Biomedical Current Insights
DOI: https://doi.org/10.63626/fr5mby05
ISSN Online: XXXX-XXXX
ISSN Print: XXXX-XXXX

Research Article

Cytotoxic effects of alkyl-phospholipids (Erufosine and Perifosine) in combination with cell cycle check point inhibitor (ATM) in breast cancer cells

Kinzah Kanwal[1], Adeela Batool[2], Marriyam Nasim[3]

1. Medical Laboratory Technology, Riphah International University, Islamabad, Pakistan
2. Institute of Biomedical & Allied Health Sciences, University of Health Sciences, Lahore, Pakistan
3. Center of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan

Abstract

Background: Breast cancer is the most common cancer in females with continuously growing incidence over the last two decades. In advanced stages, the disease is difficult to manage and imposes a major morbidity and mortality burden. Available treatment options are limited with a moderate capacity to cure. Situation demands to look for alternative therapeutic compounds. Alkyl-phospholipids (ALPs) are attractive options, and the two latest generations of this class (erufosine and perifosine) have shown substantial anticancer potential against cancer. In addition, ATM, an important factor during DNA damage/response, has come up for its corresponding therapeutic relevancy. Combining the two above-mentioned classes can lead to effective therapeutic options against breast cancer.

Methods: Toxic effects of the selected compounds on breast cancer cell lines were determined by MTT dye reduction assay. The selected cell lines (MDA-MB-231 and MCF-7) were cultured in 96-well plates and exposed to various concentrations of the compounds for three different time intervals (24, 48 and 72 hours). Following the exposure of cells with the agents, expression modulations in CDKN family of genes were monitored at transcriptome level via real-time PCR methodology.

Results: Exposure with the ALPs and ATM inhibited cell proliferation of the two breast cancer cell lines. Overall, ALPs were more capable of inhibiting cell proliferation. Combination of ALPs and ATM worked synergistically and reduced the cellular proliferation more effectively especially in MCF-7 cells. ALPs and the selected ATM inhibitor induced the expression of CDKN family members. Overall, the induction was maximally seen in CDKN1A gene with a maximum induction level of 40fold in MCF-7 cells.

Conclusion: ALPs and ATM have the potential to inhibit proliferation of breast cancer cells and can support each other synergistically. Important cell cycle inhibitor genes like CDKN family can be induced by using above mentioned compounds.

Key Words: Cancer, Alkyl-phospholipids, Erufosine, Perifosine, ATM, Anti-proliferative, Treatment

Corresponding Author: Kinzah Kanwal
Email: kinzah.kanwal@riphah.edu.pk

INTRODUCTION

Breast cancer is the term that refers to a disease that affects the mammary glands, that often develops in the cells that line milk ducts and the lobules that feed milk to the ducts [1]. When both genders are considered, breast cancer is the second most prevalent cancer. On physiological and clinical basis, breast cancer is a disorder that has multiple identified histology and molecular subtypes each with its own etiology, risk factors, treatment response and diagnosis [2]. Each year, around 1.5 million of women (that is about 25 percent of all cancer patients) are diagnosed with breast cancer [3]. Multiple types of therapies (i.e., targeted, hormonal, radiation, chemotherapy) along with surgery are some of the treatment options that have been used to treat breast cancer patients. Treatment options are limited, mainly in advanced stages of disease. The heterogeneity of this disease, as well as the present treatment's adverse effects and limited therapeutic choices, are the major hurdles in its management [4].

Cell cycle regulation is done by the presence of specific check-points and restriction points that ensures the integrity of DNA and appropriate cellular environment for a cell to enter in the division phase [5]. These check point pathways are frequently affected in cancerous cells and triggered in response to DNA damage [6]. DNA damage response (DDR) is complex network of mechanisms that sense any damage to DNA and send out signals for further processing in order to keep the genome intact [7]. Defective DDR is one of the hallmarks of cancer,

targeting DNA repair pathways have been recognized as a viable area of study for anticancer therapy [8, 9]. Ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are two core routes through which DDR is initiated. These are protein kinases that have a wide range of physiological functions. They are mainly involved in DNA damage response regulation to survival of cell, its proliferation, cellular metabolism and differentiation [10]. When double standard breaks are made, ATM is a core transducer that phosphorylates multiple effectors in downstream pathway and plays its role in DNA damage repair and autolysis [11], while in case of single-stranded DNA (ssDNA) breaks, ATR is triggered that leads to repair this break [12].

Many chemotherapeutic medicines and radiation have an anti-tumor impact by inducing DNA damage in cancer cells; hence, stimulation of DNA damage response (DDR) pathways is regarded as a significant factor influencing treatment resistance. When some DDR pathways are inactivated, the inhibition of other DDR pathways can induce cancer-specific synthetic lethality. As a result, DDR pathways are seen as attractive options for cancer treatment [13-15]. For cancer treatment ATM and ATR are two promising therapeutic targets due to their functions in regulating responses to DNA mutilation. Chemotherapeutic activity and radiation resistance are both hampered by the activation of ATM signalling [16, 17]. Inhibition of ATM activity is crucial for improving a patient's responsiveness to anti-cancer medication. Many types of cancer have been successfully treated with chemotherapeutic medicines in combination with inhibitors of ATM/ATR or combination of these ATM/ATR inhibitors with radiation therapy.

