

# Wip1 Inhibition Enhances the Therapeutic Efficacy of Palbociclib in Luminal A Breast Cancer by Modulating Cell Cycle Progression and Apoptosis

(luminal A breast cancer / CDK4/6 inhibitors / Wip1 phosphatase / cell cycle arrest / apoptosis)

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**Abstract.** Cyclin-dependent kinase 4/6 (CDK4/6) inhibitors such as palbociclib have improved the treatment of hormone receptor-positive, luminal A breast cancer; however, therapeutic resistance remains a major challenge. Wild-type p53-induced phosphatase 1 (Wip1) is a negative regulator of the p53 pathway and is often over-expressed in luminal breast cancers. This study aimed to determine whether Wip1 inhibition enhances the anti-proliferative and pro-apoptotic effects of the CDK4/6 inhibitor palbociclib in luminal A breast cancer cells and to elucidate the underlying

cell cycle-related mechanisms. MCF-7 cells were treated with palbociclib and Wip1 inhibitor GSK2830371, alone or in combination. Cell viability was assessed using the WST-1 assay, cell cycle distribution was analysed by flow cytometry, and apoptosis was evaluated using Annexin V/7-aminoactinomycin D staining. Expression of cell cycle regulators (CDK2, CDK4, CDK6, cyclin D1/D3, Rb, phospho-Rb) and p53-related proteins (p53, phospho-p53 Ser15, p21, p27) was determined by Western blot analysis. The combined treatment produced a concentration-dependent reduction in cell viability and a marked increase in both early and late apoptotic populations compared with monotherapies. While palbociclib alone induced G1 arrest, co-treatment with GSK2830371 shifted cells toward G2 accumulation. This was accompanied by enhanced phosphorylation of p53, up-regulation of p21 and p27, and dephosphorylation of Rb, indicating dual checkpoint engagement. These findings demonstrate that Wip1 inhibition augments palbociclib-mediated cell cycle arrest and apoptosis through modulation of the p53-Rb axis. The dual blockade of Wip1 and CDK4/6 may represent a promising therapeutic strategy for p53-proficient luminal A breast cancer.

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Abbreviations: ATM – ataxia telangiectasia mutated, CDK – cyclin-dependent kinase, DDR – DNA damage response, ECL – enhanced chemiluminescence, ER – oestrogen receptor, FBS – foetal bovine serum, FDA – Food and Drug Administration, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, GSK – GSK2830371 (Wip1 inhibitor), HER2 – human epidermal growth factor receptor 2, HRP – horseradish peroxidase, IgG – immunoglobulin G, PBS – phosphate-buffered saline, PR – progesterone receptor, PVDF – polyvinylidene difluoride, Rb – retinoblastoma, SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis, TNBC – triple-negative breast cancer, TP53 – tumour protein p53, WST-1 – water-soluble tetrazolium 1.

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## Introduction

Breast cancer is the most prevalent malignancy among women worldwide, representing a complex disease arising from the interplay of genetic and environmental factors. Current epidemiological data indicate that, as of 2022, the global incidence of breast cancer has reached approximately 2.3 million cases and 685,000 deaths, making it the most frequently diagnosed malignant neoplasm among women worldwide (Bray et al., 2024).

Projections by the International Agency for Research on Cancer suggest that the incidence will exceed 3 million cases annually by 2050, underscoring the urgent need to reinforce comprehensive global strategies for prevention, early detection, and treatment (Wild et al., 2020). Based on the immunohistochemical expression of hormone receptors, breast cancer can be classified into four subtypes: oestrogen receptor positive (ER<sup>+</sup>), progesterone receptor positive (PR<sup>+</sup>), human epidermal growth factor receptor positive (HER2<sup>+</sup>), and triple-negative breast cancer (TNBC), which is characterized by the absence of any of these receptors (Shaath et al., 2021). ER-positive breast cancer can be further divided into various pathological subtypes, including ductal or mixed ductal and lobular, mucinous, and tubular carcinomas, collectively referred to as luminal breast cancers (Ignatiadis and Sotiriou, 2013). Although major progress has been achieved in breast cancer treatment in recent years, the biological heterogeneity of the disease and treatment-induced selective pressure still make long-term disease control difficult. In the metastatic stage, the development of endocrine resistance and the adaptive response of the tumour microenvironment restrict the effectiveness of current targeted therapies. Thus, new treatment strategies aimed at overcoming resistance mechanisms are still needed (Ambere et al., 2025).

Cell cycle checkpoints play a critical role in cancer pathogenesis, and their dysregulation facilitates uncontrolled cell division and development of malignancies (Almalki, 2023). The G1/S transition, a crucial step in cell cycle progression, is regulated by the balance between cyclin-dependent kinases (CDKs) and tumour suppressor protein p53. CDK4 and CDK6 promote progression from the G1 phase to the S phase by hyperphosphorylating the retinoblastoma (Rb) protein (Matthews et al., 2022). CDK4/6 inhibitors (CDK4/6i) selectively inhibit cyclins 4 and 6, helping to restore normal cell cycle regulation and block cell proliferation in various cancers, including breast cancer (Sofi et al., 2022; Almalki, 2023). Three CDK4/6 inhibitors – palbociclib, ribociclib and abemaciclib – have received Food and Drug Administration (FDA) approval so far, changing the treatment landscape (Abdelmalak et al., 2022). Although CDK4/6 inhibitors have provided a significant improvement in progression-free survival in HR-positive/HER2-negative advanced breast cancer, overall survival outcomes remain heterogeneous across different agents, and some patients experience early resistance. Mechanisms such as RB1 loss, cyclin E-CDK2 activation, and reprogramming of the PI3K/MAPK pathway have been associated with primary or acquired resistance to these drugs. In addition, the absence of reliable predictive biomarkers and the limited durability of response pose major challenges to treatment optimization (Ambere et al., 2025). Despite their significant benefits, the limited efficacy of CDK4/6 inhibitors in breast cancer underscores the need for ongoing research to understand resis-

