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Role of Senescence Induced by CDK4/6 Inhibitor Palbociclib in Breast Cancer Treatment Response

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ABSTRACT

Breast cancer is the most common cancer in women globally and a leading cause of cancer-related deaths. Despite advances in early detection and targeted therapy, the 5-year survival rate drops below 25% when the cancer spreads to nearby tissues or distant organs. Breast cancer is classified into molecular subtypes, with luminal subtypes (ER/PR-positive) being the most prevalent. Luminal B tumors, which may or may not express HER2, are often treated with endocrine therapy (ET), targeting the estrogen receptor. However, resistance to ET is common, and combining ET with CDK4/6 inhibitors, like Palbociclib, has shown to improve progression-free survival. Despite this, nearly all patients experience relapse after 2-3 years of treatment. CDK4/6 inhibitors induce cell cycle arrest, leading to quiescence, which can eventually progress to senescence. Senescent cells exhibit metabolic and phenotypic changes that enable them to resist apoptosis, and there is emerging evidence suggesting that these cells may

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escape senescence upon treatment withdrawal, regenerating the tumor. This study investigated the effects of long-term senescence induced by Palbociclib in breast cancer cells. After several months of treatment, cells reached 100% senescence, but upon drug withdrawal, some cells resumed proliferation. Protein expression analysis revealed changes linked to resistance mechanisms, including altered levels of HER2 and CDK4/6 inhibitors. These findings suggest that the escape from senescence may contribute to breast cancer relapse after treatment withdrawal.

Keywords: Breast Cancer, CDK4/6 Inhibitors, Endocrine Therapy Resistance, Senescence Escape, Palbociclib Treatment

INTRODUCTION

Breast cancer remains the most commonly diagnosed cancer in women worldwide and a leading cause of cancer-related deaths. According to the International Agency for Research on Cancer (IARC), in 2018, there were approximately **2.1 million new cases** of breast cancer globally, and by 2020, this figure had risen to an estimated **2.3 million** new cases, making up nearly **11.7%** of all new cancer diagnoses (IARC, 2020). Projections for 2025 suggest that breast cancer incidence will continue to rise, with **2.5 million new cases** expected, driven by factors such as population aging and lifestyle changes, particularly in high-income countries. Mortality rates have similarly increased, with **685,000 deaths** recorded in 2019, and this number is projected to approach **750,000 by 2025** (Cancer Research UK, 2020). While survival rates are improving in high-income countries due to advancements in early detection and treatment, mortality remains higher in low- and middle-income nations, where access to healthcare and early diagnosis is often limited. For example, in the United States, the **5-year relative survival rate** for localized breast cancer exceeds **99%,** but survival for metastatic breast cancer drops to around **28%** (American Cancer Society, 2020). In contrast, countries like India and Brazil, where healthcare access may be more restricted, face significant challenges, with **India** reporting around **160,000 new cases** in 2020 and **Brazil** seeing approximately **66,000 new cases** (Global Cancer Observatory, 2020). Despite these disparities, breast cancer continues to be a leading health concern globally, with factors like age, genetics, and lifestyle choices such as obesity and late childbirth contributing to the rising incidence. These trends highlight the urgent need for improved prevention, early detection, and treatment strategies, particularly in regions with limited healthcare resources. Epidemiology Breast cancer is the most common malignant tumor in women in the world. Breast cancer patients account for as much as 36% of oncological patients. An estimated 2.089 million women were diagnosed with breast cancer in 2018 (Nardin et al., 2020). The incidence of this malignant tumoris increasing in all regions of the world, but the highest incidence occurs in industrialized countries. Almost half of the cases on a global scale are in developed countries. This trend is mainly due to the so-called Western lifestyle, associated with a poor diet, nicotinism, excessive stress and little physical activity (Bellanger et al., 2018).

The breast tissue is composed of stromal and glandular tissues. Stromal cells represent adipocytes, fibroblast among other cells that surrounds lobes of the breast. Lobes can

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be divided intosmaller ones called lobules, responsible to produce milk. Lobes are connected through a series of ductsthat allows breast milk to escape through the nipple, encompassing glandular tissues (Kumar, 2020). Breast cancer is characterized by its high heterogeneity. Histologically, breast cancer can be categorizedinto carcinoma in situ or invasive carcinoma. Carcinoma in situ represents a bunch of abnormal cellsthat have not invade yet surrounding tissues of the breast, being covered with a layer of myoepithelialcells around the tumor. On the other hand, invasive carcinoma can spread to nearby tissues, being moreaggressive and life-threatening (Shea et al., 2020).

The primary aim of this study was to investigate the role of senescence induced by the CDK4/6 inhibitor Palbociclib in breast cancer treatment response, particularly in estrogen receptor-positive (ER+) breast cancer. The specific objectives were:

To evaluate the induction of senescence in breast cancer cell lines (MCF-7 and BT-474) treated with Palbociclib and assess the potential for senescence escape upon drug withdrawal.

To analyze molecular changes associated with Palbociclib resistance, including alterations in protein expression (e.g., HER2, Cyclin D1, p27) and signaling pathways.

To explore the role of the senescence-associated secretory phenotype (SASP) in promoting tumor progression and resistance to therapy.

To investigate the effectiveness of combination therapies involving CDK4/6 inhibitors and other targeted agents in overcoming resistance and improving treatment outcomes.