Synthetic chemicals like alkyl-phospholipids (ALPs) have been shown to have anti-cancer and anti-proliferative properties. Perifosine is a second-generation ALP that has exhibited toxicity against a variety of cancer cell lines. It inhibits many important signal transduction pathways in human malignancies, including PI3K/AKT; which is one of the most important pathway for cancer cell proliferation [18]. Erufosine is a third-generation ALP molecule with 22 carbon additional chain and has been shown to be effective against leukaemia, multiple myeloma, breast, prostate, 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 [19].

In the present study, we investigated cytotoxic effects imposed by ATM inhibitor and erufosine/perifosine alone and in combinations against breast cancer cells. In addition, expressional analysis of three important genes of CDKN family (CDKN1A, CDKN1B and CDKN2B) were assessed by real time PCR methodology after exposing the cells with the test compounds alone and in combination for comparisons. The findings will be helpful to further evaluate potential utilization of erufosine/perifosine and ATM inhibitor as a part of combinational therapeutic approach to treat breast cancer in future.

METHODS

Cell Cultures

Human breast cancer cell lines (MDA-MB-231 and MCF-7), free of pathogenic contamination, were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamate, streptomycin (100µg/ml) and penicillin (100IU/ml). Cells were incubated at standard incubation conditions (5% CO_2, 37˚C, humidified environment).

Growth Curves of the Cells

Selected cell lines were cultured in 96-well culture plates (1000-6000 cells/well/100µl complete medium), followed by incubation for three different time points (24, 48 and 72 hours) at standard cell culture incubation conditions. At the end of each point, MTT solution (10mg/ml in PBS) was added (10µl/well) followed by another incubation period of 3 hours. The purple crystals of formazan were formed by viable cells. These crystals were dissolved by adding 50µl/well of DMSO. Optical densities were measured by microplate reader (540/690 nm) and growth curves were generated with time intervals on X-axis and absorbance values on Y-axis.

MTT Dye Reduction Assay (Single Agent Exposure)

MTT dye reduction assay is a colorimetric assay used to assess cell viability and proliferation. To perform this assay, selected breast cancer cell lines (MDA-MB-231 and MCF-7) were seeded at pre-determined cell densities (4000cells/well) in 96-well plates (100µl medium/well). Plates were incubated overnight at standard incubation conditions (5% CO_2, 37˚C, humidified environment) and cells were allowed to grow and attach to the surface of the well. Next day, cells were exposed with various concentrations (1.56-50µM) of erufosine, perifosine and ATM inhibitor (Abcam, Ab219506) for three time points i.e., 24, 48 and 72 hours. Following the exposure intervals, MTT solution was added, and optical densities were determined as explained above. Cell survival rates were calculated as percentage of control (untreated) while inhibitory concentrations (IC) were calculated by GraphPad Prism 10.3 software.

MTT Dye Reduction Assay
(Exposure with Combinations of Compounds)

The cells were seeded in 96-well plates as described above and were treated with combination of ATM inhibitor and erufosine/perifosine as per inhibitory concentrations (IC) generated from above-mentioned experiment (Table 2) for three-time points i.e., 24, 48 and 72 hours. Following the treatment intervals, MTT assay was performed, and viable cell population was determined as explained above. In both cases (single agent or combinational treatment), the assays were performed in triplicate and the results were obtained by at least two independent experiments. Results were compared with untreated control cells growing in parallel.

Expressional Profiling
Treatment with selected compounds

The selected breast cancer cell lines were seeded (200,000cells /2ml complete medium/well) in 6-well plates. Plates were incubated overnight at standard incubation conditions (5% CO_2, 37˚C, humidified environment) and cells were allowed to grow and attach to the surface of the well. Next day, the cells were exposed with various inhibitory concentrations of erufosine, perifosine and ATM alone (IC25 and IC50) and in combinations (IC25+IC25) (Table 3). After 48 hours of incubation, media was discarded from each well, the cells were washed with 500 µl of PBS, trypsinized and cell palettes were collected by centrifugation at 3000rpm for 5 minutes and stored at -80˚C.

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

Cell Lines	Erufosine (µM)	Perifosine (µM)	ATM (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 + ATM		Perifosine + ATM	
	Erufosine	ATM	Perifosine	ATM
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			Combinational Treatment
MDA-MB-231	Erufosine IC25	Perifosine IC25	ATM IC25	Erufosine IC25+ ATM IC25
	Erufosine IC50	Perifosine IC50	ATM IC50	Perifosine IC25+ ATM IC25
MCF-7	Erufosine IC25	Perifosine IC25	ATM IC25	Erufosine IC25+ ATM IC25
	Erufosine IC50	Perifosine IC50	ATM IC50	Perifosine IC25+ ATM IC25

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the control and treated cells by using commercially available extraction kit (Thermo Fisher Scientific: Cat#K0731). The extracted RNA was quantified and stored at -80°C immediately for further use. A total of 1000ng extracted RNA/samples was used to synthesize cDNA by using the reverse transcriptase kit by Thermofisher scientific (cat # K1622). To verify the prepared cDNA samples, PCR based amplification of a reference gene (HPRT1) was performed. Afterwards, a total of 10µl of the amplified product and was loaded on 2.5% agarose gel and visualized by gel electrophoresis.