tance mechanisms and optimize treatment strategies (Kishino et al., 2020; Cetin et al., 2022). This situation underscores the clinical importance of investigating complementary targets to CDK4/6 blockade.

Wild-type p53-induced phosphatase 1 (Wip1), encoded by *PPM1D*, is a serine/threonine phosphatase that dephosphorylates key DNA damage response (DDR) proteins including ataxia telangiectasia mutated (ATM), Chk1/2 and p53, thereby turning off checkpoint signalling and enabling cell cycle re-entry after DNA repair (Freeman and Monteiro, 2010). Over-expression or amplification of *WIP1* is commonly observed in cancers (approximately 8–10 % of breast cancers) and is generally associated with poor prognosis (Freeman and Monteiro, 2010; Portman et al., 2020). In luminal A (and B) breast cancer subtypes, *WIP1* amplification frequently co-occurs with intact TP53 and increased oestrogen receptor activity (Nahta and Castellino, 2021). Wip1 over-expression suppresses tumour suppressors such as p16/INK4A, facilitating the G1/S transition (Nahta and Castellino, 2021). WIP1 facilitates exit from the G2 checkpoint by suppressing the p53/p21 pathway following DNA damage, whereas its inhibition delays the G2/M transition, leading to p53/p21-dependent accumulation of cells in G2 and their subsequent entry into senescence (Pechackova et al., 2016). In addition, Wip1 negatively regulates p53 and other cell cycle checkpoints. Wip1 inhibition enhances cell cycle arrest by increasing p53 activity and Rb dephosphorylation (Emelyanov and Bulavin 2015; Nahta and Castellino, 2021). These observations underscore the Wip1's potential role in promoting cell cycle progression and endocrine therapy resistance in luminal breast cancers. Given that Wip1 is particularly involved in luminal-type cancers, these tumours are considered primary targets for Wip1-specific therapies.

Wip1 not only facilitates the G1/S transition by suppressing p16/INK4A and p53/p21 signalling but also promotes cell cycle re-entry by dephosphorylating p53 and Chk1/2 following DNA damage, thereby enabling G2/M transition (Freeman and Monteiro, 2010; Pechackova et al., 2016). Therefore, while CDK4/6 inhibition blocks cells at the G1/S checkpoint, Wip1 inhibition interferes with recovery from the G2/M checkpoint through sustained activation of the p53 pathway. The concomitant blockade of both checkpoints – G1/S via CDK4/6 inhibition and G2/M via Wip1 suppression – may synergistically restrict proliferation in p53-wildtype, Rb-proficient luminal A breast cancer cells.

Here we aim to examine the impact of Wip1 phosphatase inhibition on the efficacy of CDK4/6 inhibitor palbociclib in luminal A breast cancer cells and to assess the potential contribution of Wip1 suppression to this therapeutic strategy. In particular, by using palbociclib in combination with the Wip1 inhibitor GSK2830371 in MCF-7 cells, we aim to determine whether Wip1 inhibition can enhance the anti-proliferative and pro-apoptotic effects of CDK4/6 inhibitors.

## Material and Methods

### Cell culture and drug treatments

The human breast cancer cell line MCF-7 (ATCC<sup>®</sup> HTB-22<sup>™</sup>) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all obtained from Gibco/Thermo Fisher Scientific, Waltham, MA). The cells were maintained at 37 °C in a humidified incubator with 5 % CO<sub>2</sub>. Palbociclib and GSK2830371 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell viability assay (MTT)

Cell viability was assessed using the water-soluble tetrazolium salt assay. MCF-7 cells were exposed to palbociclib and GSK2830371 for 72 hours. Dimethyl sulphoxide (DMSO) was used as the solvent control. Following the incubation period, 10 µl of water-soluble tetrazolium 1 (WST-1) solution was added to each well and further incubated for 2 hours at 37 °C in a humidified incubator with 5 % CO<sub>2</sub>. The absorbance was then measured at 450 nm using a Multiskan Spectrum microplate reader (Thermo Labsystems, Waltham, MA). Cell viability was calculated according to the formula  $\text{Viability (\%)} = [(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}})] \times 100$ .

### Cell cycle analysis

The Muse Cell Cycle Assay Kit was utilized to analyse the cell cycle following the manufacturer's instructions. Briefly, cells were exposed to the specified concentrations of palbociclib or GSK2830371 for the indicated time points in triplicate. After incubation, the cells were collected, washed with 1 × phosphate-buffered saline (PBS) and fixed overnight in 70 % ethanol. Subsequently, the cells were treated with 200 µl of Muse Cell Cycle reagent (Millipore, Austin, TX), and cell cycle distribution was assessed using the Muse Cell Analyzer (Millipore).

