Biomedical Current Insights
DOI: https://doi.org/10.63626/5pa72886
ISSN Online: XXXX-XXXX
ISSN Print: XXXX-XXXX

Research Article

Effects of synthetic alkyl-phospholipid (Perifosine) on survival and cell cycle relevant genes of hepatocellular carcinoma cells

Muhammad Masood[1], Afraz Numan[1], Saqib Mahmood[2]
1. Institute of Biomedical and Allied Health Sciences, University of Health Sciences, Lahore, Pakistan
2. Allied Health Sciences and Nursing, NUR International University, Lahore, Pakistan

Abstract
Background: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality globally, particularly in Asia. Limited treatment options highlight the needs for novel therapies. Alkyl-phospholipids (ALPs) offer a promising alternative to traditional chemotherapy by targeting cell membrane integrity and signaling pathways rather than DNA replication. Perifosine, a synthetic ALP, has shown cytotoxic effects in cancer cells, but its mechanisms on cell cycle regulation in HCC require further investigation.
Methods: This *in vitro* study evaluated the effects of perifosine on HepG2 liver cancer cells. Cytotoxicity of perifosine was assessed using the MTT assay while applying various concentrations over 24-, 48-, and 72-hours intervals. Gene expression analysis of cell cycle regulator genes (CCNA1, CCNA2, and CCND1) was conducted using qRT-PCR after treating the HepG2 cancer cells with perifosine at IC25, IC50, and IC75 concentrations for 48 hours.
Results: Perifosine demonstrated time and dose dependent cytotoxicity, with increased cell death at higher concentrations and prolonged exposure. Perifosine exposure imposed discrete expression modifications in CCNA1, CCNA2 and CCNDI genes. Maximum de-regulation was observed for CNNA1 (8fold) and CCNA2 (-3.5fold) in response to perifosine exposure.
Conclusion: Perifosine exhibited cytotoxic effects and imposed de-regulation in cell cycle related genes in liver cancer cells.
Key Words: Hepatocellular carcinoma, Perifosine, Cytotoxic, Cell cycle, Cyclins

Corresponding Author: Saqib Mahmood
Email: saqib.mahmood@fmhcmd.edu.pk

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major global concern and one of the major contributors to cancer-related deaths across the globe with limited treatment options. It is the most prevalent liver cancer subtype and represents 75% of all cases of liver cancer. According to GLOBOCAN estimates, liver cancer is listed as the 6th most prevalent types of cancer and is the 3rd most common cause of cancer mortality [1]. In 2020, 72.5% of all the reported liver cancer cases and 73.3% of liver cancer mortality were reported from Asian countries [2]. HCC are typically treated with chemotherapy and immunotherapy. Synthetic ALPs present a promising alternative, potentially offering targeted treatment with reduced toxicity and side effects [3].

Cancer is a complex disorder characterized by many genetic and molecular changes that promote unchecked cell proliferation. Dysregulation of the cell cycle, a strictly controlled precise mechanism that ensures normal cell division and maintain genetic integrity across cellular generations, is an important hallmark of cancer [4,5]. There are many factors which regulate the cell cycle, but two important key regulators are cyclins and cyclin-dependent kinases (CDKs), which control the cell cycle processes through its different phases. Tumor proliferation is often caused by disruptions of the cell cycle regulators, such as loss of CDK inhibitors or overexpression of cyclins or CDKs [6]. In cancer, the regulatory network can get disturbed, thus allowing abnormal cell division to take place. For example, overexpression of cyclins or CDKs might promote cell cycle advancement despite the lack of stimuli for cell growth. Conversely, failure in expression of CDK inhibitors might interfere with key checkpoints, allowing cancer cells to continue replicating [7].

Given the central role of the cell cycle in cancer development, targeted therapeutic strategies are being developed to exploit these dysregulated pathways. Among these strategies are the alkyl-phospholipids (ALPs). It is a novel class of anticancer drugs which target the cell cycle. ALPs are synthetic substances that structurally resemble natural phospholipids but possess some alterations that give

their anticancer qualities. Structural modifications in various members of ALPs have generated new compounds with reduced side-effects and high specificity overtime. Contrary to conventional chemotherapeutic drugs, which mainly target mitotic spindle assembly or DNA replication, ALPs work by interfering with signaling networks and cell membrane integrity and cellular processes like apoptosis, cell cycle regulation and signal transduction pathways [8]. This membrane-targeted mode of action is especially interesting since it gets around some of the typical defense's tumors build up against DNA-damaging agents. Perifosine belongs to 2nd generation of ALPs that have been produced over time and has unique structural characteristics and therapeutic applications [9].

