and compounds in combinations

Cell Lines	Erufosine + ATR		Perifosine + ATR	
	Erufosine	ATR	Perifosine	ATR
MDA-MB-231	IC25	IC25	IC25	IC25
MCF-7	IC25	IC25	IC25	IC25

Table 3: Exposure with single agents and combinations for real-time PCR analysis

Cell Lines	Single Agent Treatment			Combination Treatment
MDA-MB-231	Erufosine IC25	Perifosine IC25	ATR IC25	Erufosine IC25+ ATR IC25
	Erufosine IC50	Perifosine IC50	ATR IC50	Perifosine IC25+ ATR IC25
MCF-7	Erufosine IC25	Perifosine IC25	ATR IC25	Erufosine IC25+ ATR IC25
	Erufosine IC50	Perifosine IC50	ATR IC50	Perifosine IC25+ ATR IC25

Table 4: Primer sequence for amplification of selected genes

Gene	Forward Primer	Reverse Primer
CDKN1A	GCTTCATGCCAGCTACTTCC	CTGTGCTCACTTCAGGGTCA
CDKN1B	CCGGCTAACTCTGAGGACAC	TGCAGGTCGCTTCCTTATTC
HPRTI	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT

RESULTS

Cytotoxic Effects of Compounds

The compounds (erufosine, perifosine and ATR) imposed the anti-proliferative effects in breast cancer cells. Effects of the two ALPs (erufosine and perifosine) were comparable while inhibiting the viability of breast cancer cells. At lower concentrations ($\leq 3.13\mu\text{M}$), there were some stimulatory effects on growth. While at further higher concentrations, the decline in viable cells were observed. At highest concentrations ($50\mu\text{M}$), a steep decline in viability was observed ($\geq 80\%$) in both breast cancer cell lines. As far as ATR inhibitor is concerned, growth inhibitory effects were concentration dependent. However,

these effects were less prominent in both cell lines while comparing with ALPs. With the highest concentration ($50\mu\text{M}$) of ATR inhibitor, a maximum of $\sim 50\%$ growth inhibition was observed (Figure 1, 2). When considered combinations of ALPs (erufosine and perifosine) with ATR inhibitor, synergistic impact on inhibition of viability was observed (Figure 3, 4). These synergistic effects were witnessed in both breast cancer cell lines. Furthermore, the effects were clearer at initial time intervals (24 hours) as compared to later time intervals (48 and 72 hours). All in all, erufosine and perifosine when combined with ATR inhibitor, showed synergism in inhibiting cell viability (Figure 3, 4).