Primer Designing and Optimization

Primers of the selected genes (CDKN1A, CDKN1B and CDKN2B) were designed by choosing gene sequence from NCBI Genbank and using Primer3 software. Primer sequences are given in Table 4. The designed primers were optimized by gradient PCR methodology in which three different annealing temperatures were tested (56, 59 & 62°C). The amplified products were analyzed by using gel electrophoresis. The annealing temperature was selected by observing the quality of bands on the gel.

Real Time PCR

Quantitative real time PCRs (qRT-PCRs) were performed for the selected genes (CDKN1A, CDKN1B and CDKN2B) by using SybrGreen florescence dye (Thermo Fisher Scientific, Cat#K0221), prepared cDNA samples from the two breast cancer cell lines treated with different concentrations of the compounds alone or in combination along with gene specific primers. All the samples were amplified in triplicate, while Quantstudio3™ machine was used for real-time PCR. For normalization of qRT-PCRs data, the expression levels of (HPRT1) gene were used as reference and the fold changes of the selected genes were calculated by the Livak (2-ΔΔCT) method.

Data Analysis

Cytotoxicity data generated from MTT assay was presented as frequency percentages. For real-time PCR analysis, fold changes were calculated by Livak $2^{-\Delta\Delta CT}$ method by comparing Cq values of treated and untreated control samples.

Table 4: Primer sequence for amplification of selected genes

Gene	Forward	Reverse
CDKN1A	GCTTCATGCCAGCTACTTCC	CTGTGCTCACTTCAGGGTCA
CDKN1B	CCGGCTAACTCTGAGGACAC	TGCAGGTCGCTTCCTTATTC
CDKN2B	GACCGGGAATAACCTTCCAT	AAACCCTGAAAAGCAAACGA
HPRT1	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT

RESULTS

Growth Curve

MTT dye reduction assay was done to optimize the cell densities for subsequent experiments. For this purpose, the cells were cultured in 96-well culture plates (1000-6000 cells/well/100µl medium) for 24, 48 and 72 hours at standard cell culture incubation conditions. After adding DMSO, formazan crystals produced purple color of different shades, showed the presence of viable cells. Optical densities were determined by an ELISA plate reader (540/690nm) and growth curves were generated with number of cells on X-axis and absorbance values on Y-axis shown in Figure 1. Continuous proliferation of breast cancer cell lines (MDA-MB-231 and MCF-7) was observed for 4000cells/well for all three-time intervals, while for further higher numbers (5000 and 6000 cells/well), there was a clear reduction in proliferation especially for 72 hours. This reduction in later time intervals was likely due to overabundance of cell population having limited space for attachment and further growth. In addition, lack of nutrients and production of waste could be additional limiting factors in case of these higher cell numbers/well.

Cytotoxic Effects of Erufosine, Perifosine, ATM inhibitor in breast cancer cell lines

Erufosine, perifosine and ATM inhibitor induced substantial anti-proliferative effects in MDA-MB-231 and MCF-7 cells (Figure 2). The most prominent cytotoxic effects were observed in MCF-7 cells; this in turn shows that triple positive breast cancer cells are more prone towards exposure of these compounds. The effects were almost time dependent as the more inhibition of cell viability was observed for a longer period of exposure with the compounds. Additionally, the effects were concentrations dependent (beyond 3.10µM) as more inhibition of cellular growth was witnessed with increasing concentrations. Overall, the effects in response to erufosine and perifosine were comparable, while ATM inhibitor induced fractionally low effects in comparison to ALPs.

Cytotoxic Effects of Erufosine, Perifosine, ATM inhibitor in combinations

As far as effects of the combinations are concerned, almost no synergistic impact of growth inhibition was observed while combining the two alkyl-phospholipids (erufosine and perifosine) in combination with ATM in MDA-MB-231 cells. However, fractional synergism was observed for ATM and erufosine combination only for 24 hours, which shows a short-term better control when the two compounds are combined. A prominent

synergism was observed in MCF-7 cells as compared to MDA-MB-231 cells. Synergistic effects of ALPs in combination with ATM were continuous in MCF7 cells for the three-time intervals. This indicates that combination of erufosine and perifosine with ATM inhibitor is more effective against triple positive breast cancer cells (Figure 3).