MATERIALS AND METHODS

Cell Culture and Treatment

Cell Lines: MCF-7 and BT-474 breast cancer cell lines were used. Media was prepared with 10% FBS, 1% penicillin-streptomycin, and 1% glutamine. For specific experiments, F-12 media was used to stimulate cell growth. Cells were passaged when they reached 70-80% confluency. Trypsin was used to detach cells, which were then resuspended in fresh media and seeded into new plates or flasks. Cells were treated with Palbociclib at concentrations of 1.5 µM, 2.5 µM, and 5.5 µM for varying durations (24h, 48h, 72h, and 1 week).

Western Blotting

Cells were lysed using a lysis buffer containing protease inhibitors. The lysate was centrifuged, and the supernatant was collected for protein quantification. The Bradford assay was used to measure protein concentration. Samples were loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked with 5% milk and incubated with primary antibodies against Cyclin D1, p27, HER2, and PHER2 overnight at 4°C. Secondary antibodies were used for detection. Membranes were washed with TBST and developed using an ECL reagent. Protein bands were visualized and quantified using imaging software.

Buffers for Western Blot

Preparation included mixing 100ml of 10% SDS with 900ml of Milli-Q distilled water. TBST was prepared by combining 100ml of 10x TBS with 1ml of Tween and 900ml of Milli-Q distilled water.

Proliferation Assay

Cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The

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dye was solubilized with 10% acetic acid, and absorbance was measured at 590 nm to quantify cell proliferation.

Mathematical Calculations

The concentration of Palbociclib was calculated using the formula Ci×Vi=Cf×VfCi \times Vi = Cf \times VfCi×Vi=Cf×Vf, where CiCiCi represents the initial concentration, ViViVi is the initial volume, CfCfCf is the final concentration, and VfVfVf is the final volume. For example, to prepare a final concentration of 1.5 µM in 1.5 mL of media, the initial volume was determined using the equation Vi=(Cf×Vf)/CiVi = (Cf \times Vf) / CiVi=(Cf×Vf)/Ci. Substituting the values, Vi=(1.5 µM×1500 µL)/100 µMVi = (1.5 \, \text{µM} \times 1500 \, \text{µL}) / 100 \, \text{µM}Vi=(1.5µM×1500µL)/100µM, which results in 22.5 µL.

Preparation of 20% Dimethyl Sulfoxide

To prepare 5ml of solution, we use 4ml of normal media and 1ml of Dimethyl Sulfoxide (DMSO).

Outer Membrane Vesicles (OMVs) Treatment

OMVs derived from Vibrio cholerae were used. Two different types were added at different time points: Delta HAPA (lacking metal protease HAPA) and the wild-type. The treatment was stopped by adding PBS, and plates were placed on ice for pellet collection.

Pellet Collection Protocol

Cells were washed with 1ml of cold PBS per well, followed by aspiration. A complete protease inhibitor was added. Cells were scraped, transferred into an Eppendorf tube, and centrifuged at 1200 rpm for 5 minutes at 4°C. The pellet was frozen at -80°C for further analysis.

Quantification and Lysis of Pellet

Lysis buffer (100–130µl) was added to frozen pellets. Samples were vortexed, incubated at 4°C for 30 minutes, centrifuged, and transferred to new tubes for protein quantification using the Bradford assay.

Electrophoresis and Western Blotting

The membrane was washed three times with TBST, incubated with a secondary antibody for one hour, and developed for visualization. Cells were treated with Abemaciclib by replacing the old media with fresh media containing the drug.

Mathematical Calculation

Vi(Cii.Vii)/Ci

Vi = (5.12 µl * 1500) / 100 = 76.5 µl

Preparation of Media

5ml of Gultamax, sodium pyruvate, and penicillin were added along with fetal bovine serum (FBS). The amount of FBS depended on the required percentage: 25ml for 5% and 50ml for 10%.

Plasmid Extraction

Media for MCF7 cells was changed, and they were treated with Palbociclib at concentrations of 76.5µl and 82.5µl.

Western Blot Incubation

Membranes were incubated with the secondary antibody at a 1:2000 dilution. After one

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hour, the membrane was washed three times with TBST before revealing the results.

β-Galactosidase Assay

Cells were fixed with 0.5% glutaraldehyde, washed, and incubated in staining solution (X-Gal) at 37°C for 24-48 hours.

Protocol for β-Galactosidase Assay

Cells were fixed, stained with 0.5ml of staining solution, and incubated at 30°C for 24 hours.

Qiagen Plasmid Midi Kit

Cells were treated normally with Palbociclib, passages were performed, and electrophoresis loading markers were prepared.

Electrophoresis Procedure

Loading markers were retrieved from -80°C. The gel was loaded with a buffer and run at 110V for 75 minutes. The gel was transferred to a membrane using the iBlot system and developed for analysis.

Methodology for Proliferation Assay Using Crystal Violet

Cells were seeded in a 96-well plate at 5,000–10,000 cells per well. After incubation, cells were fixed with 4% paraformaldehyde. 0.5% crystal violet solution was used for staining. 10% acetic acid was used to solubilize the dye. Measured at 570 nm using a microplate reader. The proliferation rate was calculated relative to control wells.

Western Blot for BT474 and MCF7 Cells

Western blot experiments were conducted using Cyclin D1, P27, PHER2, and HER antibodies. Samples were incubated overnight, washed, and developed.

Cell Seeding and Palbociclib Treatment

Cells were seeded and treated with Palbociclib for various experiments, including Western blotting and proliferation assays.