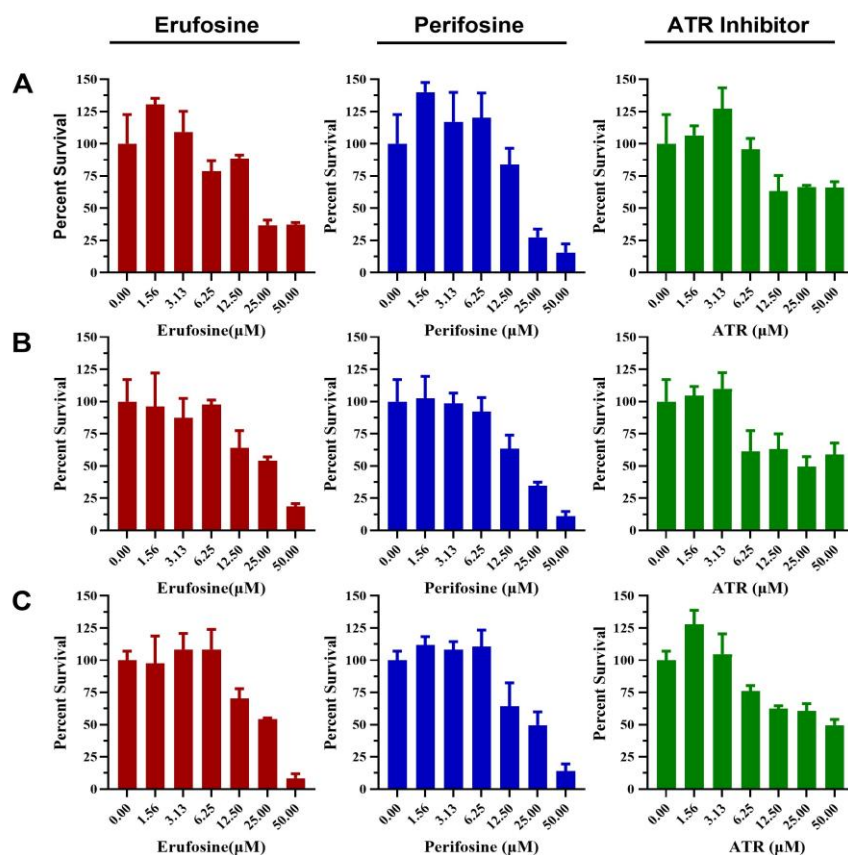


Figure 1: MTT results of breast cancer cell line (MDA-MB-231) after treatment with selected compounds (erufosine, perifosine, ATR inhibitor). The cells were cultured and exposed to compounds as single agents' treatments. Afterwards, MTT dye reduction assay was performed to evaluate the effects on cell viability.

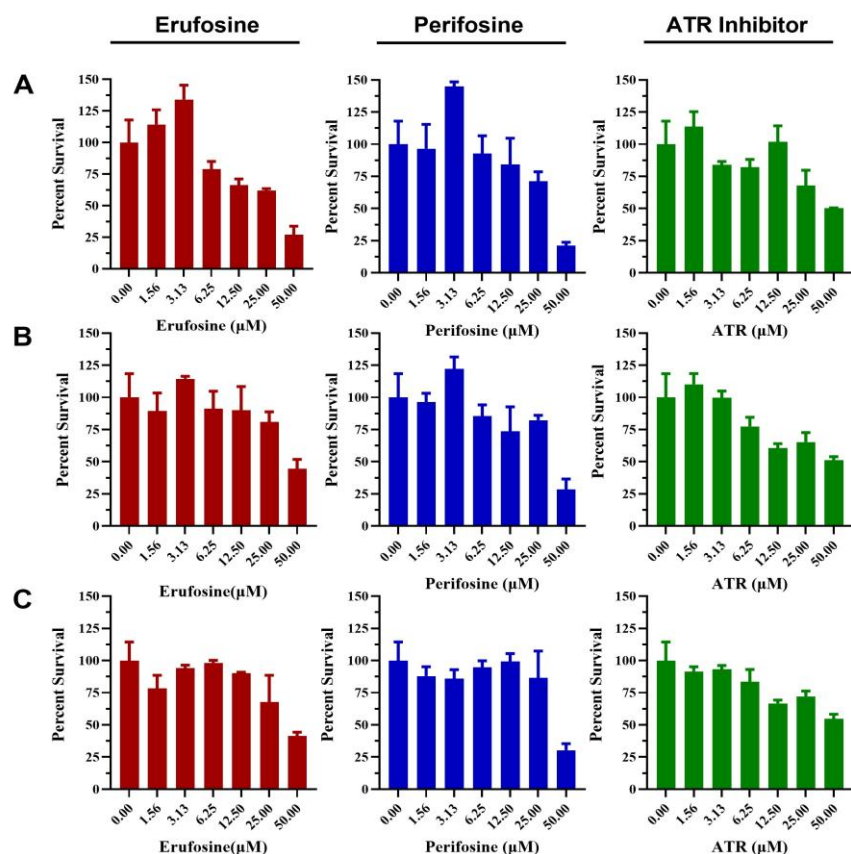


Figure 2: MTT results of breast cancer cell line (MCF-7) after treatment with selected compounds (erufosine, perifosine, ATR inhibitor). The cells were cultured and exposed to compounds as single agents' treatments. Afterwards, MTT dye reduction assay was performed to evaluate the effects on cell viability.

