The power of combining PROTAC degraders targeting cyclin-dependent kinases (CDK4/CDK6) with AlphaLISA *SureFire Ultra* screening technology for the study of cell cycle regulation in oncology.

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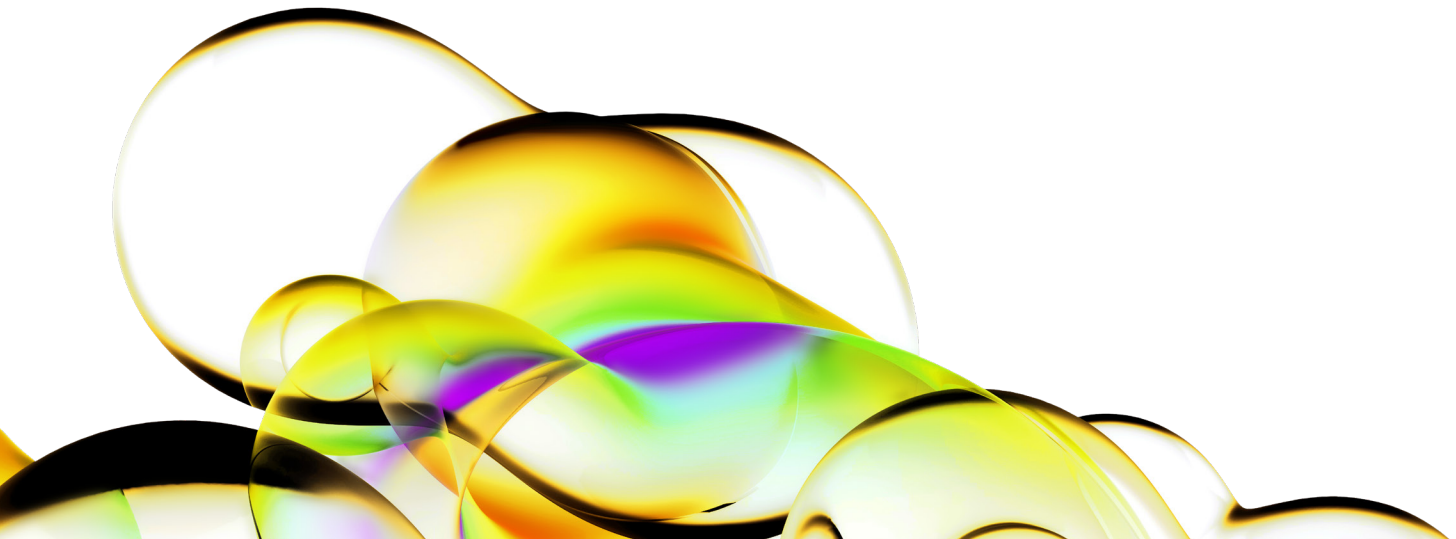
Introduction

The cell cycle is intricately regulated by a network of proteins, with Cyclin D1 playing a pivotal role in initiating cell cycle progression. Cyclin D1 forms complexes with Cyclin-Dependent Kinase 4 (CDK4) and CDK6 during the G1 phase, promoting the phosphorylation of the retinoblastoma protein (Rb).[1] This phosphorylation releases the inhibitory influence of Rb on the E2F transcription factors, allowing the expression of genes necessary for cell cycle entry. Subsequently, Cyclin E1 associates with CDK2, forming the Cyclin E/CDK2 complex, further contributing to Rb phosphorylation and progression through the G1 phase into the S phase.[2] Cyclin A2 is active in both the S and G2 phases, and continues the phosphorylation of Rb, coordinating DNA synthesis and preparation for mitosis.[3] Finally, during the G2 and M phases, Cyclin B1 associates with CDK1 to drive the final stages of cell cycle progression and mitotic entry.[4] The orchestrated activities of these cyclins and their associated CDKs ensure precise regulation of cell cycle transitions. Errors in the regulation of these key cell cycle proteins, particularly overexpression or mutations in Cyclin A2, Cyclin B1, Cyclin D1, and Cyclin E1, have been implicated as prognostic biomarkers in various cancers.[5,6] Aberrant activation of these cyclins and their associated CDKs can lead to uncontrolled cell proliferation, evading normal regulatory checkpoints and contributing to the development and progression of cancer.



AlphaLISA *Surefire Ultra* technology.