The mechanistic hypotheses for ALPs share a common factor: their presence in the cell membrane. One hypothesis posits that ALPs alter membrane particulate size and distribution [10]. Another assumption suggests that ALPs cause biological and physical disruptions, obstructing phospholipid metabolism, cell division, and associated signaling, while promoting apoptosis through various pathways [11]. Additionally, ALPs may integrate into the membrane and undergo endocytosis via lipid rafts with the help of the ATP-dependent enzyme Flippase, contributing to cell lysis [12]. Perifosine works by targeting the pleckstrin homology domain of Akt, blocking its membrane translocation however clinical validation is still needed to confirm this hypothesis [13].

Among the cell cycle associated genes, there are various members with discrete functions. CCNA1 (Cyclin A1) is involved in controlling the G1 and G2 checkpoints by associating with CDK2 and CDC2 kinases, thus facilitating the progression through these phases. CCNA2 (Cyclin A2) activate CDK2 kinase and promotes the advancement of the cell cycle, which drives the progression from the G1 phase to S phase and from G2 to M phase. Cyclin D1, or CCND1, interacts with the Rb protein to control the progression of the cell cycle and is necessary for the transition from the G1 phase to S phase [14]. Alterations in either expression or structure of CCND1 are commonly observed in various neoplasia, highlighting its role in cancer development. Studying the effects of synthetic ALPs on hepatocellular carcinoma cells, especially on cell cycle-relevant genes, is of vital significance. The present study was designed to understand the potential effects of perifosine in HCC cells and corresponding implications on cell cycle related genes.

METHODS

Cell Culture and Perifosine

HepG2 (a human liver cancer cell line) cells, obtained from Center of Excellence in Molecular Biology (CEMB), Punjab University, were grown in DMEM medium containing 10% fetal bovine serum (FBS), 2mM L-glutamine, and antibiotics (100 µg/ml streptomycin, 100 IU/ml penicillin) to inhibit microbes. In 5% CO2 atmosphere cells were incubated at 37°C passaged biweekly to ensure logarithmic growth. An alkyl-phospholipid "Perifosine" was selected for the study and was purchased from Sigma Company (Sigma Aldrich, Cat#SML0612).

Cytotoxicity of Perifosine

To assess the toxic effects of this compound, the MTT dye reduction assay was performed. In 96-well plates HepG2 cells were seeded at 3000 cells per well and were treated with perifosine at concentrations ranging from 0.78-50 µM, and incubated for 24, 48, and 72 hours. After each interval, the MTT solution poured into each well and incubation of 3 hours was given. To resolve formazan crystals, 50µl of DMSO was add to each well then optical density was recorded at 560/690 nm wavelength via ELISA reader. The inhibitory concentration (IC) was calculated with GraphPad Prism 6 software.

RNA Extraction and Reverse Transcription

In 6-well plates, HepG2 cells were seeded maintaining 150,000 cells per well for expressional analysis, each plate was treated with IC25, IC50, and IC75 concentrations of perifosine for 48 hours, while the IC values were calculated based on MTT assay results. Utilizing the RNeasy Mini Kit (Qiagen, Cat# 74104), RNA was extracted, and its concentration along with purity were measured using Nanodrop ND2000 spectrophotometer. Complementary DNA (cDNA) was prepared via RevertAid cDNA Synthesis Kit (Thermo-Scientific, Cat#K1622) using final 200ng of RNA with the following incubation conditions, annealing at 42°C for 60 minutes, extension at 72°C for 5 minutes. For validation of cDNA synthesis, PCR amplification was performed using GAPDH.