### Apoptosis analysis (Annexin V/7-AAD assay)

Apoptotic cell death was evaluated using the Muse<sup>®</sup> Annexin V and Dead Cell Assay Kit (Luminex/Millipore). After 72 h of treatment with palbociclib and/or GSK2830371, both adherent and floating MCF-7 cells were collected, washed once with Ca<sup>2+</sup>-containing PBS and resuspended at 1–5 × 10<sup>5</sup> cells/ml. Each sample (100 µl) was mixed with an equal volume of Annexin V/7-AAD reagent and incubated for 20 min at room temperature, protected from light. Samples were analysed within 1 h using the Muse Cell Analyzer (Luminex/Millipore), and populations of viable, early-apoptotic, late-apoptotic and necrotic cells were quantified automatically. Results were expressed as the mean ± SD from three independent experiments.

### Protein extraction and Western blot analysis

After drug treatments, cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Complete, Roche, Mannheim, Germany) and 1 mM Na<sub>3</sub>VO<sub>4</sub>. The lysates were centrifuged at 12,000 × g for 10 min at 4 °C to remove the debris. Total protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce<sup>™</sup> BCA Protein Assay Kit, Thermo Fisher Scientific) with bovine serum albumin (BSA) as a standard. Absorbance was measured at 562 nm, and sample concentrations were calculated from the standard curve. Equal protein amounts (20–30 µg) were mixed with Laemmli sample buffer, denatured at 95 °C for 5 min, separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting as previously described (Nahta and Castellino, 2021). Briefly, the membranes were incubated overnight at 4 °C with primary antibodies following standard procedures. The primary antibodies used included mouse anti-Wip1, mouse anti-p53 and mouse anti-p21 (1 : 1000 dilution), along with horseradish peroxidase (HRP)-conjugated secondary antibodies such as anti-mouse IgG-HRP or anti-rabbit IgG-HRP, obtained from Santa Cruz Biotechnology. Polyclonal antibodies such as rabbit anti-CDK2, anti-CDK4, anti-p21, anti-p27Kip1, anti-CyclinD1, mouse anti-CDK6, anti-CyclinD3, anti-p18 and rabbit Phospho-Rb (Ser807/811) mAb and rabbit Phospho-p53 (Ser15) (all 1 : 1000 dilution) were sourced from Cell Signaling Technology (Danvers, MA). Mouse anti-GAPDH was acquired from ProteinTech (Rosemont, IL). The enhanced chemiluminescence (ECL) reagent was purchased from Bio-Rad (Hercules, CA), and membrane visualization was performed using the ChemiDoc-ItR2 Digital Imager (UVP, UK) system.

### Preparation of graphics and statistical analysis

Graphical representations were generated using Origin 8.0 software. Data are expressed as the mean ± standard deviation (SD) from at least three independent experiments performed in triplicate. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons among treatment groups. A P value of ≤ 0.05 or ≤ 0.01 was considered statistically significant.

## Results

### Combined inhibition of Wip1 and CDK4/6 reduces MCF-7 cell viability in a concentration-dependent manner

To investigate the role of the oncogenic Wip1 phosphatase on the efficacy of CDK4/6 inhibitors and to assess the potential contribution of Wip1 suppression to this therapeutic strategy, we used the MCF-7 cell line harbouring an amplified PPM1D/Wip1 (Gilmartin et al., 2014). The allosteric small molecule inhibitor GSK 2830371 and palbociclib were used to provide inhibition to Wip1

and CDK4/6, respectively (Emelyanov and Bulavin, 2015; Abdelmalak et al., 2022). MCF-7 cells were incubated for 72 h with different concentrations of GSK2830371 (GSK) and palbociclib (palbo) either alone or in combination. Subsequently, cell viability was assessed using the WST-1 assay. As seen in Figure 1, palbo treatment applied for 72 hours caused a significant decrease in cell viability at high doses (200, 250, 500 nM), while low-dose palbo (50, 100, 150 nM) and GSK (1, 2.5, 5  $\mu$ M) treatments had no significant effect (Fig. 1).

However, combined treatments of GSK and palbo significantly decreased cell viability after 72 h exposure. The lowest viability rate (52 %) was noted in the palbo 250 nM + GSK 2.5  $\mu$ M combination group. Analysis of the drug-drug interaction patterns using the Bliss independence framework revealed condition-dependent synergistic effects between palbociclib and the GSK\* compound. Notably, the combinations pal250 + gsk2.5 and pal100 + gsk5 yielded  $\Delta$ Bliss values of approximately +16.5 and +13.5, respectively, indicating measurable synergistic enhancement under these specific concentration pairs. In contrast, the pal250 + gsk5 combination produced a  $\Delta$ Bliss value of +1.4, consistent with an interaction profile close to additivity and suggesting only a mild synergistic tendency. These findings suggest that combination synergy occurs only at certain dose ranges, not across all doses. Consequently, at certain dose combinations, administration of GSK plus palbo resulted in a more pronounced increase in efficacy compared to single agents.

MCF-7 cells were treated with increasing concentrations of palbociclib (100–500 nM) and/or GSK2830371 (1–5  $\mu$ M) for 72 h. Cell viability was assessed using the WST-1 colorimetric assay. Data are presented as mean  $\pm$  SD from three independent biological replicates, each performed in triplicate. Drug-drug interactions were assessed using the Bliss independence model. The pal250 + gsk2.5 and pal100 + gsk5 combinations exhibited clear synergy, with  $\Delta$ Bliss values of +16.5 and +13.5, respectively, whereas pal250 + gsk5 showed a  $\Delta$ Bliss of +1.4,

consistent with an interaction pattern close to additivity. Synergistic combinations ( $\Delta$ Bliss > 10) are marked with † on the graph.