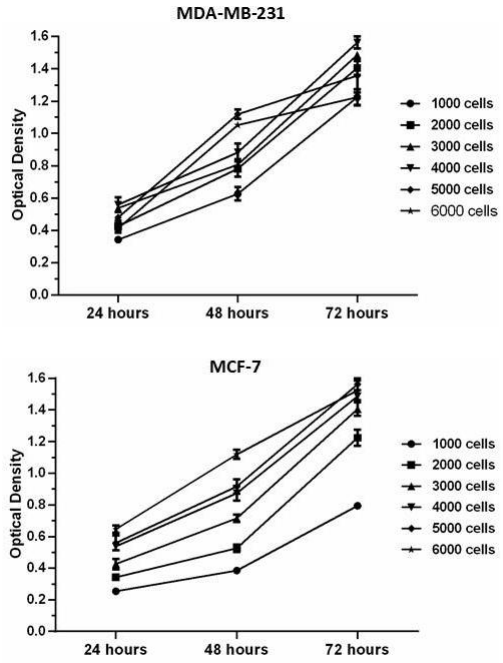

Figure1: Growth curve generation for three different time points

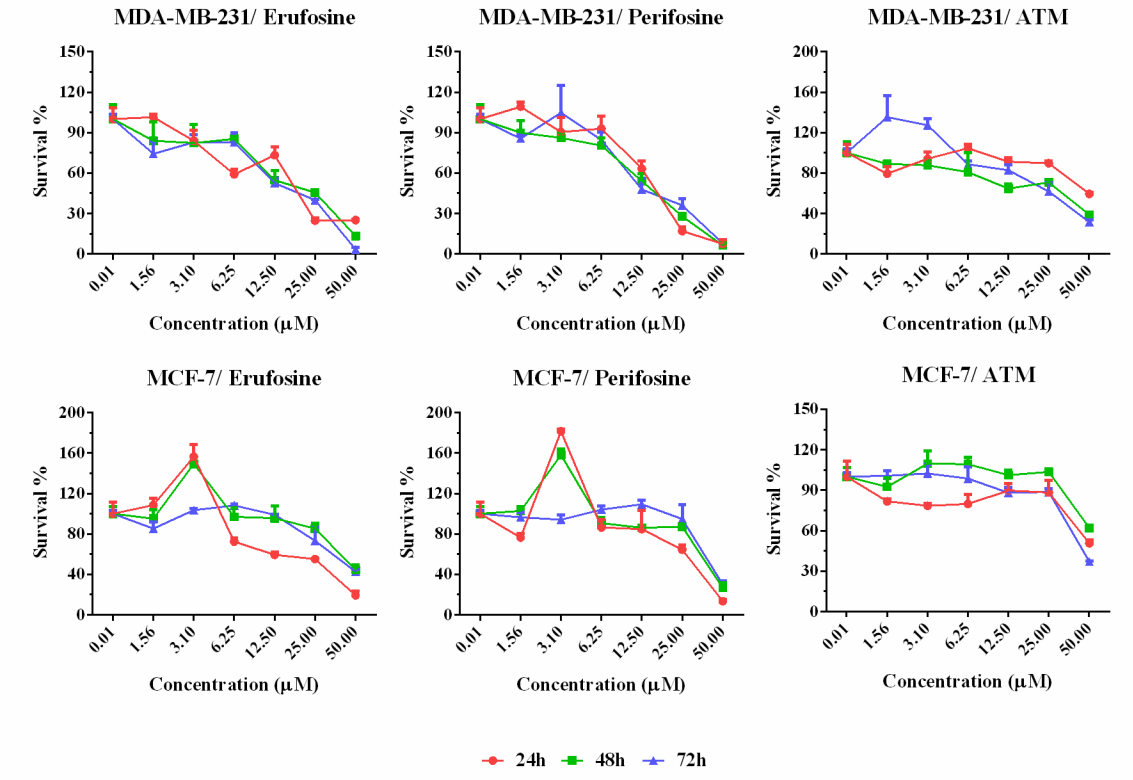

Figure 2: MTT results of breast cancer cell lines (MDA-MB-231 and MCF-7) after treatment with selected compounds (erufosine, perifosine, ATM inhibitor)

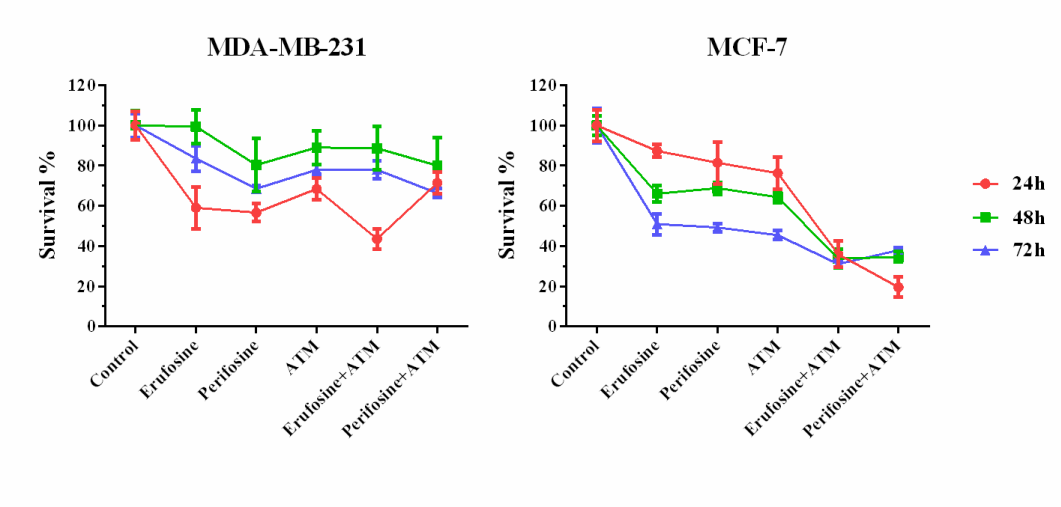

Figure 3: MTT results of breast cancer cell lines (MDA-MB-231 and MCF-7) after treatment with selected compounds (erufosine, perifosine, ATM inhibitor) alone and in combinations