Primer Designing and Real Time PCR

Primers for the target genes were designed using the sequences from NCBI database using Primer3Plus software. The sequences of the CCNA1 primers were 5′-TAGACACCGGCACACTCAAG-3 (forward stand), 5′-CTACCAGCATAGGGGAAACTGT-3 (reverse stand), with 312 bp amplicon size. For CCNA2, the sequences were 5′-CCTCCTTGGAAAGCAAACAG-3′ (forward stand), and the reverse was 5′-TCAAGAGGGACCAATGGTTT-3′ (reverse stand), with 173 bp product size. For CCND1, primer sequences were 5′-GGGGGCGTAGCATCATAGTA-3′(forward), and 5′-

GTGGTGGCACGTAAGACACA-3′ (reverse stand), with 114 bp amplicon size. Expressional analysis was done via real-time PCR using SYBR Green qPCR Master and cDNA templates. The data was normalized using housekeeping gene (HPRT). Livak method (2^ΔΔCT) was employed to determine the relative expression of genes.

RESULTS

Perifosine Mediated Cytotoxic Effects in Liver Cancer Cells

HepG2 cells were exposed to varying concentrations (0.75-50µM) of perifosine over 24, 48, and 72 hours. Cytotoxic effects were evaluated using the MTT assay, with results analyzed via GraphPad Prism 6. The compound exhibited concentration- and time-dependent cytotoxicity, with increasing concentrations leading to greater toxicity (Figure 1). Furthermore, the effects were more prominent over longer time intervals (48 and 72 hours) as there were greater inhibitory effects on the similar concentrations applied. Thus, IC values decreased over time, indicating enhanced cytotoxicity with prolonged exposure. Perifosine was effective in reducing the number of viable cells as shown by a steep decline in the curve. IC values obtained after the selected time intervals are shown in Table 1.

Expressional Analysis of Cell Cycle Genes

Expression levels of the three selected genes i.e., CCNA1, CCNA2 and CCND1 were identified in HepG2 cells by real-time PCR methodology. For this purpose, the cells were exposed to perifosine followed by cDNA synthesis. After normalization of the data, fold changes were identified by $2^{\Delta\Delta CT}$ method to compare the experimental (treated) and control (untreated) groups. In response to perifosine exposure, effects were not concentration dependent. For the initial low concentrations of perifosine (IC25), there was a slight inhibition (-1.5fold) of CCNA1 expression. In contrast, for higher concentrations of perifosine i.e., IC50 and IC75, substantial induction of CCNA1 expression was observed as shown by 4.7 and 8.6fold increase at mRNA levels (Figure 2). A uniform response in expressional pattern of CCNA2 gene was observed when HepG2 cells were exposed to the ALP. Selected compound induced negligible change in expressional levels of CCNA2 gene at lower concentrations (IC25) when compared to untreated controls cells. In contrast, substantial inhibition was observed at higher concentrations of the compounds as shown in Figure 2. More specifically, perifosine inhibited the expression of CCNA2 gene by -1.3 and -3.4fold when the cells were exposed to IC_{50} and IC_{75} concentrations.

Perifosine induced the expression of CCND1 gene in HepG2 cells in a concentration dependent format. A uniform response in expressional pattern of CCND1 gene was observed when HepG2 cells were exposed to perifosine. Tested compound induced minimal changes in expressional levels of CCND1 gene at lower concentrations (IC25) as shown by 1.2fold induction. Higher concentrations of tested compound substantially induced CCND1 gene expression in the target cells as shown by a maximum 2.5fold induction in response to the exposure. Interestingly, there were almost no differences when fold changes were compared for IC50 and IC75 concentrations of the tested compound. This, in turn, shows a plateau stage where HepG2 cells did not show further response to the test compound as far as induction of CCND1 gene is concerned. All in all, perifosine induced discrete expression alterations in HepG2 cells.