### Dual inhibition of Wip1 and CDK4/6 induces G1 and G2 phase accumulation in MCF-7 cells

To determine how Wip1 and CDK4/6 inhibition influences cell cycle progression, MCF-7 cells were treated with GSK2830371 (1–5  $\mu$ M), palbociclib (100–250 nM), or their combinations for 72 hours and analysed using the Muse Cell Cycle Assay (Fig. 2).

As expected, palbociclib treatment caused a dose-dependent accumulation of cells in the G1 phase with a concomitant decrease in S and G2 populations, consistent with CDK4/6 inhibition. GSK treatment alone caused a modest increase in the proportion of cells in the G0/G1 phase, particularly at 2.5  $\mu$ M, consistent with Wip1 inhibition-mediated activation of p53-p21 signalling. However, this increase was not as pronounced as that observed with palbociclib. When combined, palbociclib and GSK2830371 did not further increase the G1 arrest caused by palbociclib alone but instead led to a distinct accumulation of cells in the G2 phase, most evident at 200 nM palbociclib plus 5  $\mu$ M GSK. These findings indicate that while CDK4/6 inhibition primarily enforces a G1 block, additional Wip1 inhibition shifts cells toward G2 accumulation, implying engagement of a second checkpoint through p53 activation.

Cells were exposed to palbociclib (100–500 nM) and/or GSK2830371 (2.5–5  $\mu$ M) for 72 h, fixed in 70 % ethanol and stained using the Muse® Cell Cycle Assay Kit. The DNA content was analysed by the Muse Cell Analyzer. Histograms show representative results and bar graphs display the distribution of cells across cell cycle phases. Values represent mean  $\pm$  SD from three independent experiments (N = 3). Statistical analysis was performed using one-way ANOVA with Bonferroni's post-hoc correction (\*P < 0.001).

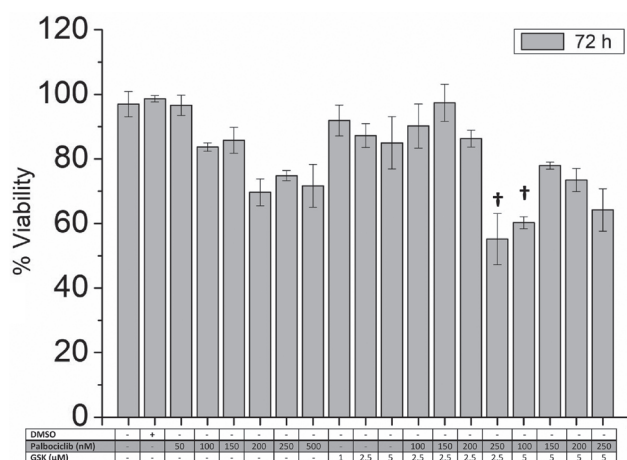


Fig. 1. Combined inhibition of Wip1 and CDK4/6 reduces MCF-7 cell viability in a concentration-dependent manner.

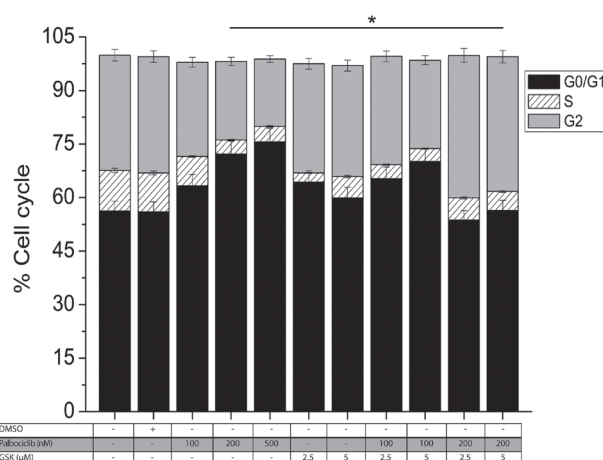


Fig. 2. Dual inhibition of Wip1 and CDK4/6 induces G1 and G2 phase accumulation in MCF-7 cells.

### *Wip1 inhibition enhances palbociclib-induced apoptosis in luminal A breast cancer cells*

Since we found that Wip1 inhibition enhanced the palbo-mediated decrease in MCF-7 cell viability, we measured whether the decrease in viability was due to cell death. Therefore, we performed an Annexin V/7AAD analysis to examine the apoptotic cell death in response to different concentrations of palbo and/or GSK treatment for 72 h. Increasing concentrations of GSK treatment resulted in different rates of apoptosis: the highest apoptosis rate detected was 17.03 % in response to 5  $\mu$ M GSK treatment for 72 h. Similar results were also obtained in response to palbo treatment. According to the results, combined treatment of GSK and palbo significantly enhanced apoptosis compared to palbo or GSK treatment alone for 72 h. The combination of palbo and GSK treatment enhanced apoptosis rates in a concentration-dependent manner. As shown in Figure 3A and B, the highest rates of apoptosis were detected in response to palbo 250 nM + GSK 2.5  $\mu$ M and palbo 250 nM + GSK 5  $\mu$ M combination (Fig. 3A–B). These findings highlight the potential of GSK and palbo combinations to enhance apoptosis rates in a concentration-dependent manner.