Data Analysis

Graphs and Statistical Analysis: Data were plotted using GraphPad Prism. Statistical significance was determined using Student's t-test or ANOVA, with a p-value < 0.05 considered significant.

RESULTS

This study aims to examine differences in the expression of key senescence and cell cycle-related proteins, including p21, p16, CDK4/6, and other markers, to better understand resistance mechanisms in MCF-7 and BT-474 cell lines. Western blot analysis is a widely used technique for detecting specific proteins in biological samples. In this study, Western blot was performed on MCF-7 and BT-474 breast cancer cell lines to compare protein expression between control cells and those that escaped from senescence after Palbociclib treatment (MCF7rPalbociclib and BT474rPalbociclib). Palbociclib is a CDK4/6 inhibitor used to induce cell cycle arrest and senescence in cancer cells. However, some cells develop resistance and escape from this senescent state, leading to acquired resistance. By analyzing protein expression through Western blot, we can identify key molecular changes associated with this escape mechanism.

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Western Blot MCF-7 and BT-474 control vs resistant cell lines

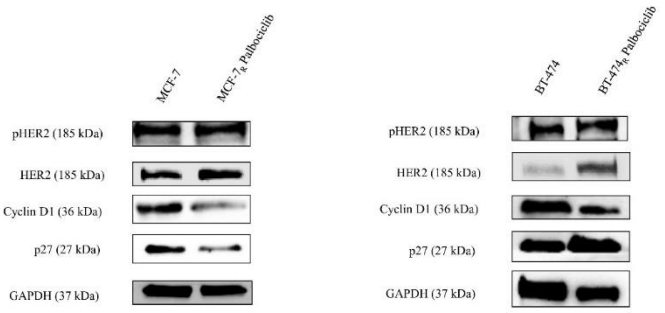

Fig. No. 3.1:

Western Blot MCF-7 and BT-474 control vs cell lines that escaped from senescence (MCF7rPalbociclib; BT474rPalbociclib)

Western Blot Analysis of MCF-7 and BT-474 Cell Lines in Response to Palbociclib Resistance

To investigate the molecular alterations associated with Palbociclib resistance, we performed Western blot analysis on MCF-7 and BT-474 breast cancer cell lines, comparing control (sensitive) and Palbociclib-resistant (R) counterparts. The expression levels of HER2, phosphorylated HER2 (pHER2), p27, Cyclin D1, and GAPDH were analyzed, and band intensities were quantified using GraphPad Prism, normalizing all target proteins against GAPDH.

pHER2 and HER2 Expression

HER2 (185 kDa) was highly expressed in BT-474 and BT-474 R cells, consistent with the HER2-positive status of this cell line, whereas MCF-7 and MCF-7 R showed minimal or no HER2 expression. Interestingly, pHER2 levels were significantly elevated in BT-474 R compared to BT-474 ($p < 0.05$), suggesting an increase in HER2 phosphorylation as a potential resistance mechanism. In contrast, MCF-7 cells exhibited negligible pHER2 expression in both sensitive and resistant conditions.

p27 Downregulation in Resistant Cells

p27 (27 kDa), a cyclin-dependent kinase inhibitor, was highly expressed in Palbociclib-sensitive cells (MCF-7 and BT-474), aligning with its role in CDK4/6 inhibition. However, in resistant cells (MCF-7 R and BT-474 R), p27 expression was significantly reduced ($p < 0.01$). This downregulation suggests a loss of cell cycle inhibition, allowing resistant cells to proliferate despite CDK4/6 blockade by Palbociclib.

Cyclin D1 Upregulation in Resistant Cells

Cyclin D1 (36 kDa), a key regulator of the G1-S phase transition, was downregulated

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in Palbociclib-sensitive cells due to the drug's inhibitory effects on the CDK4/6-Cyclin D1 axis. However, in MCF-7 R and BT-474 R, Cyclin D1 expression was significantly upregulated ($p < 0.001$). This suggests that resistant cells bypass CDK4/6 inhibition by maintaining high Cyclin D1 levels, a well-documented resistance mechanism.

Normalization with GAPDH

GAPDH (37 kDa) was used as a loading control, and its expression remained consistent across all conditions, ensuring equal protein loading for comparative analysis.

Summary of Key Findings

Protein	MCF-7	MCF-7 R	BT-474	BT-474 R	Resistance Mechanism
pHER2	Low	Low	High	**Higher ($p < 0.05$)**	Increased HER2 phosphorylation in resistance
HER2	Low	Low	High	High	HER2 amplification contributes to resistance
p27	High	**Low ($p < 0.01$)**	High	**Low ($p < 0.01$)**	Loss of CDK4/6 inhibition
Cyclin D1	Low	**High ($p < 0.001$)**	High	**Higher ($p < 0.001$)**	Cyclin D1 overexpression drives resistance
GAPDH	Control	Control	Control	Control	Ensures equal loading