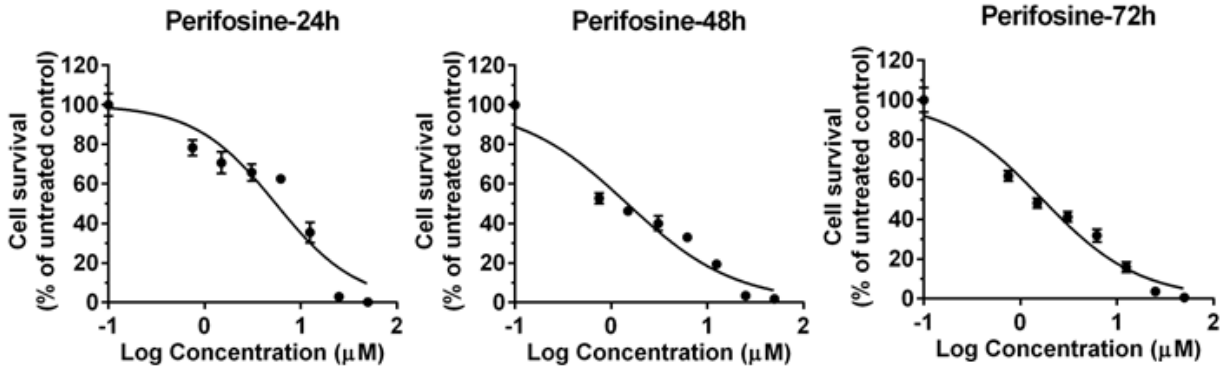

Figure 1: Perifosine induced substantial toxic effects in HepG2 cells as shown by a decline in growth response curves. The effects were calculated by using MTT assay and results were analyzed by using GrapPad Prism v.6 software.

Table 1: IC values of Perifosine against liver cancer cells (HepG2)

Perifosine	IC25	IC50	IC75
24 h	1.9 µM	5.5 µM	16 µM
48 h	0.4 µM	1.5 µM	6.0 µM
72 h	0.5 µM	1.7 µM	6.0 µM

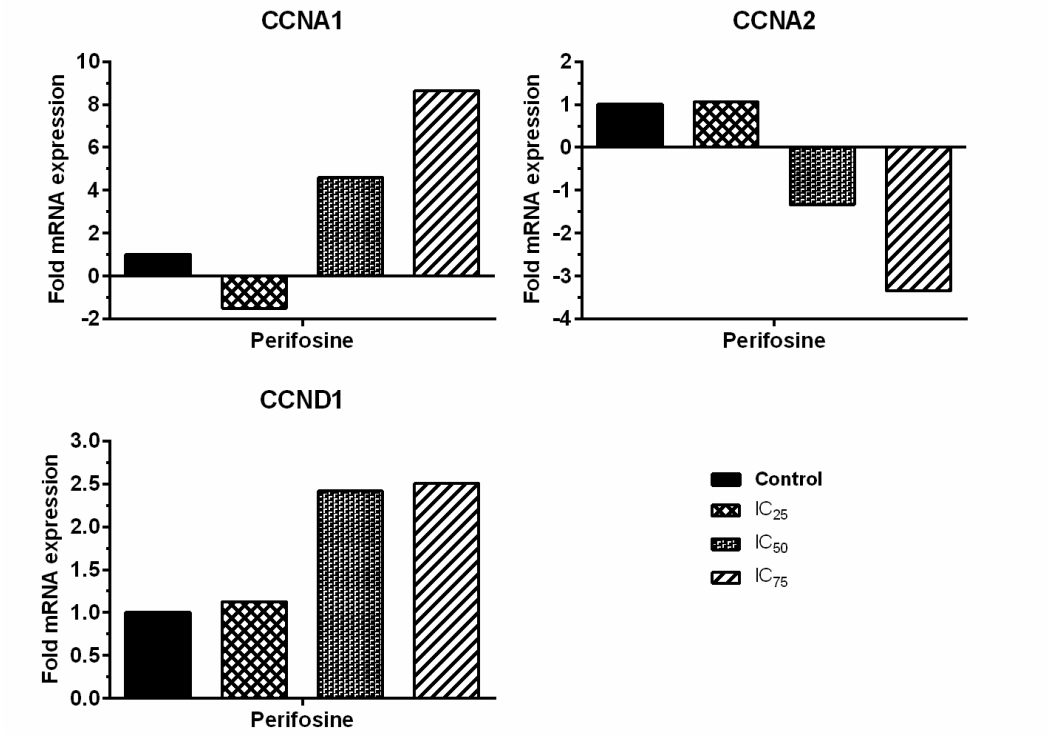

Figure 2: Expressional changes in cell cycle related genes in response to exposure with Perifosine. Exposure with the selected compound de-regulates the expression of genes in discrete fashion as determined by real-time PCR analysis.