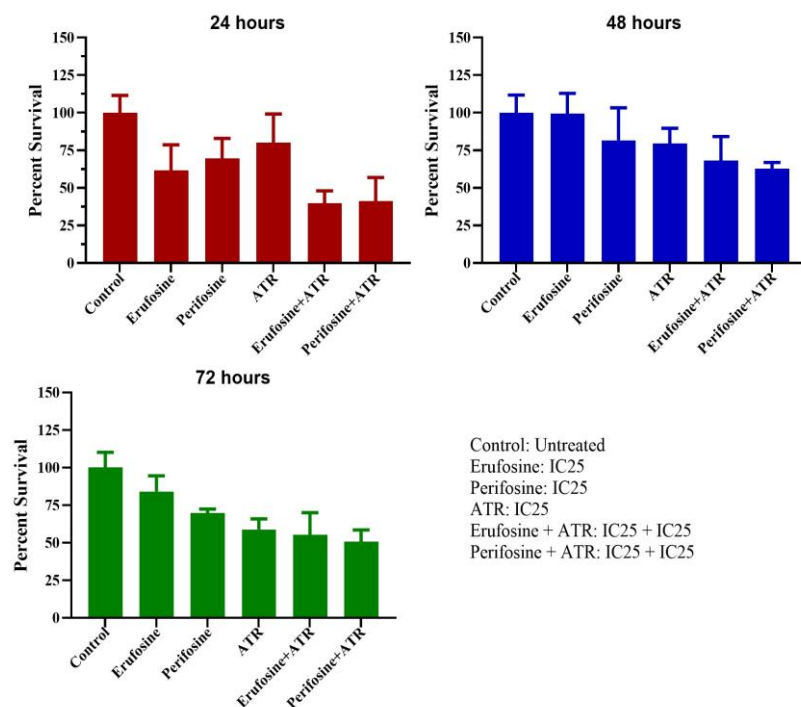


Figure 3: MTT results of breast cancer cell line (MDA-MB-231) after treatment with selected compounds (erufosine, perifosine, ATR inhibitor). The cells were cultured and exposed to compounds as combinational treatments. Afterwards, MTT dye reduction assay was performed to evaluate the effects on cell viability.