These findings indicate that Palbociclib resistance is associated with increased HER2 phosphorylation (pHER2), downregulation of p27, and upregulation of Cyclin D1 in resistant cells. The observed loss of p27 expression and Cyclin D1 overexpression suggest that resistant cells circumvent CDK4/6 inhibition, allowing uncontrolled proliferation. Furthermore, in BT-474 R, elevated pHER2 levels indicate potential HER2 pathway activation, which may contribute to Palbociclib resistance. A Western Blot analysis was performed to compare MCF-7 and BT-474 control cell lines with their respective Palbociclib-resistant counterparts (MCF7rPalbociclib and BT474rPalbociclib), which have escaped senescence. This experiment aimed to assess differences in protein expression between the parental and resistant cell lines, providing insights into the molecular changes associated with Palbociclib resistance. By analyzing key signaling pathways and markers related to cell cycle regulation, senescence, and resistance mechanisms, the Western Blot data can help identify potential targets for overcoming resistance in breast cancer treatment. Palbociclib is a potent and highly selective CDK4/6 inhibitor. In vivo trials revealed antitumoractivity in a variety of tumors, including BC, although Palbociclib was found to be inactive in Rb- negative BC tumors (Fry et al., 2004). Among a large panel of BC cell lines, ER+ cell lines, includingluminal HER2+, showed the greatest sensitivity in contrast to basal subtypes. In addition to luminal markers, sensitive cell lines showed overexpression of RB1 and cyclin D1, as well as under expressionof p16 (Finn et al., 2009). Ribociclib is a selective CDK4/6 inhibitor with preclinical activity via the induction of cytostasis and senescence (Rader et al., 2013). Enhanced tumor growth inhibition in BC was observed

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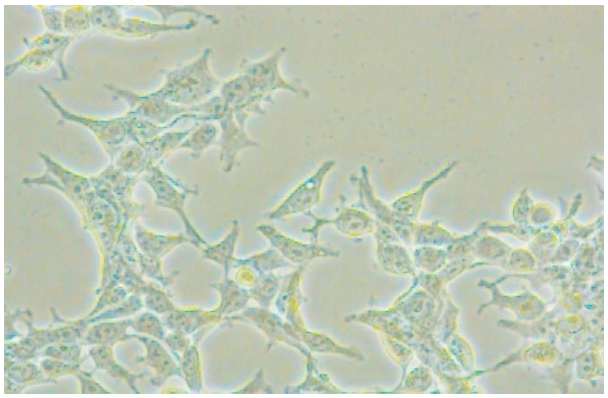
when combined with ET, and was further improved with PI3K inhibitor triple combination (O'Brien et al., 2014). Abemaciclib is a selective CDK inhibitor with a higher selectivity towards CDK4 than towards CDK6. Abemaciclib activity was observed only in Rb-proficient cells, inducing reversible cell cycle arrest and senescence. Abemaciclib has been shown to cross the blood- brain barrier in a more effective way than Palbociclib (Raub et al., 2015).

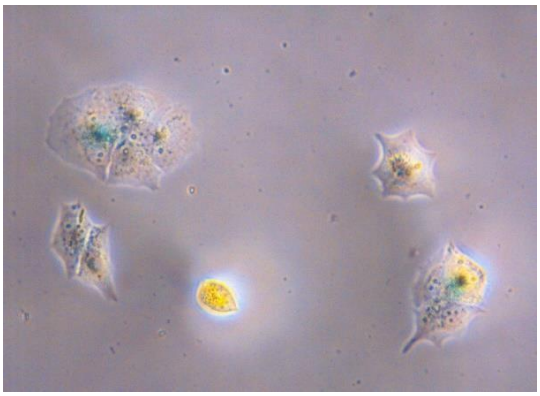
Effect of Palbociclib on BT474 Breast Cancer Cells

The images represent BT474 breast cancer cells treated with different concentrations of Palbociclib, a CDK4/6 inhibitor used in cancer therapy. The control sample (untreated cells) serves as a baseline, while the treated samples (R1 to R4) correspond to increasing doses of Palbociclib (1.5 µM, 2.5 µM, and 5.5 µM). As the concentration increases, cellular responses such as changes in morphology, proliferation inhibition, and possible cell death become more pronounced. These images help assess the drug's effectiveness and its dose-dependent impact on BT474 cells.

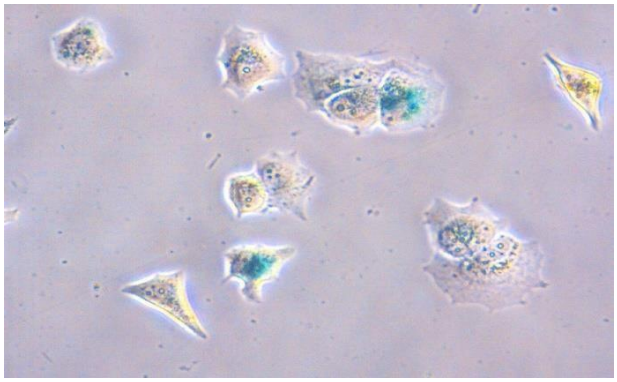
Bt474 Palbociclib (R1)



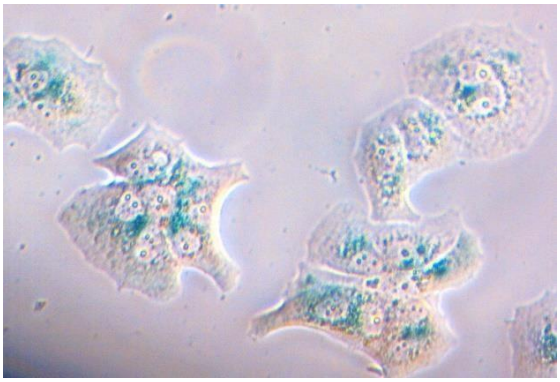
Bt474 Palbociclib 1.5µM (R2)



Bt474 Palbociclib 2.5 µM (R3)



Bt474 Palbociclib 5.5 µM (R4)



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BT474 Palbociclib (Control)

