APPLICATION NOTE

Cyclin dependent kinase signaling in oncology: Evaluation of CDK4/6 inhibition in breast cancer cell lines via HTRF immunoassays.

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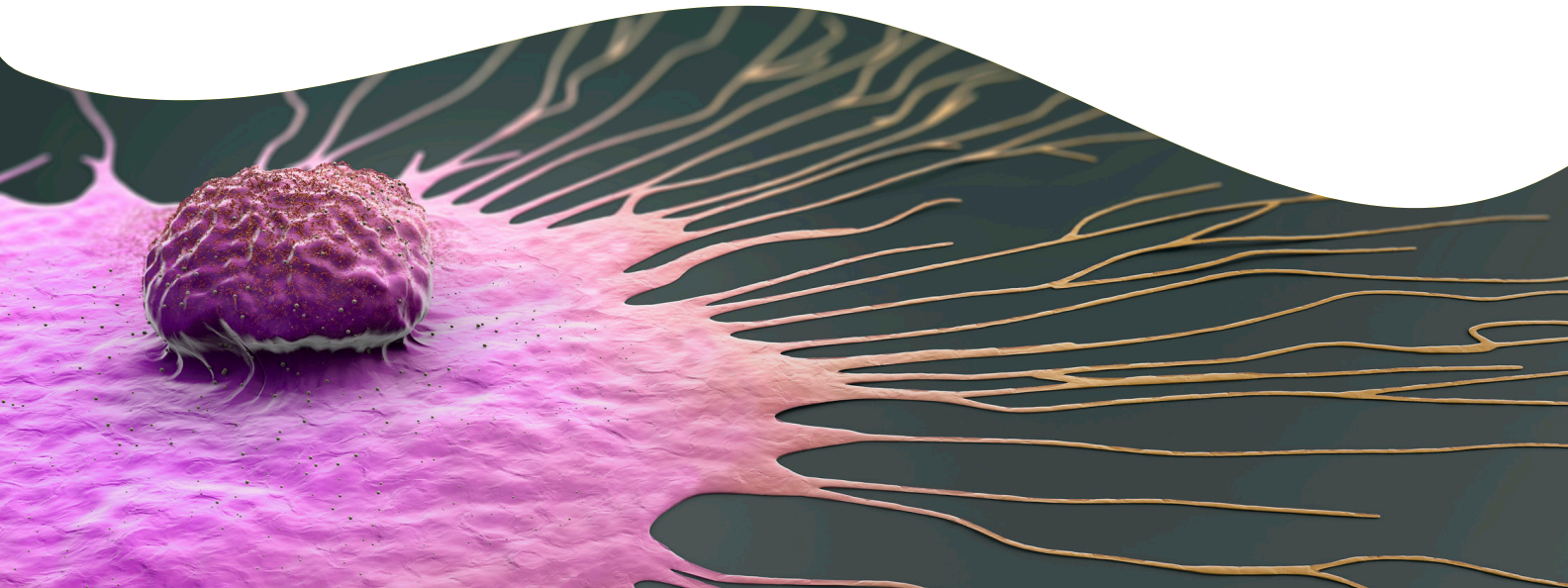
Introduction

Cyclin dependent kinases (CDKs) are key regulatory enzymes involved in cell proliferation by controlling cell cycle checkpoints and transcriptional events in response to extracellular and intracellular signals. CDKs bind to unique regulatory sub-units, known as cyclins, to form an active holoenzyme capable of modulating cellular proliferation by phosphorylating target proteins. The D-type cyclins and their partner kinases, CDKs 4 and 6, play a particularly important role in breast cancer. Cyclin D1-CDK4/6 complexes are critical regulators of the cell cycle transition from the G1 to S phase (Figure 1). To proceed through these phases, a cell must pass a restrictive checkpoint, tightly regulated in this case by the retinoblastoma tumor suppressor protein (Rb). The phosphorylation of Rb protein by CDK/Cyclin complexes is necessary for passage through this restriction checkpoint.[1,2]

Uncontrolled cell proliferation in many solid tumor types, including breast cancer, is caused by dysregulation of the cell cycle or genetic alterations in cell cycle regulatory proteins overriding many safeguards within the cell. Cyclin D1-CDK4/6 is the key controller of Rb protein phosphorylation to promote cellular proliferation.[3] Palbociclib, also known as PD0332991, is a small-molecule kinase inhibitor that blocks Cyclin D1-CDK4/6- mediated phosphorylation of the Rb protein to prevent E2F driven transcription of genes that commit the cell to DNA replication and cell division. This application note explored the effect of palbociclib treatment in two breast cancer cell lines (MCF-7 and HCC1187). The amount of phosphorylated Rb protein and Cyclin D1 protein levels were monitored by HTRF® immunoassays to examine the effect of CDK4/6 inhibition and Cyclin D1 regulation.