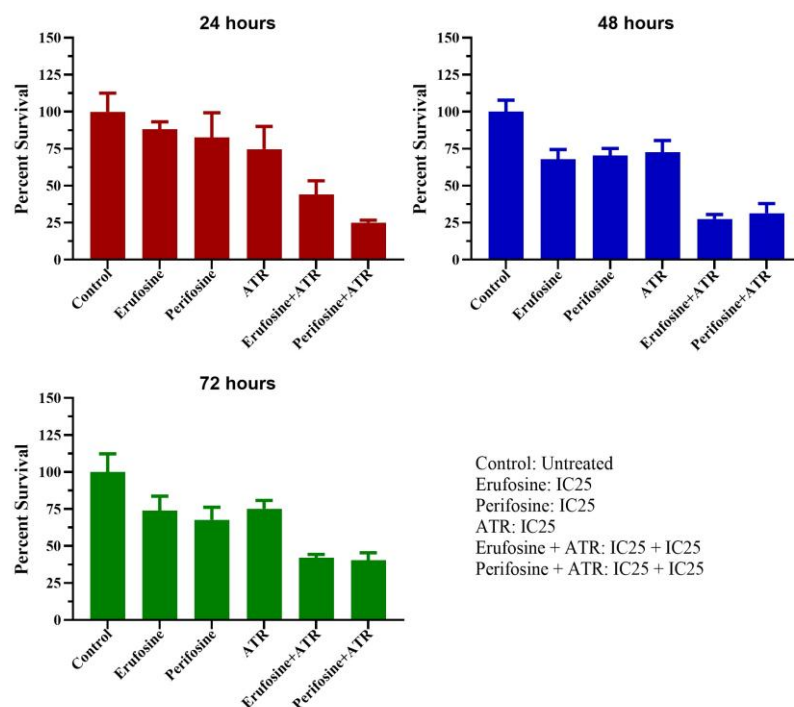


Figure 4: MTT results of breast cancer cell line (MCF-7) after treatment with selected compounds (erufosine, perifosine, ATR inhibitor). The cells were cultured and exposed to compounds as combinational treatments. Afterwards, MTT dye reduction assay was performed to evaluate the effects on cell viability.

Expressional Profiling of Genes

The cells were exposed to compounds (single and combinations) followed by evaluation of expression modifications via real-time PCR method. Single agent treatments uniformly induced the CDKN1A and CDKN1B genes in MDA-MB-231 cells. However, these effects were more intense in response to perifosine (up to 14fold) followed by erufosine (up to 11fold) and ATR inhibitor (up to 2fold). Furthermore, these inducing effects were concentration dependent as maximum up regulation of the genes was observed with IC50 concentrations of compounds. Considering the combinational effects, again there was induction of the gene in response to erufosine/ATR and perifosine/ATR combinations. Surprisingly, these agents imposed a more pronounced induction relatively as single agent when compared to combined effects of any two compounds as shown in Figure 5.

In MCF-7 cells, CDKN1A gene was highly up-regulated after erufosine exposure (up to 40fold) followed by perifosine and ATR exposures (up to 10fold). These inductions were concentration dependent as perifosine, and ATR inhibitor induced maximum up regulation with highest applied concentration (IC50). Conversely, erufosine did not follow this pattern as more pronounced effects were seen with IC25 concentration. In CDKN1B gene, there was a mild induction (up to 3fold) after exposure to erufosine and ATR inhibitor, while there were negligible effects after perifosine exposure. As far as combinations are concerned, overall induction in both genes was witnessed in MCF-7 cells. However, this induction was more pronounced in CDKN1A gene as compared to CDKN1B gene (Figure 6).