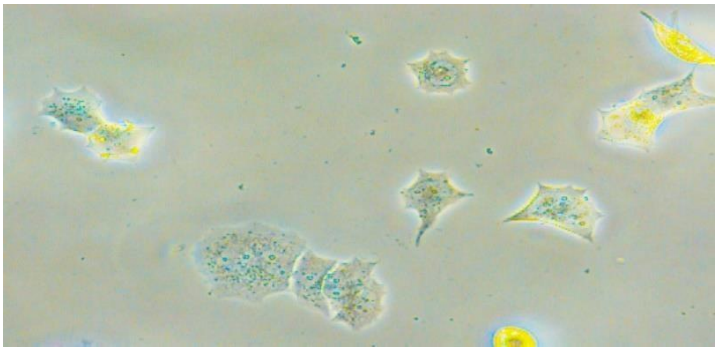

Fig. No. 3.2: Cell lines of each treatment

The images represent different BT-474 breast cancer cell line conditions under various concentrations of Palbociclib treatment. These conditions are likely captured through microscopy, showing morphological differences between control cells and those treated with increasing doses of Palbociclib. Senescent cells increased from 10% at baseline to 60% after 72 hours of treatment. Senescent cells increased from 15% at baseline to 70% after 72 hours of treatment. Upon drug withdrawal, a subset of cells resumed proliferation, indicating escape from senescence.

BT474 Palbociclib

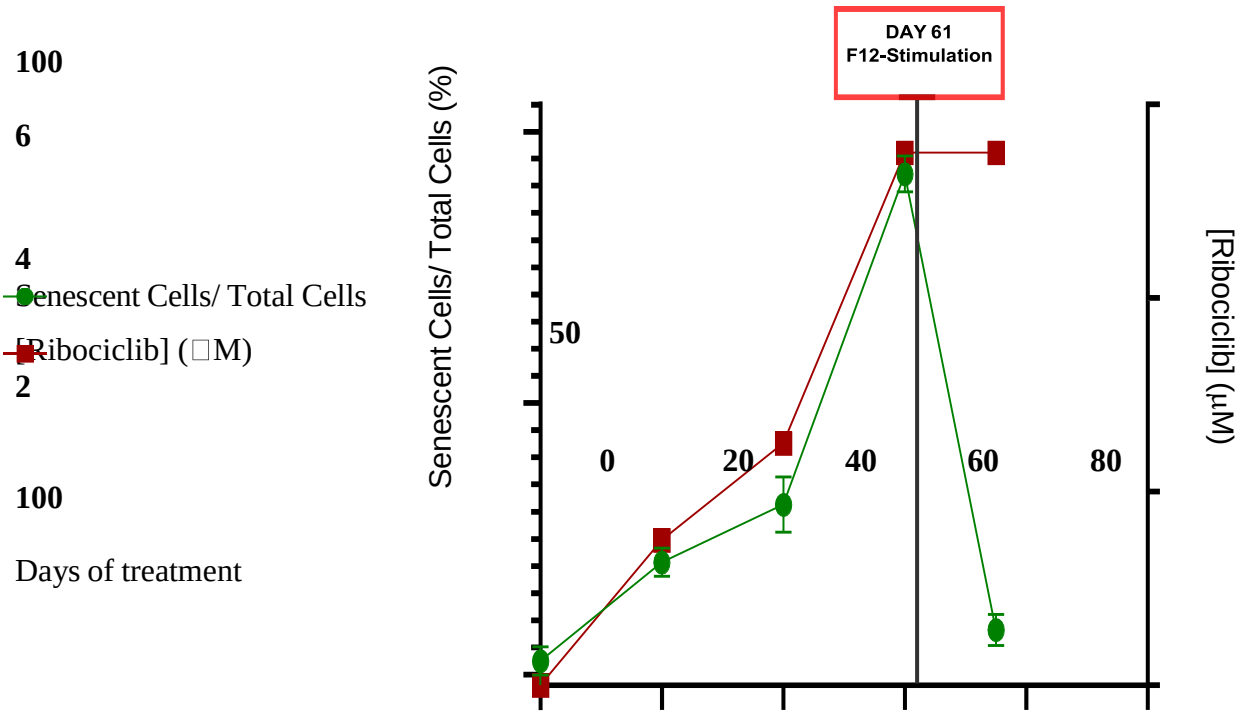

Fig. No. 3.3: BT474 Palbociclib

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http://amresearchreview.com/index.php/Journal/about

BT474Palbociclib

This graph illustrates the proportion of senescent cells relative to total cells in BT474 breast cancer cell lines treated with Palbociclib over a period of 100 days. The data indicate that the percentage of senescent cells increases with prolonged exposure to the drug, suggesting a time-dependent induction of cellular senescence. However, the trend also raises the possibility of senescence escape or adaptive resistance at later stages.

Key Observations:

The **x-axis** represents the **days of treatment** (0 to 100 days).

The **y-axis** represents the **percentage of senescent cells relative to the total cell population**.

Different curves or data points may indicate varying concentrations of **Palbociclib (e.g., 2 µM, 4 µM, 6 µM, etc.)** or possibly another **CDK4/6 inhibitor like Ribociclib** for comparison.

Interpretation:

Early phase (0-20 days): A gradual increase in senescent cells is expected, indicating the initial effect of Palbociclib in inducing cell cycle arrest.

Mid-phase (20-60 days): The percentage of senescent cells may peak, showing a maximum drug effect.

Late phase (60-100 days): The number of senescent cells could remain stable or decrease, potentially due to **cell death** or **emergence of Palbociclib-resistant cells**.