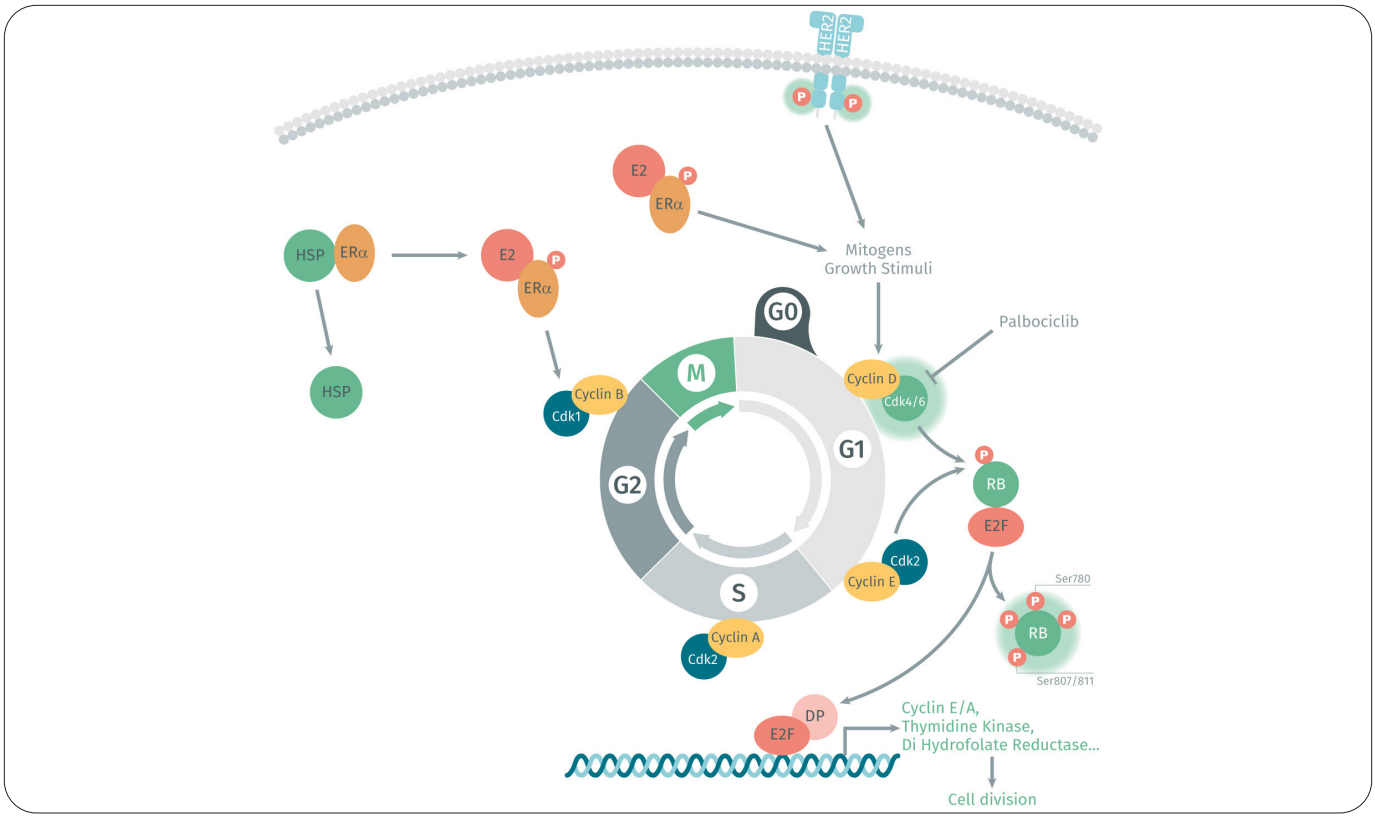


Figure 1: The Cyclin D/CDK4 and CDK6/Rb Pathway and Cell Cycle. Tumor suppressor retinoblastoma protein (Rb) is involved in regulating the transition from G1 to S phase.When activated by upstream signaling such as mitogenic growth factors, CDK4 and CDK6 form a complex with Cyclin D1 and phosphorylate Rb protein. Hyper-phosphorylation of Rb protein relieves its inhibition of E2F and allows transcription to occur and cells to progress into S phase. Palbociclib is a small-molecule CDK4/6 inhibitor which prevents phosphorylation of Rb protein and inhibits cell-cycle progression from G1 to S phase causing decreased proliferation.

Materials and methods

Cell-based materials: MCF-7 cells (ATCC, HTB-22), HCC1187 cells (ATCC, CRL-2322), Eagle's Minimum Essential Medium (EMEM) (ATCC, 30-2003), RPMI Media (ATCC, 30-2001), recombinant human insulin (Santa Cruz, sc-360248), Fetal Bovine Serum (FBS) heat inactivated (Thermo Fisher, 10082147), and Palbociclib (MedChemExpress, HY-50767).

HTRF assays: HTRF is a homogeneous time-resolved fluorescence immunoassay technology that provides a simple, no-wash strategy to detect and quantify proteins. It combines standard fluorescence resonance energy transfer (FRET) technology with time-resolved measurement of fluorescence, eliminating short-lived background fluorescence. For a sandwich assay, two antibodies that recognize a protein of interest are used, with one antibody coupled to a donor, and the other to an acceptor. If the two antibodies recognize the analyte, the donor will emit fluorescence upon excitation and the energy will be transferred to the nearby acceptor, giving specific acceptor fluorescence (Figure 2). Phosphorylated Rb (Ser780) was assayed with an HTRF detection kit (Revvity, 64RBS780PEG). Cyclin D1 was measured with HTRF detection kits for both Total Cyclin D1 (Revvity, 64CYCD1TPEG) and Phosphorylated Cyclin D1 