DISCUSSION

ALPs have been utilized therapeutically since the mid-twentieth century, with continual structural changes to improve function. This study looked at cytostatic and cytotoxic effects of perifosine in HepG2 cells derived from hepatocellular carcinoma. The exact mechanism through which ALP's are cytotoxic for cancer cells is not clear yet but ALPs are known for blocking for phosphocholine in multiplying cells [15]. The current study was designed to demonstrate insights about the potential effects of perifosine on functional and molecular aspects of liver cancer cells (HepG2).

Perifosine, a 2nd generation ALP, has been shown to suppresses cell survival and trigger apoptosis in neoplastic lung cells by disrupting mTOR signaling and promoting autophagy [16]. Perifosine has also shown cytotoxic effects in cancer cell lines such as HepG2 causing cell cycle arrest and P21 upregulation, triggering apoptosis via caspase and JNK pathways [17]. Cytotoxic effects of perifosine were highlighted by using MTT assay strategy. Following the exposure with compound, substantial inhibition of proliferation was induced. Our results showed that the drug concentration required to inhibit cell growth (IC values) were higher for shorter incubation periods (24h vs. 48h and 72h). Similar kind of growth inhibitory effects of the test compound. For instance, perifosine treatment led to a decrease in hepatoma cell proliferation in a time and dose dependent approach, as measured by the MTT assay [18].

Cancer cells possess multiple genetic defects, which is consistent with the two-hit hypothesis. These cells can be targeted by various mechanisms, as their growth relies on cell division governed by the cell cycle. Because cell growth depends on cell division controlled by the cell cycle, checkpoints in the cycle generally stop mitosis when errors such as DNA damage occur. However, these checkpoints are

impaired in cancer cells and lead to uncontrolled proliferation. DNA damage response systems play a key role in preventing cancer growth, for example, the Mre11-Rad50-Nbs1 complex detects DNA double-strand breaks and activates ATM kinase, which phosphorylates CHK2, thereby stopping the G1 checkpoint to prevent cell division [18, 19].

The effects of Perifosine on the cell cycle were evaluated by analyzing the expression of genes coding for CCNA1, CCNA2, and CCND1, which regulate different stages of the cell cycle. CCNA1 plays a key role at the G1 and G2 checkpoints, where it interacts with CDK2 and CDC2 kinases to facilitate the cell's progression through these phases. HepG2 cells treated with IC25, IC50, and IC75 concentrations of perifosine showed altered expression of CCNA1, CCNA2, and CCND1. Up regulation of CCNA1 suggests a halt in the G1 or G2 phase of the cell cycle. The downregulation of CCNA2 at all concentrations further supports these cell cycle arrests. This inhibition aligns with the role of cyclin A2 (CCNA2) in the regulation of mitotic progression and its potential involvement in cellular stress responses [20]. The concentration-dependent inhibition observed in this study suggests that the compound may affect mitotic progression or induce cell cycle arrest, which could contribute to their anti-cancer effects.

The expression of CCND1 was also influenced by perifosine, with a noticeable increase at higher concentrations. The CCND1 gene, encoding cyclin D1, plays a pivotal role in regulating the transition from G1 to S phase of the cell cycle. The observed minimal changes at lower concentrations followed by significant induction at higher concentrations suggest a potential saturation point or maximum response level for these compounds. This pattern is consistent with the reported role of cyclin D1 in tumorigenesis and its modulation in response to cytotoxic agents [21]. Additionally, the 2.5-fold increase in CCND1 expression in IC50 and IC75-treated cells indicates a halt specifically in the G1 phase, as CCND1 interacts with the Rb protein to regulate this checkpoint.