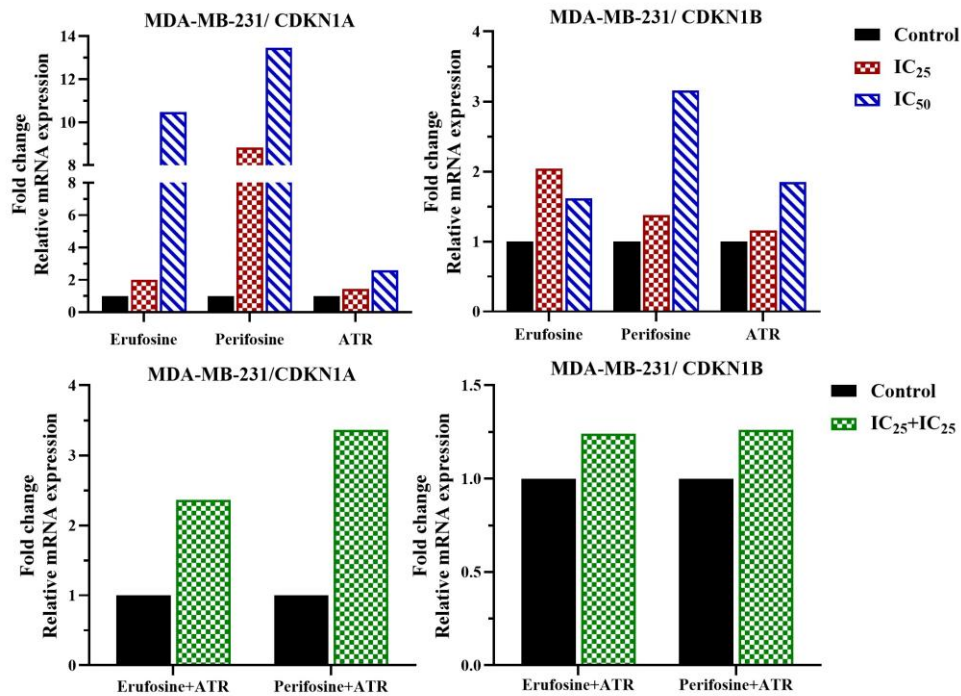


Figure 5: Expressional changes in CDKN1A and CDKN1B gene in MDA-MB-231 cells. The cells were exposed to erufosine, perifosine and ATR inhibitor as single agent or combinations followed by expression analysis by real-time PCR methodology.

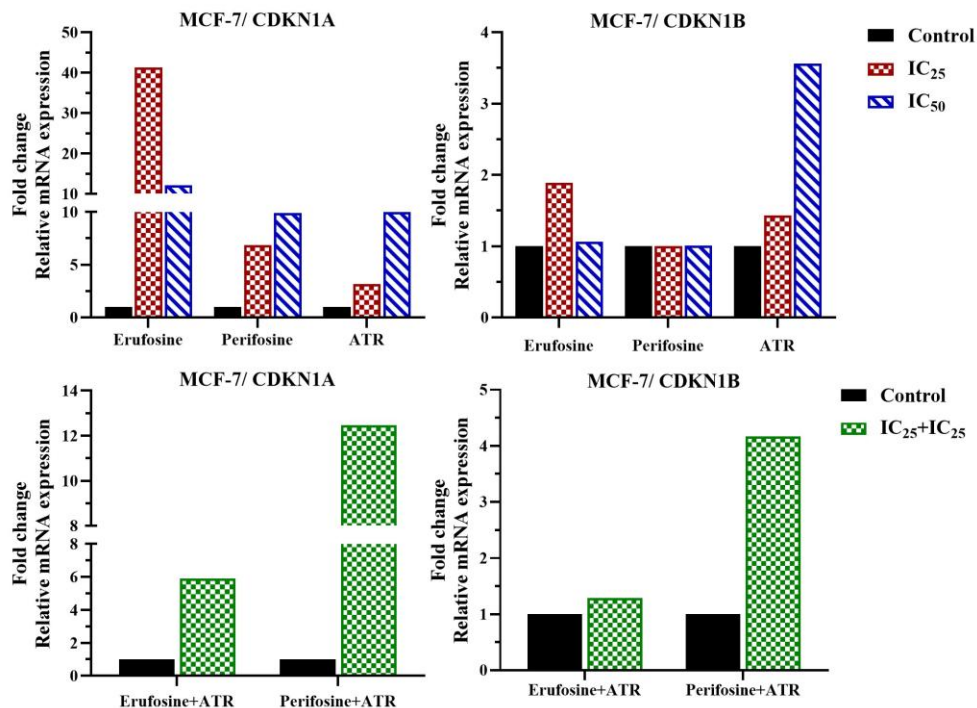


Figure 6: Expressional changes in CDKN1A and CDKN1B gene in MCF-7 cells. The cells were exposed to erufosine, perifosine and ATR inhibitor as single agent or combinations followed by expression analysis by real-time PCR methodology.