Due to their role in the activation of the cell cycle master regulator Rb, CDK4/6-specific inhibitors have been studied as treatments for many types of cancer, including metastatic breast cancer.[7] Interestingly, a relatively new class of hetero-bi-functional molecules known as PROteolysis TArgeting Chimeras (PROTACs) have been identified and successfully developed as oncotherapeutics.[8,9] Unlike conventional small molecule inhibitors that block enzymatic activity, PROTACs bind to target proteins and form a ternary complex with an E3-ubiquitin ligase. The target protein is then ubiquitinylated and subsequently proteolyzed by the proteasome, thereby effectively removing it from the cell.

Compounds or biomolecules that lead to cell cycle dysregulation can be used to study related pathologies. For example, Neuregulin-1 (NRG1) is a protein that functions as a pleiotropic growth factor and is known to stimulate nerve cell differentiation, neurite outgrowth, and synaptogenesis. However, NRG1 has also been linked to the development of certain diseases in the nervous system, cardiac system, and epithelial cells, as well as the development of several cancers, including breast cancer.[10,11]

This technical note examines the effect of treating MCF7 cells with the PROTAC BSJ-03-123 and NRG1 by measuring the levels of the cell cycle regulators Cyclin D1, CDK4/6, total and phospho-Rb, Cyclin A2, B1, E1, and CDK2 using AlphaLISA™ *SureFire™ Ultra™* (ALSU) Immunoassay Kits (Revvity). A diagram of the cell cycle regulatory network and the effect of PROTAC and NRG1 treatment on MCF7 cells is provided in Figure 1. The results from this work demonstrate that ALSU technology is capable of detecting changes in the expression and phosphorylation status of key cell cycle regulators in response to treatment with PROTAC and NRG1. These findings highlight the versatility and sensitivity of ALSU technology in elucidating the intricate molecular responses associated with PROTAC and growth factor treatments, contributing to a deeper understanding of cell cycle regulation and potential therapeutic strategies.

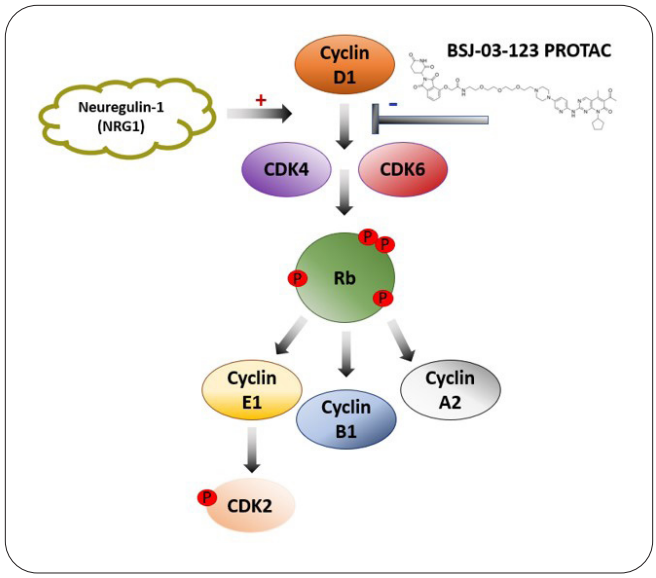


Figure 1: A simplified diagram of the cell cycle regulatory protein network depicting the expression level relationship between each of the proteins analyzed in this tech note. The effect of the CDK6 PROTAC BSJ-03-123 and pleiotropic growth factor, NRG1 is also shown.

Materials and methods

Cell culture, treatment, and lysis

MCF7 cells (ATCC, HTB-22) were grown at 37 °C with 5% CO2 in Minimum Essential Medium (MEM, Gibco 11095) supplemented with 10% FCS, 1% penicillin/streptomycin (Gibco, 15140122). MCF7 cells (passage 13) were seeded at a density of 40,000 cells/well in a 96-well plate and incubated overnight at 37 °C with 5% CO_2.

To evaluate the effect of CDK4 and CDK6 degradation, cells were treated with a dose range of BSJ-03-123 PROTAC (MedChemExpress, HY-11556) for 24 hours in complete MEM medium containing 10% FCS at 37 °C and 5% CO_2.

To assess the upregulation of the cell cycle proteins, MCF7 cells were treated with 20 µM Wortmannin (PI3K/AKT inhibitor, Calbiochem 681676) for 3 hours in serum free MEM, and then further stimulated with a dose range of NRG1 (Abcam, ab282401) for 24 hours in serum free MEM at 37 °C and 5% CO_2.

Following treatments, the media was removed and cells were lysed in 200 µL of Lysis Buffer following the protocol described in the ALSU Guide, resulting in ~2,000 cells/data point. A total volume of 50 µL of Lysis Buffer was added to the wells used for measuring CDK6 due to low expression levels in MCF7 cells resulting in ~8,000 cells/data point.