### *Combined Wip1 and CDK4/6 inhibition modulates the p53-Rb axis and cell cycle regulatory proteins*

In order to identify the mechanisms underlying the effect of the combined use of GSK and palbo, we examined the expression of cyclin-dependent kinases 2, 4, 6 and the regulatory proteins p21, p27, p18, Rb and p53, which are involved in cell cycle regulation and apoptosis, respectively. In addition, we checked the expression of Wip1 to ensure its inhibition as well as the expression of GAPDH as a control for equal loading. As illustrated in Figure 4, treatment of MCF-7 cells with 2.5 or 5  $\mu$ M of GSK or with any combination of GSK and palbo resulted in the inhibition of Wip1 expression. Palbo treatments of MCF-7 cells at any concentration did not result in reduction in the levels of Wip1 protein. It is notable that the levels of the CDK2 and CDK6 proteins were reduced in response to palbo treatment, either alone or in combined treatments with GSK. No reduction was observed in CDK4 levels in response to palbo, GSK, or combination treatments (Fig. 4). Furthermore, the total and phosphorylated levels of p53 were examined. It was observed that the levels of total p53 were elevated with GSK treatment. It is noteworthy that palbo treatment, either alone or in combination with GSK, resulted in the phosphorylation of p53 (Fig. 5). It was also detected that p21 levels remained unaltered in response to palbo treatment alone; however, a notable increase was evident when palbo was combined with GSK. GSK treatment alone also resulted in an increase in p21 protein levels (Fig. 5). Furthermore, a slight increase in p18 levels was observed in response to 200 and 500  $\mu$ M of palbo treatment, whereas combination with GSK did not induce any change

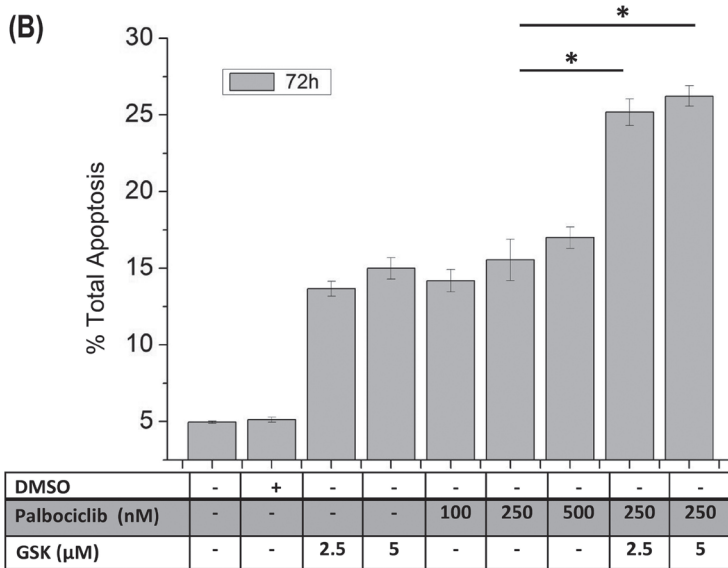
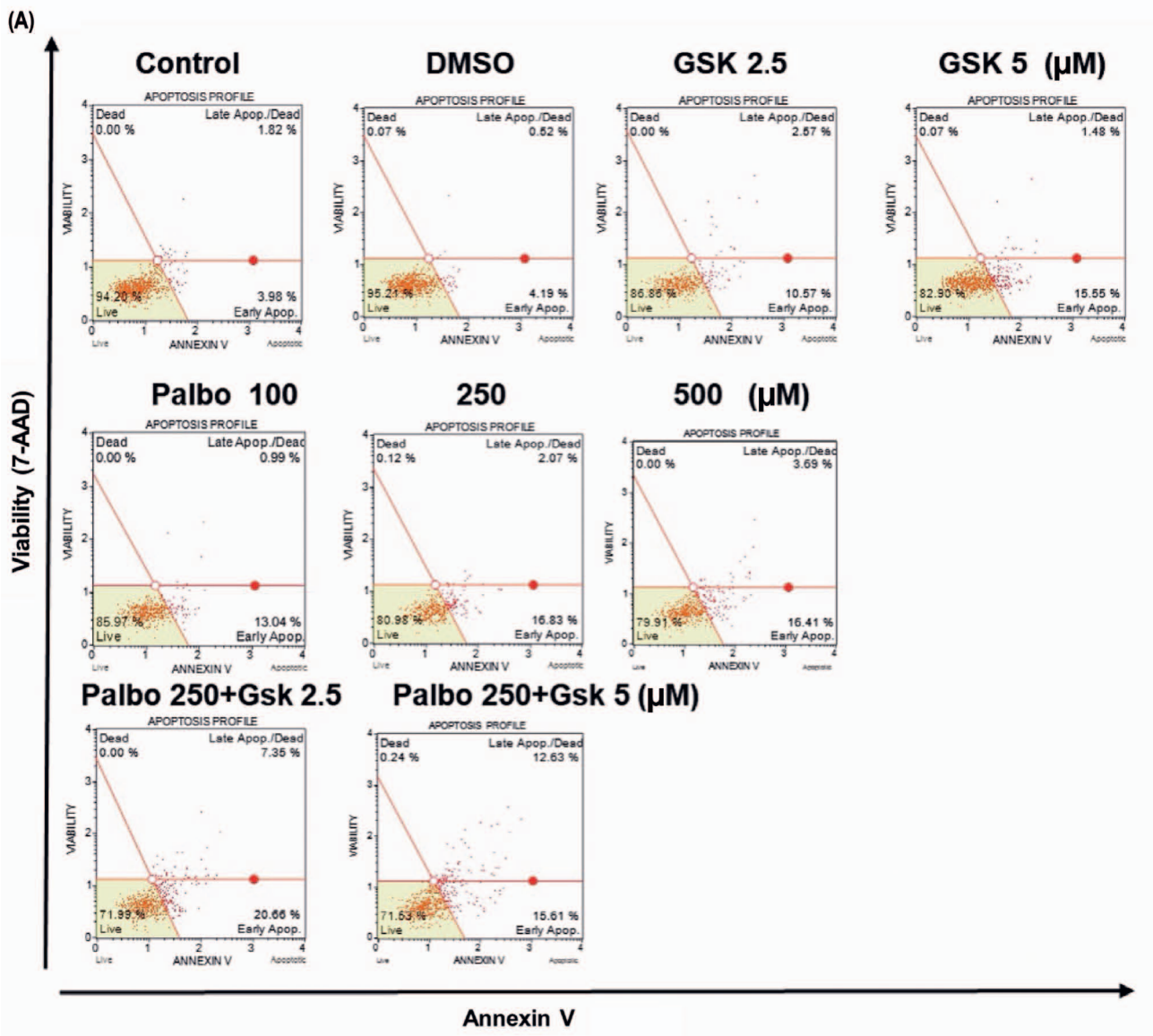
compared to the control. Notably, p27 levels increased in response to 200 and 500  $\mu$ M of palbo treatment and any combination treatments with GSK. Additionally, the phosphorylation of Rb protein was found to be decreased with the GSK treatment, either alone or in combination with palbo (Fig. 5). Thus, the results demonstrate that the combination of GSK and palbo treatments led to a marked reduction in the protein levels of Wip1, CDK2 and CDK6. While Wip1 activity was directly inhibited by GSK2830371, and CDK6 by palbociclib, the decrease in CDK2 levels likely reflects a secondary effect associated with p53-p21-mediated cell cycle regulation rather than direct inhibition. Furthermore, combination treatments resulted in the phosphorylation of p53 and dephosphorylation of the Rb protein, as well as the modulation of cell cycle regulatory proteins, including p18, p21 and p27. Hence, it is proposed that the combination of Wip1 and CDK4/6 inhibition, which leads to decreased expression of Wip1, CDK2, and CDK6 and modulation of p21 and p18, contributes to enhanced apoptosis induction. Quantification of the Western blot band intensities is provided separately in the study, and the corresponding data are presented in Supplementary Figure 1 and Supplementary Figure 2.