DISCUSSION

Cancer treatment is challenging with the availability of limited therapeutic options. Furthermore, treatment of cancer with available anticancer compounds imposes a huge burden of side effects in the patients. In these conditions, it is inevitable to find the novel anticancer compounds which can be used for the treatment of cancer patients with better physiological profile and safety. Considering this, the present study was designed to evaluate the effects of three compounds named erufosine, perifosine and ATR inhibitor as a single agent and in combinations. Erufosine and perifosine are ALPs and have shown substantial anticancer effects in pre-clinical settings. On the other hand, ATR inhibitor (VE-821) is a commercially available ATP competitive inhibitor. Among the selected compounds, ALPs interact with cell surface membranes, especially the lipid rafts and induce tumor cell specific lethal effects. These effects have been validated *in vitro* and *in vivo* against multiple cancers which indicate the broad-spectrum anticancer potential of the two compounds [26-29]. On the other end, ATR is a promising therapeutic target as it is involved in multiple cellular functions and facilitates responses including cell cycle progression, cell stress and DNA damages. Clinical utilization of ATR inhibitors is an open secret, and further research is being reported to highlight the utilization of these targets [30-32]. As we know, combining the anticancer compounds for clinical utilization often led to better control of tumor progression with minimal side-effects. On this ground, combining the selected ALPs and ATR inhibitor was a promising area of research. In this study, the breast cancer cell lines of different molecular subtypes (MCF-7: ER⁺/PR⁺, MDA-MB-231:ER⁻/PR⁻/HER2⁺) were exposed to the compounds as single agent and combinational treatment followed by the determination of cytotoxic profile of the compounds. Afterwards, the cells were exposed to compounds and expression change in two important cell cycle inhibitor genes (CDKN1A, CDKN1B) were evaluated by using the real time PCR method.

For the said purpose, breast cancer cell lines were cultured in 96-well plates and treated with erufosine, perifosine and ATR inhibitor alone and in combinations. Afterwards, effects on viability of cells were assessed by MTT dye reduction assay (Table 1 and 2). The results highlighted that ALPs were more potent while inhibiting viability of breast cancer cells in comparison to ATR inhibitor (Figure 1 and 2). These effects were uniform in both cancer cell lines which were distinct from each other on molecular grounds. A similar type of trend has been observed in the breast cancer cell lines treated with ATM inhibitor in combination with the selected alkyl-phospholipid [33]. As far as combinational effects are concerned, the

cells were more responsive towards the combination of alkyl-phospholipids with ATR inhibitor as more prominent inhibition of the cancer cell growth was observed after treatment with the combinations of compounds. This shows that combining the two classes with lower concentrations impose substantial antitumor effects, which can be instrumental while treating cancers with minimal side effects.

Deregulation of genetic expression is a common phenomenon observed during carcinogenic processes. Among these deregulations, pro-tumor genes are often upregulated while antitumor genes are downregulated. Among the important genes, cell cycle inhibitors like CDKN family are master regulators and often are inhibited during cancer cell progression [34, 35]. Considering the importance of this gene family, we selected the two important members (CDKN1A, CDKN1B) and evaluated the impact of selected compounds on expression profile in breast cancer cell lines. The cells were exposed to the compounds as single or combination treatments and resulting effects on expression profile were determined via real-time PCR method. When exposed to single agents, the two genes were upregulated in two cell lines. However, these induction levels vary among the cell lines and genes themselves. Specifically, CDKN1A was overall induced more prominently in both cell lines as compared to CDKN1B. In addition, the inductions were more intense in receptor negative (MDA-MB-231) cells as compared to counterparts. This shows the cell line and gene specific modifications by the selected compounds. Surprisingly, when treated with combinations of the compounds (alkyl-phospholipids and ATR inhibitor), the effects were less pronounced overall as compared to single agent treatments. Thus, there was no synergism among the compounds while inducing the CDKN family in breast cancer cells, which reflect the possible involvement of additional feedback loops working in different directions. Nevertheless, the compounds showed potential of inducing cell cycle inhibitor genes in breast cancer cells thus can be used as significant cytostatic agents.

To conclude, findings suggest that combining ALPs (erufosine and perifosine) with ATR inhibitor can be instrumental to impose anti-proliferative effects in breast cancer cells. Furthermore, the agents can deregulate the key master regulators of cell cycle (CDKN family) while imposing the cytostatic effects. Further research is required to understand the potential of these agents while using in combinations to treat breast cancer to obtain optimal results.

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