The study demonstrated the anticancer potential of synthetic ALP perifosine against HepG2 liver cancer cells. The compound caused significant toxicity and altered expression of key cell cycle genes, suggesting its potential as antineoplastic and cytostatic agents. These findings warrant further investigation to explore perifosine effects in HCC cells, while including additional *in vitro* and *in vivo* studies to support future clinical evaluation. Provided with further evidence, perifosine may be a new addition to treatment modalities against liver cancer.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021;71(3):209-49.
2. Zhang C-h, Cheng Y, Zhang S, Fan J, Gao Q. Changing epidemiology of hepatocellular carcinoma in Asia. Liver International. 2022;42(9):2029-41.
3. Anwanwan D, Singh SK, Singh S, Saikam V, Singh R. Challenges in liver cancer and possible treatment approaches. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer. 2020;1873(1):188314.
4. Motofei IG, editor Biology of cancer; from cellular and molecular mechanisms to developmental processes and adaptation. Seminars in Cancer Biology; 2022: Elsevier.
5. Almalki SG. The pathophysiology of the cell cycle in cancer and treatment strategies using various cell cycle checkpoint inhibitors. Pathol Res Pract. 2023;251:154854.
6. Ding L, Cao J, Lin W, Chen H, Xiong X, Ao H, et al. The Roles of Cyclin-Dependent Kinases in Cell-Cycle Progression and Therapeutic Strategies in Human Breast Cancer. International journal of Molecular Sciences. 2020;21(6):1960.
7. Galbraith MD, Bender H, Espinosa JM. Therapeutic targeting of transcriptional cyclin-dependent kinases. Transcription. 2019;10(2):118-36.
8. Markova AA, Plyavnik NV, Morozova NG, Maslov MA, Shtil AA. Antitumor phosphate-containing lipids and non-phosphorus alkyl cationic glycerolipids: chemical structures and perspectives of drug development. Russian Chemical Bulletin. 2014;63(5):1081-7.
9. Mollinedo F, Gajate C. Mitochondrial targeting involving cholesterol-rich lipid rafts in the mechanism of action of the antitumor ether lipid and alkylphospholipid analog edelfosine. Pharmaceutics. 2021;13(5):763.
10. de Castro Spadari C, Borba-Santos LP, Rozental S, Ishida K. Miltefosine repositioning: A review of potential alternative antifungal therapy. Journal of Medical Mycology. 2023:101436.
11. Salucci S, Aramini B, Bartoletti-Stella A, Versari I, Martinelli G, Blalock W, et al. Phospholipase Family Enzymes in Lung Cancer: Looking for Novel Therapeutic Approaches. Cancers. 2023;15(12):3245.
12. Çetinel ZÖ, Bilge D. Investigation of miltefosine-model membranes interactions at the molecular level for two different PS levels modeling cancer

cells. Journal of Bioenergetics and Biomembranes. 2024:1-13.
13. Gills JJ, Dennis PA. Perifosine: update on a novel Akt inhibitor. Curr Oncol Rep. 2009;11(2):102-10.
14. Wood D, Endicott J. Structural insights into the functional diversity of the CDK-cyclin family. Open Biol 8: 180112. 2018.
15. Kaleağasıoğlu F, Zaharieva MM, Konstantinov SM, Berger MR. Alkylphospholipids are signal transduction modulators with potential for anticancer therapy. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2019;19(1):66-91.
16. Taniguchi K, Suzuki T, Okamura T, Kurita A, Nohara G, Ishii S, et al. Perifosine, a bioavailable alkylphospholipid akt inhibitor, exhibits antitumor activity in murine models of cancer brain metastasis through favorable tumor exposure. Frontiers in Oncology. 2021;11:754365.
17. Yosifov DY, Dineva IK, Zaharieva MM, Konstantinov SM, Berger MR. The expression level of the tumor suppressor retinoblastoma protein (Rb) influences the antileukemic efficacy of erucylphospho-N, N, N-trimethylpropylammonium (ErPC3). Cancer biology & therapy. 2007;6(6):930-5.
18. Fei HR, Chen G, Wang JM, Wang FZ. Perifosine induces cell cycle arrest and apoptosis in human hepatocellular carcinoma cell lines by blockade of Akt phosphorylation. Cytotechnology. 2010;62(5):449-60.
19. Bian L, Meng Y, Zhang M, Li D. MRE11-RAD50-NBS1 complex alterations and DNA damage response: implications for cancer treatment. Molecular Cancer. 2019;18(1):169.
20. Gong D, Ferrell JE. The Roles of Cyclin A2, B1, and B2 in Early and Late Mitotic Events. Molecular Biology of the Cell. 2010;21(18):3149-61.
21. Chen S, Li L. Degradation strategy of cyclin D1 in cancer cells and the potential clinical application. Frontiers in Oncology. 2022;12:949688.

Ethics Approval: Not Applicable
Author Contributions: MM performed the experiments. AN helped in analysis. SM supervised the manuscript 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.