Thr286 (Revvity, 64CYCD1T6PEG). HTRF 96-well low volume assay plates (Revvity, 66PL96025) were read on an EnVision® 2105 Multimode Plate Reader using laser excitation with APC 665 nm and Cy5 620 nm emission filters, and the LANCE/DELFIA Dual bias mirror. Both the 665 nm and 620 nm signals were collected, and the HTRF Ratio of acceptor-to-donor signal provided in the results was calculated as (665 nm/620 nm) x 10^4.

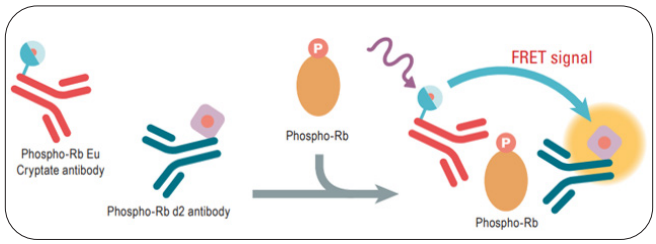


Figure 2: HTRF Assay Schematic. The schematic shows the detection of Phosphorylated Rb (Ser780) in a sandwich assay format using two labeled antibodies, Eu Cryptate donor and a d2 acceptor. FRET signaling only occurs if both antibodies are bound to the protein of interest and are in proximity to one another.

Cell-based experiments: phosphorylated RB protein

Detection: 25,000 cells/well were plated in 75 µL volume in a ViewPlate-96 well black, clear bottom assay plate (Revvity, 6005182) and allowed to adhere overnight. MCF-7 cells were plated in EMEM + 10% FBS + 0.01 mg/mL recombinant human insulin and HCC1187 cells were plated in RPMI + 10% FBS. Serial dilutions of palbociclib were first prepared in DMSO then diluted into each appropriate culture media at 4X final concentration and 25 µL was added to each well. Cells were stimulated overnight for approximately 20 hours. The 2-plate assay format for suspension cells was followed for both cell types. 70 µL of the stimulation media was removed from the assay plate leaving a volume of 30 µL in each well. Next, 10 µL of 4X supplemented lysis buffer was added to the cells which were incubated for 30 minutes at room temperature on an orbital shaker set to 350 rpm. Visual inspection confirming lysis was conducted using a light microscope. The HTRF assay was performed following the standard protocol in which 16 µL of lysate sample was added to a separate 96-well low volume HTRF assay plate for testing.