## **Discussion**

The present study demonstrates that the inhibition of Wip1 enhances the therapeutic effects of CDK4/6 inhibitor palbociclib in luminal A breast cancer cells. The combination treatment resulted in significantly greater reduction in cell viability and higher induction of apoptosis. Our findings strongly suggest that suppression of Wip1, particularly when combined with CDK4/6 inhibitors such as palbociclib, can enhance therapeutic efficacy.

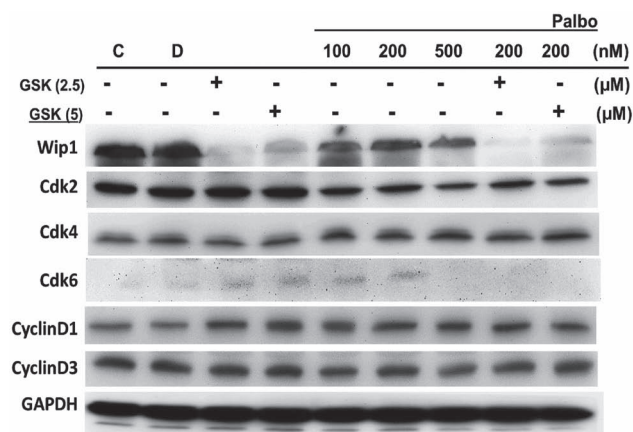
This conclusion is supported by several lines of evidences. First, the combination of palbociclib and GSK2830371 significantly suppressed the cell viability of MCF-7 breast cancer cells. Second, in apoptosis analyses, the combination treatment increased cell death in a concentration-dependent manner. Third, in cell cycle analyses, the combination treatment induced accumulation of cells in the G2 phase, demonstrating effective modulation of the cell cycle. Fourth, protein analysis findings indicated that Wip1 suppression was effective through mechanisms such as CDK2/6 inhibition, phosphorylation of p53 and dephosphorylation of Rb.