Expressional Analysis of CDKN1A and CDKN1B Genes in MDA-MB-231 cells

A concentration dependent induction of CDKN1A gene was observed in MDA-MB-231 breast cancer cell line after exposure with selected ALPs and ATM inhibitor. An induction of 10.5, 13.5 and 10.3fold was observed in response to IC50 for erufosine, perifosine and ATM inhibitor respectively. The induction of CDKN1B gene was inversely proportional to the applied concentrations for erufosine i.e., maximum induction (2fold) at low concentration (IC25) while declining slightly (1.6fold) at high concentration (IC50). Perifosine and ATM inhibitor induced expression of CDKN1B gene in dose dependent format. The two compounds induced minimal effects at lower concentration (IC25) as shown by maximum induction of 1.9fold. At higher concentration (IC50), perifosine and ATM inhibitor induced the expression by 3.2 and 2.8fold respectively. The combination of erufosine (IC25) with ATM inhibitor induced 1.9fold expression, while combination of perifosine (IC25) with ATM (IC25) inhibitor induced relatively more effective expression of almost 3.4fold in CDKN1A gene. Overall, reduced modulation by combinational approach was observed in CDKN1B gene as compared to CDKN1A gene in MDA-MB-231breast cancer cell line (Figure 4).

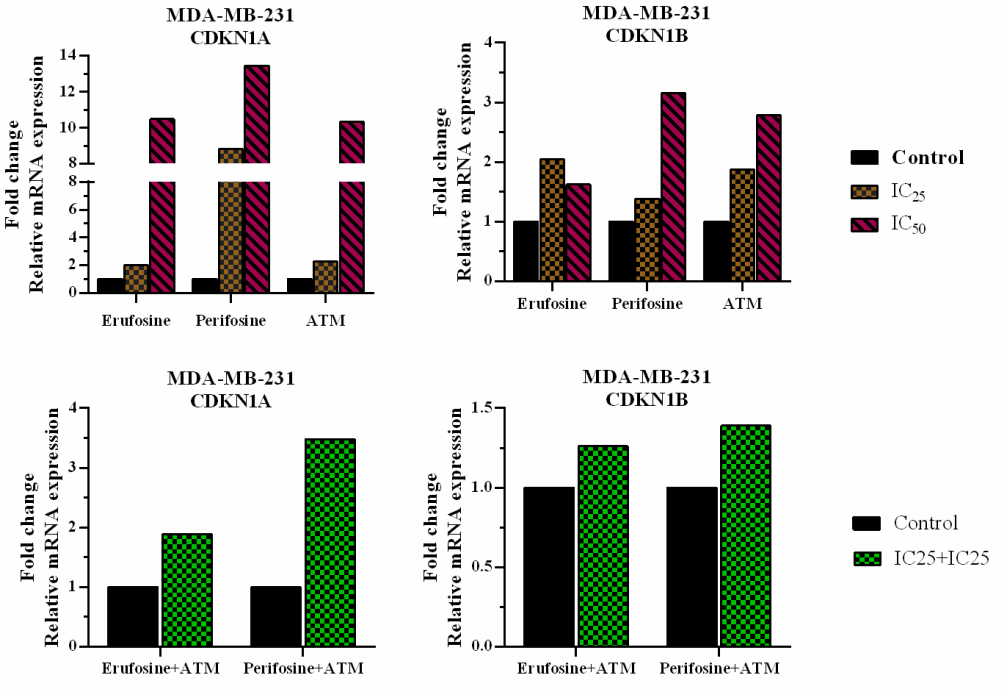

Figure 4: Expressional changes in CDKN1A and CDKN1B gene in MDA-MB-231 cells

Expressional Analysis of CDKN1A, CDKN1B and CDKN2B Genes in MCF-7 cells

Exposure with the selected compounds induced more substantial expressional modulations in MCF-7 cells as compared to MDA-MB-231 cells. In MCF-7 cell line, the gradual decline in induction of CDKN1A gene was observed with increasing ICs of erufosine. Precisely, maximum induction (41.4fold) was observed at IC25 while lowest induction (12.1fold) was observed at IC50 exposure. In response to perifosine, the expression was dose dependent as the effects intensified (6.9 and 9.9fold) with increasing concentration (IC25 and IC50 respectively). The CDKN1A expression in response to ATM inhibitor is quite similar with erufosine. Maximum activation (31.3fold) was observed with IC25, while reduced induction (7.8fold) was witnessed with higher concentration (IC50). Overall, it can be claimed that the compounds altered the expression levels of CDKN1A more effectively in MCF-7 cells.