This graph is crucial for understanding how BT-474 cells respond to long-term **Palbociclib treatment**, particularly in assessing **senescence induction vs. resistance development** over time.

The graph depicting BT-474 breast cancer cells treated with Palbociclib provides crucial insights into the dynamics of senescence induction and potential resistance mechanisms. Initially, during the early phase of treatment (0–20 days), there is a gradual increase in the percentage of senescent cells, indicating that Palbociclib effectively induces cell cycle arrest by inhibiting CDK4/6. This suggests that a significant portion of the BT-474 cell population is responding to treatment by entering a stable state of senescence, thereby preventing uncontrolled proliferation. The extent of this increase may depend on the concentration of Palbociclib administered, with higher doses likely inducing a stronger initial response. As treatment continues into the mid-phase (20–60 days), the senescent cell population appears to reach a plateau, suggesting that the majority of susceptible cells have undergone cell cycle arrest. This confirms the effectiveness of Palbociclib in inducing a prolonged growth arrest in BT-474 cells. However, heterogeneity within the cell population may lead to variability in response, with some cells potentially remaining unaffected or beginning to develop adaptive mechanisms that allow them to survive despite treatment.

In the later phase of treatment (60–100 days), the key observation is whether the proportion of senescent cells remains stable or begins to decline. A decline in

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senescence would suggest that a subpopulation of cells has escaped from senescence and resumed proliferation, indicative of emerging resistance to Palbociclib. The mechanisms driving this resistance could involve loss of Rb pathway dependency, upregulation of alternative cyclins such as Cyclin E, or activation of compensatory signaling pathways like PI3K/AKT/mTOR. If resistance emerges, it could have significant clinical implications, as Palbociclib-resistant cells may become less responsive to CDK4/6 inhibition, necessitating alternative treatment approaches. When comparing treated cells with the control group, untreated BT-474 cells are expected to exhibit a low baseline level of senescence since they continue proliferating unchecked. In contrast, cells treated with low doses of Palbociclib (e.g., 1.5 µM) may show a slower increase in senescence, leading to only partial G1 arrest, while higher doses (e.g., 5.5 µM) may induce a stronger and more immediate response. However, higher doses could also exert selective pressure on the cell population, accelerating the emergence of resistant clones over time.

The biological implications of these findings emphasize the dual role of senescence in cancer therapy. While inducing stable senescence is a desirable outcome, as it prevents tumor cell proliferation, the possibility of senescence escape poses a major challenge. If a significant number of cells bypass senescence and resume division, Palbociclib monotherapy may become insufficient in the long term. This underscores the need for combination strategies, such as integrating Palbociclib with CDK2 inhibitors to target resistant clones or PI3K/mTOR inhibitors to counteract survival pathways that promote resistance. Additionally, incorporating endocrine therapies like Fulvestrant or Tamoxifen may help enhance treatment efficacy, particularly in ER+/HER2+ breast cancer models like BT-474.

Future research should focus on characterizing the resistant cell populations to determine whether they exhibit stem-like properties or undergo epithelial-to-mesenchymal transition (EMT), which could make them more aggressive and invasive. Biomarker analysis, including p16, p21, and SA-β-gal expression, could provide further confirmation of senescence levels, while RNA sequencing may help identify key pathways driving resistance. Investigating alternative CDK inhibitors, such as Abemaciclib, or testing Palbociclib in combination with targeted therapies, could offer potential solutions to overcoming resistance.

The analysis of senescence induction in BT-474 cells treated with Palbociclib highlights both the therapeutic potential and limitations of CDK4/6 inhibition. While Palbociclib effectively halts cell cycle progression and induces senescence, the emergence of resistance over time remains a concern. A deeper understanding of the mechanisms underlying senescence escape will be essential in designing more effective treatment strategies to improve long-term outcomes in breast cancer patients.