In previous studies, it was shown that treatment of MCF-7 cells with palbociclib led to cell cycle arrest at the G1/S phase, increasing the proportion of cells in the G1 phase and decreasing the proportion in the S phase. However, no significant G2/M arrest was observed (Abdelmalak et al., 2022; Sofi et al., 2022; Hunter et al., 2023). In cases of resistance to palbociclib, it is thought that the cells bypass the G1/S checkpoint dependency, and thus the G2/M phase becomes critical (Hunter et al., 2023). In our study, we showed that palbociclib induced a G1 phase arrest, whereas GSK2830371 alone did not



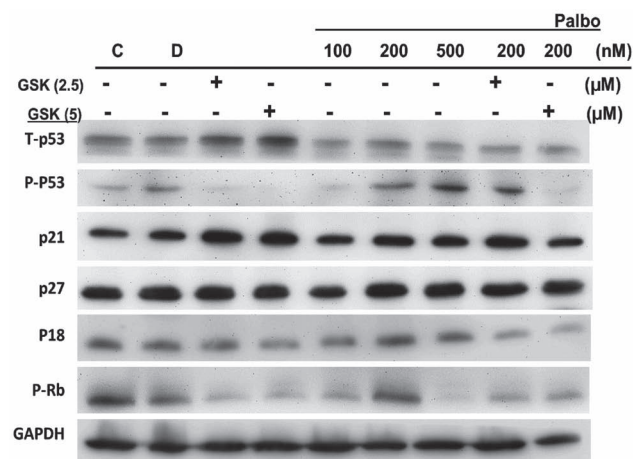
**Fig. 3.** Wip1 inhibition enhances palbociclib-induced apoptosis in luminal A breast cancer cells. **(A)** Representative Annexin V/7-AAD dot plots of MCF-7 cells treated with palbociclib (100–500 nM), GSK2830371 (2.5–5 µM), or their combination for 72 h. Populations were identified as follows: viable (Annexin V<sup>-</sup>/7-AAD<sup>-</sup>), early apoptotic (Annexin V<sup>+</sup>/7-AAD<sup>-</sup>), late apoptotic (Annexin V<sup>+</sup>/7-AAD<sup>+</sup>) and necrotic (Annexin V<sup>-</sup>/7-AAD<sup>+</sup>). **(B)** Quantitative analysis of apoptotic populations obtained from three independent biological replicates (mean ± SD). Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test (\*P < 0.0001). Combined treatment resulted in a consistent and statistically significant increase in both early and late apoptotic fractions compared with monotherapies.

DMSO	-	+	-	-	-	-	-	-	-
Palbociclib (nM)	-	-	-	-	100	250	500	250	250
GSK (µM)	-	-	2.5	5	-	-	-	2.5	5



**Fig. 4.** Combined Wip1 and CDK4/6 inhibition modulates the p53-Rb axis and cell cycle regulatory proteins.

Whole-cell lysates from MCF-7 cells treated with palbociclib (200 nM), GSK2830371 (2.5 or 5  $\mu$ M), or both for 72 h were analysed by Western blotting. Representative blots are shown for Wip1, CDK2, CDK4, CDK6, cyclin D1, cyclin D3, and GAPDH as a loading control. Band intensities were quantified by densitometry, normalized to GAPDH, and expressed as mean  $\pm$  SD of three independent experiments (N = 3).



**Fig. 5.** Combined Wip1 and CDK4/6 inhibition enhances apoptosis-related signalling in MCF-7 cells.

MCF-7 cells were treated with palbociclib (200 nM), GSK2830371 (2.5 or 5  $\mu$ M), or their combination for 72 h. Western blot analysis was performed for total p53, phospho-p53 (Ser15), p21, p27, p18, phospho-Rb and GAPDH. Quantitative densitometry data represent mean  $\pm$  SD from three independent biological replicates (N = 3).

show a significant effect. Interestingly, the combination therapy increased the accumulation of cells in the G2 phase, indicating greater modulation of the cell cycle. In a recent study, Pechackova et al. (2016) suggested that Wip1 plays a pivotal role not only by targeting p53 but also by facilitating the resolution of the DNA damage response through ATM and H2AX phosphorylation, thus

influencing G1/S and G2/M transitions. Treatment of MCF-7 cells with GSK2830371 has been shown to induce cell cycle arrest at both the G1/S and G2/M phases (Pechackova et al., 2016). Other studies have also highlighted the importance of cycle arrest at G1/S, which prevents the transition to S, or at G2/M, which inhibits mitotic proliferation of cancer cells (Finn et al. 2009; Nahta and Castellino, 2021). Our findings suggest that Wip1 inhibition may synergize with CDK4/6 inhibitors in the regulation of cell cycle progression and may serve as a potential therapeutic approach in cases of resistance to CDK4/6 inhibitors.

Notably, previous research by Finn et al. (2009) and Kishino et al. (2020) reported that palbociclib treatment of MCF-7 cells did not induce apoptosis. In contrast, a different study has shown that abemaciclib, another CDK4/6 inhibitor, can trigger apoptosis in breast cancer cells (Torres-Guzmán et al., 2017). Our findings indicate that palbociclib instigates a considerable degree of apoptosis after 72 h of treatment in MCF-7 cells in a concentration-dependent manner. Thus, data by us and others suggest that the ability of CDK4/6 inhibitors to cause apoptosis is dependent on both the concentration and the duration of exposure. Regarding Wip1 inhibition, Ogasawara et al. (2015) demonstrated that Wip1 inhibition alone is effective in promoting apoptosis. In addition, other studies have shown that Wip1 inhibition not only contributes to apoptosis but also sensitizes MCF-7 cells to doxorubicin-induced apoptosis, exerting a synergistic effect to further enhance cell death (Kong et al., 2009; Pilevneli and Kilic-Eren 2021). Likewise, our results showed that Wip1 inhibition, in combination with CDK4/6 inhibitors, significantly increased apoptosis. In particular, combination treatment with GSK2830371 and palbociclib enhanced cell death in a concentration-dependent manner. These results highlight the potential of combining GSK2830371 and palbociclib to provide improved and effective therapeutic strategies in breast cancer cells.