Almost a uniform response in expressional pattern of CDKN1B gene was observed when MCF-7 cells were exposed to the selected ALPs i.e., erufosine and perifosine. The maximum effect (1.9fold) was observed at IC25 concentration of erufosine and beyond this the effect was diluted as shown by lower expression level (1.1fold). Perifosine induced negligible change in expression levels of CDKN1B gene at both tested concentrations when compared to the untreated control cells. The pattern of expression of CDKN1B gene in response to ATM inhibitor was quite like that of erufosine. Maximum induction was 3.5fold at IC25 while it declined to 1.6fold at higher concentration IC50. All in all, CDKN1B gene expression was minimally affected in response to tested compounds.

A different expressional pattern of CDKN2B gene was observed when MCF-7 cells were exposed to the selected ALPs and ATM inhibitors as compared to the results of CDKN1A and CDKN1B genes. Erufosine induced a negligible change in the expression level of CDKN2B gene at lower concentration (IC25) when compared to the untreated control cells. In contrast, a substantial inhibition of -2.7fold was observed at higher concentration (IC50). Perifosine and ATM inhibitor inhibited expression of CDKN2B gene in dose dependent format. To be precise, there was a moderate inhibition at lower concentration (IC25) while the maximum inhibition of -4.1 and -3.5fold were observed at higher concentration (IC50).

Combination of selected ALPs with ATM inhibitor induced relatively effective alterations in CDKN1A gene in MCF-7 cells as compared to MDA-MB-231 cells. The maximum observed induction was 8.7fold when exposing the cells to the combination of erufosine (IC25) with ATM (IC25) inhibitor. Overall, a less effective modulation by combinational approach was observed in CDKN1B gene as compared to CDKN1A gene in both breast cancer cell lines. In MCF-7 cell line, the expression of the CDKN2B gene was inhibited at all tested combinations of erufosine and perifosine with ATM inhibitor. Overall modifications in the genes in response to tested compounds in MCF-7 cells are shown in Figure 5.

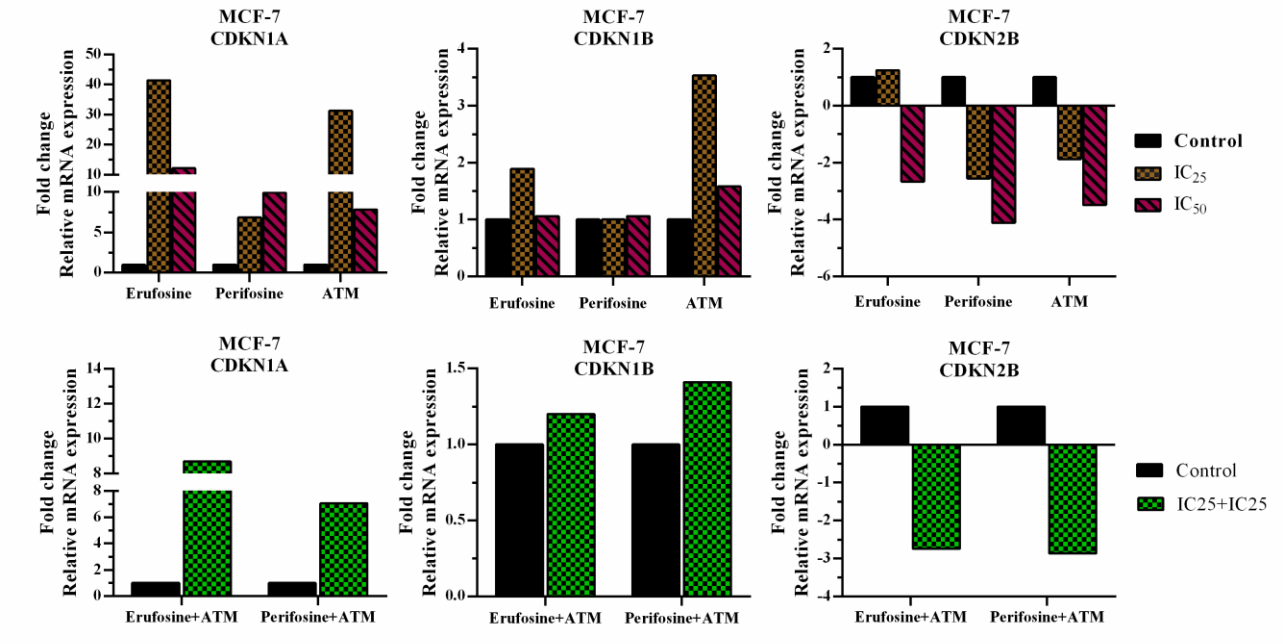

Figure 5: Expressional changes in CDKN1A, CDKN1B and CDKN2B gene in MCF-7 cells

DISSCUSION

Despite significant scientific achievements, breast cancer remains the most frequent cancer in women globally and is a serious public health issue. Female breast cancer is becoming more common every year [20]. According to literature, early screening and detection of breast cancer combined with proper treatment could greatly decrease death rates due to breast cancer. The heterogeneity of this disease, as well as the present treatment's adverse effects and limited therapeutic choices, are the major hurdles in its management [4, 21]. To enhance the present therapeutic regimens, more effective solutions are urgently needed. Immunotherapy, gene therapy, and combinational treatment approaches are among the strategies being researched to overcome the hurdles [22].