Cyclin D1 phospho/total detection: 25,000 cells/well were plated in 75 µL volume in a ViewPlate-96 well black, clear bottom assay plate (Revvity, 6005182) and allowed to adhere overnight. MCF-7 cells were plated in EMEM + 10% FBS + 0.01 mg/mL recombinant human insulin. Serial dilutions of palbociclib were first prepared in DMSO then diluted into culture media at 4X final concentration and 25 µL was added to each well. Cells were stimulated for 96 hours prior to measuring Total Cyclin D1 and Phosphorylated Cyclin D1 (Thr286). The 2-plate assay format for adherent cells was followed. The stimulation media was fully aspirated and 50 µL of 1X supplemented lysis buffer was added to the cells. Cell lysis occurred for 30 minutes at room temperature on an orbital shaker set to 350 rpm. After visual inspection under a microscope confirming lysis was complete, the ViewPlate-96 assay plates were sealed and stored at -80 oC until processing of each sample was completed. Assay plates were thawed at room temperature and 16 µL of lysate from each sample was transferred to a separate 96-well low volume HTRF assay plate for testing following the standard protocol.

Results and discussion

Palbociclib preferentially inhibits Rb phosphorylation in luminal breast cancer cells

Breast cancer can be characterized through gene expression profiling, assessment of biological properties, and morphological comparisons. In work performed by Finn *et al* 4, 47 well-characterized breast cancer cell lines were tested for sensitivity to CDK4/6 inhibition by palbociclib and they found that luminal, estrogen receptor-positive (ER+) breast cancer cell lines responded to palbociclib treatment through decreases to cell proliferation and phosphorylated Rb protein levels. However, non-luminal/basal estrogen receptor-negative (ER-) breast cancer cell lines were insensitive to palbociclib treatment. Figure 3 shows the HTRF results for phosphorylated Rb protein in two breast cancer cell lines, luminal ER+ MCF-7 cells and basal ER- HCC1187 cells. MCF-7 cells responded to drug treatment with a decrease in phosphorylated Rb (Ser780), while HCC1187 cells remained unaffected. It should be noted that HCC1187 cells natively have a reduced, yet detectable, level of total Rb as compared to MCF-7 cells.[4]

Prolonged palbociclib treatment causes accumulation of cyclin D1

While the response to palbociclib led to a rapid reduction of phosphorylated Rb protein in MCF-7 cells (Figure 3),

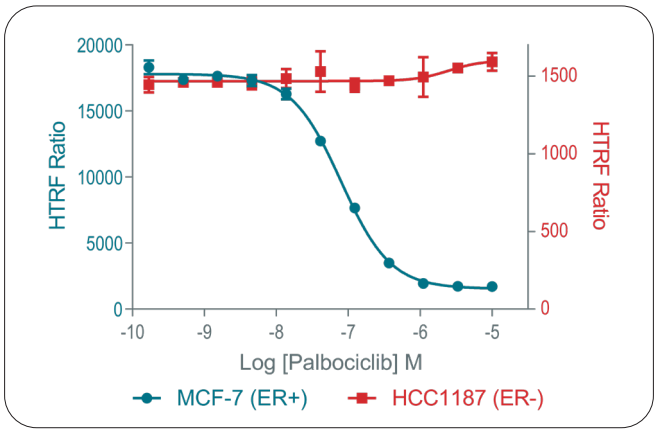


Figure 3: HTRF Phosphorylated Rb (Ser780) Assay. CDK4/6 inhibitor palbociclib was used to treat ER+ luminal breast cancer cells (MCF-7) or ER- basal breast cancer cells (HCC1187) overnight. Inhibition of Rb protein phosphorylation at Ser780 occurred preferentially in MCF-7 cells with an IC_{50} of 79.4 nM. There was no detectable effect on phosphorylated Rb protein in the HCC1187 cells with an IC_{50} of > 10 µM.