Previous data indicate that Wip1 regulates the cell cycle via p53 and p21, while Wip1 inhibitor GSK2830371 increases p53 and p21 levels in the MCF-7 cell line (Emelyanov and Bulavin 2015; Nahta and Castellino, 2021). Our results are consistent with these reports and show an increase in the levels of p53 and p21 following treatment with GSK2830371, suggesting that apoptosis and cell cycle arrest are induced as a result. We examined Wip1 protein levels to confirm target engagement by the GSK2830371 inhibitor. Although GSK2830371 is primarily an allosteric phosphatase inhibitor, previous reports have shown that it can also promote Wip1 degradation through the ubiquitin-proteasome pathway (Gilmartin et al., 2014). Consistently, a reduction in Wip1 protein levels was observed in our study, indicating effective inhibition of Wip1 activity. This was further supported by the concomitant increase in phospho-p53 (Ser15) levels, consistent with functional suppression of Wip1-mediated dephosphorylation. Whittle et al. (2020) reported an increase in cyclin D1 protein levels in MCF-7 cells treated

with palbociclib. In our study, we did not detect any increase in either cyclin D1 or D3 protein levels. Effective suppression of cell proliferation requires dephosphorylation of Rb protein. The current data in the literature highlight the necessity of intact Rb for effective CDK4/6 inhibition, supporting its utility as a biomarker (Elango et al., 2019; Ji et al., 2019). Accordingly, a low level of Rb protein expression can render cells insensitive to CDK4/6 inhibitors (Kishino et al. 2020; Cetin et al. 2022). In our study, combination therapy with Wip1 and CDK4/6 inhibitors resulted in decreased CDK2 and CDK6 protein levels, increased phosphorylation of p53 and dephosphorylation of Rb protein, and increased protein levels of p21 and p27. Thus, our data showing dephosphorylation of the Rb protein and increased p21 and p27 protein levels further support the critical role of these regulatory proteins in inducing cell cycle arrest and induction of apoptosis during combination therapy. These findings suggest that suppressing the modulatory effects of Wip1 on the DNA damage response may help to improve the efficacy of CDK4/6 inhibitors.

At the molecular level, our findings provide an insight into how Wip1 inhibition cooperates with CDK4/6 inhibition. We found that Wip1 blockade by GSK2830371 increases p53 and p21 levels, consistent with the Wip1's known role in restraining the p53-p21 axis (Emelyanov and Bulavin 2015; Nahta and Castellino 2021). Palbociclib's efficacy, on the other hand, depends on intact Rb function and leads to Rb dephosphorylation and cell cycle arrest in Rb-proficient cells (Elango et al. 2019; Abdelmalak et al. 2022). In our combination-treated cells, we indeed saw dephosphorylation of Rb along with an increase in p21 and p27, which are critical enforcers of cell cycle arrest. Additionally, we detected a decrease in CDK2 and CDK6 protein levels, which might reflect downstream consequences of prolonged p53 activation. Our findings imply that Rb hypo-phosphorylation and up-regulation of CDK inhibitors may reinforce the G1 checkpoint, while increased p53 activity can enforce the G2/M checkpoint and trigger apoptosis. Thus, our data imply that suppressing Wip1 and CDK4/6 may ensure that the cell's two major tumour-suppressive pathways (Rb and p53) are both activated. This dual activation is likely responsible for the synergistic effects observed. It is also worth noting that loss of Rb or cyclin E up-regulation (driving CDK2 activity) are known mechanisms of resistance to CDK4/6 inhibitors (Finn et al., 2009); the strong induction of p21/p27 and reduction in CDK2 we observed with the combination could counteract such resistance mechanisms by inhibiting CDK2/cyclin E-driven S-phase entry.

The present study suggests that the suppression of the modulatory effects of Wip1 on the DNA damage response can improve the efficacy of CDK4/6 inhibitors. The findings of this study indicate that by impeding the capacity of cancer cells to recover from checkpoint activation via Wip1 inhibition, these cells may be propelled towards irreversible growth arrest or demise when CDK4/6 is concurrently blocked. This strategy has the potential to

be particularly advantageous in cases where tumours develop resistance to CDK4/6 inhibitors, as it targets an alternative vulnerability (the p53-dependent checkpoint) in those cells.

## Conclusion

The study shows that inhibiting Wip1 phosphatase enhances palbociclib's effects in luminal A breast cancer cells by modulating cell cycle progression and apoptosis. Wip1 inhibition suppresses CDK2/6 activity, causing G2-phase arrest, promoting p53 phosphorylation and facilitating Rb dephosphorylation. This approach may improve CDK4/6 inhibitor efficacy in p53-wildtype luminal breast cancers, warranting further preclinical and clinical studies.

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## Competing Interests

Authors have no financial interests to disclose.

## Author Contributions

Material preparation, data collection and analysis were performed by Gokhan Çolak, Nazlican Kaygusuz and Mehtap Kılıç Eren. Nezi Meydan and Esin Oktay contributed to the data analysis. Mehtap Kılıç Eren designed the study conception and wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Data Availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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