Many chemotherapeutic medicines and radiation have an anti-tumor impact by inducing DNA damage in cancer cells. Combinational approach of chemotherapy for effective anticancer effects is a well-accepted fact as it provides an opportunity to control the disease burden by using lower dosages of the compounds with lesser side-effects. For cancer treatment, ATM and ATR are the two promising therapeutic targets due to their vital cellular functions and facilitators in regulating responses to DNA changes. Inhibition of ATM activity is crucial for improving a patient's responsiveness to anti-cancer medication. Many types of cancer have been successfully treated with chemotherapy in combination with inhibitors of ATM/ATR [14, 16, 17]. All is all, ATM and ATR are effective therapeutic targets, and their corresponding inhibitors are being investigated intensively.

ALPs, targeting cell membrane phospholipids, have anti-cancer and anti-proliferative properties against various cancers. Perifosine, a 2nd-generation ALP, inhibits key signal transduction pathways, including PI3K/AKT, and shows clinical effectiveness in combination treatments [18, 23]. Erufosine (3rd-generation ALP), is metabolically more stable, with lower hemolytic activity and less bone marrow damage, able to penetrate the blood-brain barrier and can be administered intravenously to obtain clinically meaningful doses that are not achievable with other ALPs [19]. Combining synthetic ALPs with ATM/ATR inhibitors is considered an attractive area of research.

In this study, we investigated the cytotoxic effects imposed by ATM inhibitor and erufosine/perifosine alone and in combinations in breast cancer cells. For this purpose, two BC cell lines (MDA-MB-231 and MCF-7) were selected and grown in 96-well plates in cell culture settings and exposed to various concentrations of erufosine, perifosine and ATM inhibitor alone or in combinations, followed by assessment of proliferation by MTT dye reduction assay as shown in Table 1 and 2. Based on the results obtained, it is clear that ALPs are more effective in inhibiting the proliferation of breast cancer cells as compared to ATM inhibitor. One of the potential reasons behind this phenomenon could be that ALPs interact with cellular lipids and affect multiple intracellular downstream signaling cascades. In comparison, ATM inhibitors specifically interact with their targets and induce limited overall reaction. Furthermore, development of resistance mechanisms or negative feedback loops in response to ATM inhibitor exposure cannot be figured out. As far as combinations are concerned, the approach was more effective against triple positive breast cancer cells (MCF-7) as compared to triple negative cells (MDA-MB-231). Other than receptor status, further cellular differences among the two cell lines need to be considered before just focusing on molecular subtype dependent response of breast cancer cells.

As we know, genes are the basic moderators of cellular functions, thus, to figure out mechanistic reasoning behind the observed anti-proliferative effects, a key cell cycle related gene family (CDKN) was taken up in this study. This family is comprised of four major cell cycle inhibitors which play a crucial role in cell cycle. Their expression levels can affect cell proliferation and divisions efficiently. As explained in methods, breast cancer cells were exposed to ALPs and ATM in single or combinations and expression levels of four CDKN family members (CDKN1A, CDKN2A, CDKN1B, CDKN2B) were identified via real-time PCR methodology. During the primer optimization step, CDKN2B expression was only seen in MCF-7 cells while CDKN2A did not show any expression in both selected breast cancer cell lines. As per available literature, the two members did not express in all breast cancer cell lines. Furthermore, expression of transcript variant is also possible, against which our designed primer was not compatible. To figure out the exact situation, primers against all known transcript variants of CDKN2A and CDKN2B need to be designed and check for potential expression of these two genes in the selected breast cancer cell lines.

For other two members (CDKN1A and CDKN1B), a clear expression was noticed in both breast cancer cell lines. In response to ALPs, a substantial expression (up to 14fold) was noticed in MDA-MB-231 cells. The effects were more pronounced in MCF-7 cells as a maximum of ~40fold induction was observed in response to ALPs exposure. Interestingly, the results were in line with MTT data where MCF-7 cells were more sensitive towards ALPs exposure. In comparison, the ATM inhibitor was more effective and induced a maximum of ~30fold induction in MCF-7 cells. As far as CDKN1B gene is concerned, overall, a moderate induction (up to 4fold) was observed in both cell lines. Interestingly, the ATM inhibitor was more influencing in this condition in comparison to ALPs. With

combinations of ALPs and the ATM inhibitor at lower concentrations (IC25), a substantial induction of CDKN1A and CDKN1B gene was observed in breast cancer cell lines, particularly MCF-7 cells. This in turn indicates that by using lower dosage of the ALPs and ATM inhibitor, a substantial induction of a vital cell cycle inhibitor (CDKN1A) is possible in breast cancer cells, and this is a valuable hope from clinical perspective. All in all, data suggests that combinations of ALPs and the ATM inhibitor can work synergistically to impose anti-proliferative effects. With further studies to support these findings, ALPs and ATM inhibitors can be a promising combinational therapy to control breast cancer overtime. In this context, existing FDA approved ALPs (Perifosine) can be considered immediately, while an improved ALP (Erufosine) is a promising futuristic compound.