additional biological changes can take longer to develop. Prolonged exposure of MCF-7 breast cancer cells to palbociclib resulted in an approximate 2-fold accumulation of Cyclin D1 with a 1 µM dose of palbociclib and a mild 1.2-fold increase in phosphorylated Cyclin D1 (Thr286) was seen at the same 1 µM dose (Figure 4). Increases in Cyclin D1 with prolonged exposure to palbociclib in MCF-7 and other breast cancer cell types as measured by western blot have been reported in the literature.[2,5] It is believed to be evidence of cellular adaptation and evasion mechanisms used to bypass the inhibition of CDK4/6. Interestingly, the phosphorylation of Cyclin D1 at threonine 286 is reported to be involved in targeting the protein for degradation.[6] This data suggests that we may have captured a snapshot of the balance between accumulation and degradation of Cyclin D1 with prolonged CDK4/6 inhibition.

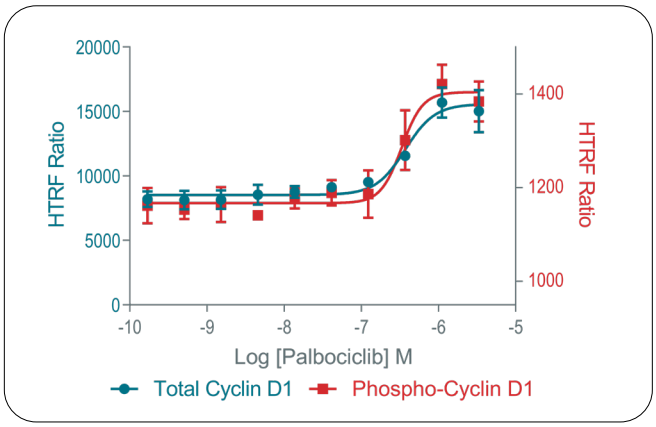


Figure 4: HTRF Total and Phospho-Cyclin D1 (Thr286) Assay. CDK4/6 inhibitor palbociclib was used to treat MCF-7 breast cancer cells for 96 hours. Total Cyclin D1 levels increased approximately 2-fold over vehicle control at 1 µM of palbociclib. Phospho-Cyclin D1 (Thr286) increased 1.2-fold over vehicle control at 1 µM of palbociclib. The changes in phosphorylated Cyclin D1 were consistent with the increase in total Cyclin D1 expression seen with drug treatment.

Summary

Numerous efforts have been made at regaining control of the cell cycle in solid tumors and other cancer cell types through the use of therapeutic drugs. Here we demonstrated the effect of palbociclib-directed CDK4/6 inhibition on two breast cancer cell types, one sensitive (MCF-7) and one resistant (HCC1187) to the drug. In the sensitive MCF-7 cells we not only observed a decrease in phosphorylated Rb (Ser780) protein with a short overnight treatment, but we also measured an accumulation of Cyclin D1 after prolonged 96 hr treatment. We also reported an increase in phosphorylated Cyclin D1 (Thr286), which is believed to be involved in the targeted degradation of the protein. Therefore, treatment with palbociclib may shift the equilibrium between the accumulation and post- phosphorylation degradation of Cyclin D1, a protein with a short half-life of approximately 30 minutes.[6] In both sets of experiments, HTRF technology allowed us to monitor the effect of the drug treatment on cells utilizing a simple, no-wash immunoassay format, measuring either total protein levels or specific phosphorylation events within the cells.

References

1. Garrido-Castro AC and Goel S. (2017) CDK4/6 Inhibitionin Breast Cancer: Mechanisms of Response and Treatment Failure. Current Breast Cancer Reports; 9(1).

2. Herrera-Abreu MT ***et al***. (2016) Early Adaptation and Acquired Resistance to CDK4/6 Inhibition in Estrogen Receptor–Positive Breast Cancer. Cancer Research; 76(8).

3. Ding L. ***et al***. (2020) The Roles of Cyclin-Dependent Kinases in Cell-Cycle Progression and Therapeutic Strategies in Human Breast Cancer. International Journal of Molecular Sciences; 21.

4. Finn RS ***et al***. (2009) PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines ***in vitro***. Breast Cancer Research; 11(5).

5. Chen F ***et al***. (2020) HSulf-1 and palbociclib exert synergistic antitumor effects on RB-positive triple-negative breast cancer. International Journal of Oncology; 27.

6. Alao JP. (2007) The regulation of cyclin D1 degradation: roles in cancer development and the potential for thera peutic invention. Molecular Cancer; 6(24).

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