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MCF7 Palbociclib

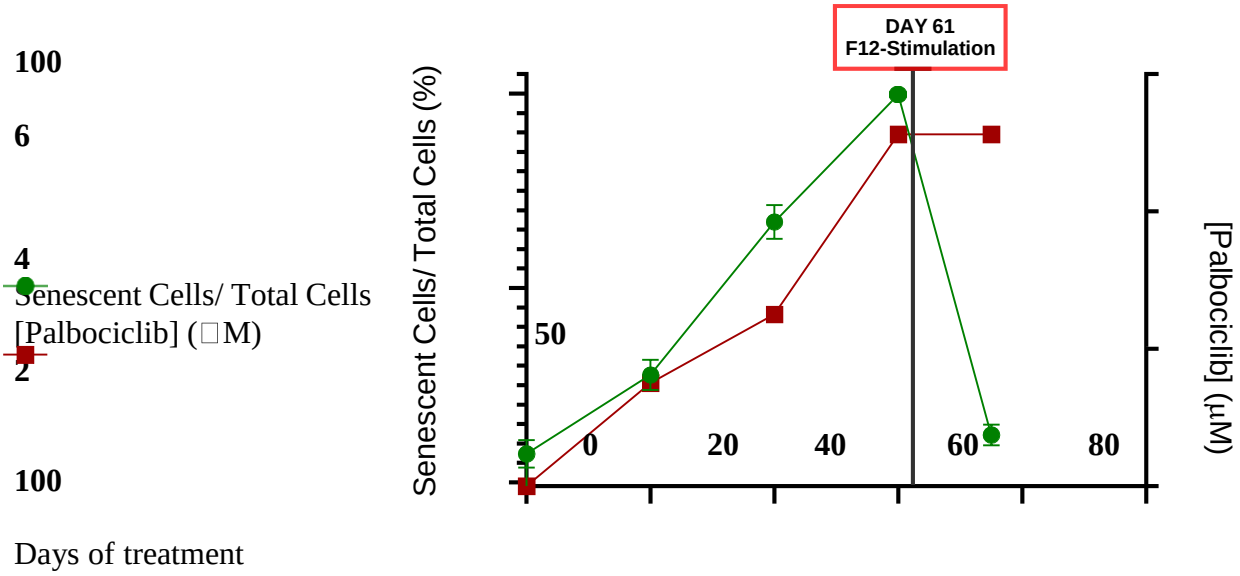

Fig. No. 3.4: MCF7 Palbociclib

The graph illustrating the response of MCF-7 breast cancer cells to Palbociclib treatment provides valuable insights into the dynamics of senescence induction over time. At the early stages of treatment (0–20 days), there is an initial increase in the proportion of senescent cells, suggesting that Palbociclib effectively induces cell cycle arrest in a significant fraction of the MCF-7 population. This is consistent with the drug's mechanism of action as a CDK4/6 inhibitor, which prevents progression through the G1 phase of the cell cycle, leading to cellular senescence. The extent of senescence induction may depend on the concentration of Palbociclib administered, with higher doses likely causing a more rapid and pronounced effect.

As treatment progresses into the intermediate phase (20–60 days), the proportion of senescent cells appears to stabilize, indicating that most susceptible cells have undergone growth arrest. This plateau phase suggests that Palbociclib maintains its efficacy in keeping the majority of cells in a senescent state. However, cellular heterogeneity within the MCF-7 population may lead to variations in response, with some cells remaining unaffected or beginning to develop adaptive mechanisms that allow them to persist despite prolonged exposure to the drug.

DISCUSSION

Senescence Induction and Cell Cycle Arrest

One of the primary mechanisms by which Palbociclib exerts its therapeutic effect is through the inhibition of CDK4/6, leading to the downregulation of Cyclin D1 and the upregulation of cell cycle inhibitors such as p27. This effectively prevents phosphorylation of the retinoblastoma protein (Rb), enforcing a G1-phase arrest and

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Volume 4, Issue 3 (2026)

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ultimately inducing senescence. The observed increase in senescent markers confirms that a significant proportion of cells enter a stable growth-arrested state upon treatment. However, senescence is not a terminal state; rather, it is a stress response that can be reversed in certain conditions, leading to the risk of tumor recurrence.

Escape from Senescence and Resistance Mechanisms

A key challenge in Palbociclib treatment is the ability of some cancer cells to escape from senescence and resume proliferation, leading to resistance. The restoration of Cyclin D1 expression in resistant cell lines suggests that these cells may bypass CDK4/6 inhibition by reactivating the cell cycle. Additionally, the upregulation of HER2 in resistant clones points to the activation of alternative signaling pathways, such as the PI3K/AKT/mTOR pathway, which can promote survival and proliferation independent of CDK4/6 inhibition. This suggests that Palbociclib resistance is not solely due to mutations in cell cycle regulators but may also involve broader changes in oncogenic signaling.

Role of SASP in Promoting Resistance

Beyond direct cell cycle alterations, the senescence-associated secretory phenotype (SASP) may also contribute to resistance. Senescent cells secrete a variety of pro-inflammatory cytokines, growth factors, and proteases that can modify the tumor microenvironment. These secreted factors can promote immune evasion, enhance angiogenesis, and even stimulate the proliferation of nearby cancer cells, ultimately facilitating tumor relapse. The presence of SASP-related factors, such as IL-6 and IL-8, in resistant cell populations suggests that these secretions may create a pro-tumorigenic niche that supports the survival of Palbociclib-resistant cells.

Implications for Therapeutic Strategies

Given the ability of ER+ breast cancer cells to escape Palbociclib-induced senescence, there is an urgent need for combination treatment strategies. One potential approach is to pair Palbociclib with inhibitors targeting compensatory pathways, such as PI3K or mTOR inhibitors, to prevent reactivation of proliferation. Additionally, blocking SASP-mediated effects using anti-inflammatory agents or senolytics (drugs that selectively eliminate senescent cells) could mitigate the pro-tumorigenic consequences of therapy-induced senescence.

Another promising strategy is the combination of Palbociclib with endocrine therapy, such as Fulvestrant or Tamoxifen, which may enhance the durability of senescence induction by inhibiting estrogen receptor signaling. Clinical studies have already demonstrated that dual inhibition of CDK4/6 and ER signaling improves progression-free survival in patients with advanced ER+ breast cancer. However, further research is needed to determine the optimal sequencing and dosing of these agents to prevent resistance.

CDK4/6 inhibitors—Palbociclib, Ribociclib, and Abemaciclib—play an eminent role in the treatment of advanced breast cancer. The pharmacokinetics, pharmacodynamics, and efficacy of the three CDK4/6 inhibitors seem to be comparable, although there are also interesting differences such asability for brain penetration, side effects, and dosing schedules. These differences between the three CDK4/6 inhibitors can be used to

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optimize selection of treatment for individual patients. Further research is needed to investigate the optimal treatment sequence of CDK4/6 inhibitors in different breast cancer settings and different subtypes and to develop (pharmacodynamics) biomarkers for selecting patients, predicting response, and to optimize the treatment schedule (Braal et al., 2021).