The following AlphaLISA *SureFire Ultra* assay kits from Revvity were used to complete these experiments:

Target	Catalog no.
Cyclin A2 (Total)	ALSU-TCYCA2-A500
Cyclin B1 (Total)	ALSU-TCYCB1-A500
Cyclin D1 (Total)	ALSU-TCYCD1-A500
Cyclin E1 (Total)	ALSU-TCYCE1-A500
Phospho CDK2 (Thr160)	ALSU-PCDK2-A500
CDK2 (Total)	ALSU-TCDK2-A500
CDK4 (Total)	ALSU-TCDK4-A500
CDK6 (Total)	ALSU-TCDK6-A500
Phospho-Rb (Ser780)	ALSU-PRB-C500
Phospho-Rb (Ser807/811)	ALSU-PRB-A500
Phospho-Rb (Thr821/826)	ALSU-PRB-B500
Rb (Total)	ALSU-TRB-A500

Data analysis

The assay plates (OptiPlate 384-well, Revvity, #6007290) were read on an EnVision 2105 Multimode Plate Reader with the default AlphaLISA settings. Results represent the mean ± SD for triplicate samples and are representative of three independent experiments. Data was analysed in GraphPad Prism (version 8, GraphPad Software Inc) using a non-linear four parameter logistic regression for the dose-response curves.

Principle of Alphalisa *Surefire Ultra* detection kits

AlphaLISA technology is a fast, highly sensitive, homogeneous, no-wash assay platform that can be performed in a microplate format. AlphaLISA assays require two bead types: Donor beads and Acceptor beads. In the AlphaLISA *SureFire Ultra* assay, Donor beads are coated with streptavidin to capture one of the detection antibodies, which is biotinylated. Acceptor beads are coated with a proprietary Capt*Sure*™ agent that immobilizes the other antibody, labeled with a Capt*Sure*™ tag. In the presence of a target protein, the two antibodies bring the Donor and Acceptor beads close together. Upon excitation at 680 nm, a photosensitizer inside the Donor bead converts ambient oxygen to an excited singlet state. Singlet oxygen diffuses up to 200 nm to produce a chemiluminescent reaction in the Acceptor bead, leading to light emission at 615 nm. If an Acceptor bead is not in proximity of a Donor bead, little to no signal is produced over background. AlphaLISA signal is measured on the EnVision 2105 Multimode Plate Reader using default settings for AlphaLISA with excitation at 680 nm and emission at 615 nm. A schematic of the AlphaLISA *SureFire Ultra* assay for a phosphorylated target is shown in Figure 2.

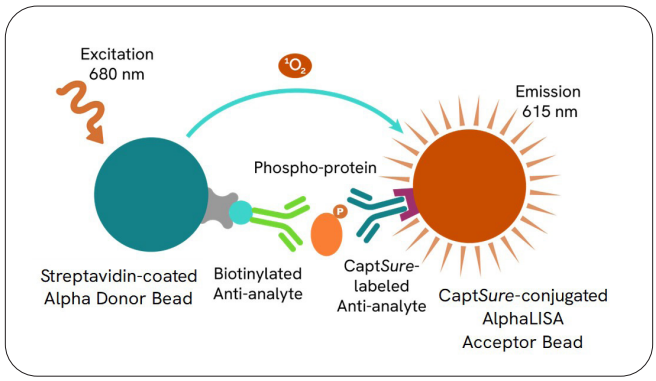


Figure 2: AlphaLISA *SureFire Ultra* Assay schematic depicting the capture of a phosphorylated protein.

Results

PROTAC BSJ-02-123 dose-response in MCF7 cells results in the downregulation of multiple cell cycle regulatory proteins

The dose-response curves for each target detected by AlphaLISA *SureFire Ultra* detection kits run on lysates of MCF7 cells treated with BSJ-03-123 PROTAC compound are provided in Figure 3. The treatment of MCF7 cells with the BSJ-02-123 CDK6 PROTAC resulted in the targeted degradation of both total CDK6 and CDK4. As expected, Cyclin D1 levels remained unaffected by the PROTAC molecule. Importantly, the phosphorylation status of Rb at key serine residues (Ser780, Ser807/811, and Thr821/826) was significantly downregulated in response to PROTAC treatment, indicating a disruption in cell cycle progression. Moreover, while total Rb levels exhibited a modest decrease at higher PROTAC concentrations, this reduction was less pronounced than the substantial downregulation observed

in the phosphorylated forms of Rb. The significant reduction in total Cyclin A2 and Cyclin B1 further underscores the impact of PROTAC treatment on critical components of the cell cycle regulatory pathway. Interestingly, Cyclin E1 levels remained unaffected. Total levels of CDK2 in MCF7 cells remained relatively flat across the PROTAC dosing range, but a significant reduction in the level of phospho CDK2 (Thr160) was observed. This result demonstrates how the removal of a key cycle regulator impacts downstream components of the cell cycle.