REFERENCES

1. Kabel AM, Baali FH. Breast cancer: insights into risk factors, pathogenesis, diagnosis and management. Breast Cancer Res Treat. 2015;3(2):28-33.
2. Pashayan N, Antoniou AC, Ivanus U, Esserman LJ, Easton DF, French D, et al. Personalized early detection and prevention of breast cancer: ENVISION consensus statement. Nat Rev Clin Oncol. 2020;17(11):687-705.
3. Sun YS, Zhao Z, Yang ZN, Xu F, Lu HJ, Zhu ZY, et al. Risk Factors and Preventions of Breast Cancer. Int J Biol Sci. 2017;13(11):1387-97.
4. Akram M, Iqbal M, Daniyal M, Khan AU. Awareness and current knowledge of breast cancer. Biol Res. 2017;50(1):33-.
5. Bertoli C, Skotheim JM, De Bruin RA. Control of cell cycle transcription during G1 and S phases. Nat Rev Mol Cell Biol. 2013;14(8):518-28.
6. Visconti R, Della Monica R, Grieco D. Cell cycle checkpoint in cancer: a therapeutically targetable double-edged sword. J Exp Clin Cancer Res. 2016;35(1):1-8.
7. Qiu Z, Oleinick NL, Zhang J. ATR/CHK1 inhibitors and cancer therapy. Radiother Oncol. 2018;126(3):450-64.
8. Gavande NS, VanderVere-Carozza PS, Hinshaw HD, Jalal SI, Sears CR, Pawelczak KS, Turchi JJ. DNA repair targeted therapy: The past or future of cancer treatment? Pharmacol Ther. 2016;160:65-83.
9. Ma J, Setton J, Lee NY, Riaz N, Powell SN. The therapeutic significance of mutational signatures from DNA repair deficiency in cancer. Nat Commun. 2018;9(1):1-12.
10. Weber AM, Ryan AJ. ATM and ATR as therapeutic targets in cancer. Pharmacol Ther. 2015;149:124-38.
11. Choi M, Kipps T, Kurzrock R. ATM mutations in cancer: therapeutic implications. Mol Cancer Ther. 2016;15(8):1781-91.
12. Lin W-Y, Brock IW, Connley D, Cramp H, Tucker R, Slate J, et al. Associations of ATR and CHEK1 single nucleotide polymorphisms with breast cancer. PLoS One. 2013;8(7):e68578.
13. Bouwman P, Jonkers J. The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. Nat Rev Cancer. 2012;12(9):587-98.
14. Curtin NJ. DNA repair dysregulation from cancer driver to therapeutic target. Nat Rev Cancer. 2012;12(12):801-17.
15. Oda K, Okada J, Timmerman L, Rodriguez-Viciana P, Stokoe D, Shoji K, et al. PIK3CA cooperates with other phosphatidylinositol 3′-kinase pathway mutations to effect oncogenic transformation. Cancer Res. 2008;68(19):8127-36.

16. Hosoya N, Miyagawa K. Targeting DNA damage response in cancer therapy. Mol Cell. 2014;105(4):370-88.
17. Matt S, Hofmann TG. The DNA damage-induced cell death response: a roadmap to kill cancer cells. Cell Mol Life Sci. 2016;73(15):2829-50.
18. Kaley TJ, Panageas KS, Mellinghoff IK, Nolan C, Gavrilovic IT, DeAngelis LM, et al. Phase II trial of an AKT inhibitor (perifosine) for recurrent glioblastoma. J Neurooncol. 2019;144(2):403-7.
19. Ansari SS, Akgün N, Berger MR. Erufosine increases RhoB expression in oral squamous carcinoma cells independent of its tumor suppressive mode of action-a short report. Cell Oncol (Dordr). 2017;40(1):89-96.
20. Bilani N, Zabor EC, Elson L, Elimimian EB, Nahleh Z. Breast cancer in the United States: a cross-sectional overview. J Cancer Epidemiol. 2020;2020.
21. Wang L. Early diagnosis of breast cancer. Sensors. 2017;17(7):1572.
22. Shafi S, Khan S, Hoda F, Fayaz F, Singh A, Khan MA, et al. Decoding novel mechanisms and emerging therapeutic strategies in breast cancer resistance. Curr Drug Metab. 2020;21(3):199-210.
23. Ríos-Marco P, Marco C, Gálvez X, Jiménez-López JM, Carrasco MP. Alkylphospholipids: An update on molecular mechanisms and clinical relevance. Biochim Biophys Acta Biomembr. 2017;1859(9):1657-67.

Ethics Approval: Not Applicable
Author Contributions: KK executed experiments and drafted manuscript. AB and MN performed data analysis and figures preparation.
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.