CDK4/6 inhibitors have been approved in combination with hormone therapy for the treatmentof patients with HR+, HER2-negative advanced or metastatic breast cancer. Despite their significant activity, approximately 10 % of patients present with inherent resistance and the rest will develop acquired resistance after 24–28 months when used as first-line therapy and after a shorter period whenused in the second line. Various mechanisms of resistance to CDK4/6 inhibitors have been described, including alterations of factors controlling cell cycle progression, such as RB loss-of-function, E2F amplification, p16 amplification, CDK4/6 amplification, and cyclin E-CDK2 amplification. Other mechanisms involve alterations in cell cycle-nonspecific mechanisms, such as aberrant FGFR signaling, aberrant PI3K/AKT/mTOR signaling, and ATM-Chk2 activation. Identifying and targeting these mechanisms can be an interesting strategy for bypassing resistance to CDK4/6 inhibitors. In clinical trials, targeting of the PI3K/AKT/mTOR pathway, as well as that of FGFR, has yielded promising results. Other interesting approaches involve combined CDK2 and CDK4/6 inhibition, MAPK inhibition and immunotherapy with immune checkpoint inhibitors (Papadimitriou et al., 2022).

CDK4/6 inhibitors—Palbociclib, Ribociclib, and Abemaciclib—have revolutionized the treatment of HR+, HER2-negative advanced breast cancer by effectively delaying disease progression when combined with endocrine therapy. While these inhibitors share similar pharmacokinetic and pharmacodynamic properties, key differences such as their ability to penetrate the blood-brain barrier, side effect profiles, and dosing schedules provide opportunities for personalized treatment strategies. For instance, Abemaciclib’s superior brain penetration makes it a potential option for patients with brain metastases, whereas Ribociclib's favorable toxicity profile may be preferable for certain patient populations. Further studies are required to determine the optimal sequencing of these inhibitors across different treatment settings and breast cancer subtypes.

Despite their clinical success, resistance to CDK4/6 inhibitors remains a major challenge, limiting long-term efficacy. Approximately 10% of patients exhibit primary resistance, while the majority develop acquired resistance within 24–28 months of first-line treatment. Resistance mechanisms can be broadly categorized into those affecting cell cycle regulation—such as RB loss, E2F amplification, and CDK4/6 or cyclin E-CDK2 amplification—and those involving alternative signaling pathways, including FGFR, PI3K/AKT/mTOR, and ATM-Chk2 activation. The complexity of these resistance pathways underscores the need for novel therapeutic strategies that either prevent or overcome resistance.

Ongoing research is focused on combination therapies to enhance treatment response and delay resistance. Inhibition of the PI3K/AKT/mTOR and FGFR pathways has shown promising results in clinical trials, suggesting that targeting these pathways could help overcome resistance to CDK4/6 inhibitors. Additionally, combined

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inhibition of CDK2 and CDK4/6 is being explored as a way to counteract resistance driven by cyclin E-CDK2 amplification. Other innovative approaches, such as MAPK inhibition and immunotherapy with immune checkpoint inhibitors, hold potential for further improving patient outcomes. To optimize the use of CDK4/6 inhibitors, there is an urgent need to develop predictive biomarkers that can guide patient selection and treatment sequencing. Identifying reliable pharmacodynamic markers will allow clinicians to tailor therapy based on individual tumor biology, ultimately improving response rates and prolonging the benefits of CDK4/6 inhibition. Future research should focus on integrating biomarker-driven strategies with combination therapies to refine treatment protocols and enhance survival outcomes in patients with HR+, HER2-negative breast cancer.

CONCLUSION

This study provides valuable insights into the mechanisms underlying Palbociclib-induced senescence and resistance in ER+ breast cancer. While CDK4/6 inhibitors have proven effective in inducing cell cycle arrest and improving patient outcomes, the persistence and eventual emergence of resistant cells highlight a critical challenge in long-term treatment efficacy. The findings underscore the need for a deeper understanding of the molecular and cellular adaptations that drive resistance, particularly the role of senescence-associated secretory phenotypes (SASP), epigenetic alterations, and compensatory signaling pathways that enable tumor cell survival and proliferation despite CDK4/6 inhibition. To improve therapeutic strategies, future research should prioritize the identification of predictive biomarkers that can help stratify patients who are more likely to develop resistance. Additionally, exploring combination therapies holds significant promise in overcoming resistance mechanisms. Approaches such as incorporating senolytic agents to selectively eliminate senescent tumor cells or leveraging immune checkpoint inhibitors to enhance anti-tumor immunity could provide synergistic effects with CDK4/6 inhibitors. Investigating the tumor microenvironment and its role in modulating response to therapy will also be crucial in refining treatment strategies.

Future Directions

To improve the efficacy of Palbociclib-based therapies, future research should focus on identifying biomarkers that predict senescence escape. Transcriptomic and proteomic profiling of resistant cell populations could reveal key drivers of resistance, enabling the development of targeted therapies. Additionally, investigating the impact of Palbociclib on the tumor immune microenvironment may provide insights into how immune-modulating strategies can be integrated into treatment regimens. Overall, these findings underscore the dual nature of Palbociclib's effects in ER+ breast cancer while it effectively induces senescence in a majority of cells, a subset can escape and develop resistance through Cyclin D1 restoration, HER2 activation, and SASP-mediated signaling. Addressing these resistance mechanisms through combination therapies and biomarker-driven approaches will be essential for improving long-term treatment outcomes.

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