This work not only illustrates the precision and sensitivity of ALSU immunoassay technology in detecting protein levels in cell lysates but also provides valuable insights into the specific alterations within the cell cycle pathway induced by the proteolytic removal of CDK4 and CDK6 upstream regulatory proteins. These results demonstrate the biological consequences of PROTAC treatment, offering potential avenues for therapeutic intervention in diseases characterized by dysregulated cell cycle progression.

Treatment of MCF7 cells with NRG1 increased the concentration of key cell cycle regulatory proteins

Dose-response curves for each target measured with AlphaLISA *SureFire Ultra* detection kits run on lysates of MCF7 cells treated with NRG1 are provided in Figure 4. Treatment of MCF7 cells with NRG1 resulted in a significant increase in Cyclin D1 and CDK6, and a modest increase in CDK4 levels, suggesting that NRG1 promotes the expression of these critical regulatory proteins, potentially enhancing cell cycle initiation. The increase in all forms of Rb indicates a heightened activity of Rb in response to NRG1, suggesting enhanced regulation of cell cycle progression. The relatively stable levels of CDK2 contrasted with a significant increase in phospho CDK2 (Thr160) at the highest NRG1 concentration, indicating a potential enhancement of CDK2 activity. This observation underscores the intricate and specific effects of NRG1 on the phosphorylation status of key cell cycle regulators. Finally, the notable increases in Cyclin A2, Cyclin B1, and Cyclin E1 levels further support the notion that NRG1 stimulation induces a comprehensive upregulation of cell cycle regulatory proteins.

BSJ-03-123 PROTAC dose response in MCF7 cells

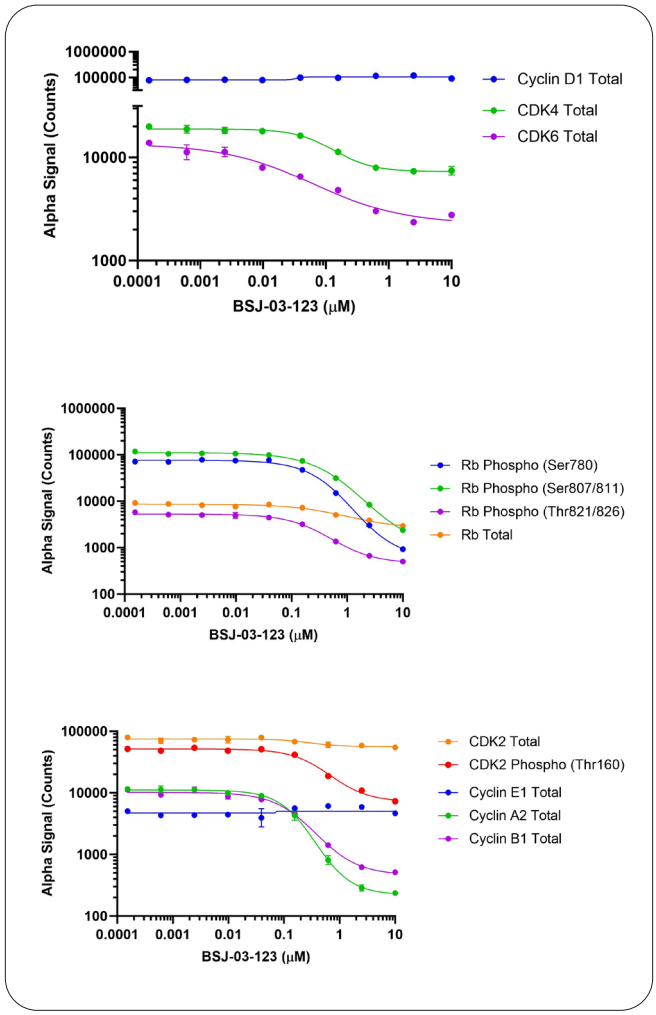


Figure 3: Dose-response curve of cell cycle regulatory proteins measured in MCF7 cell lysates after treatment with BSJ-03-123 PROTAC.

Neuregulin-1 (NRG1) dose response in MCF7 cells

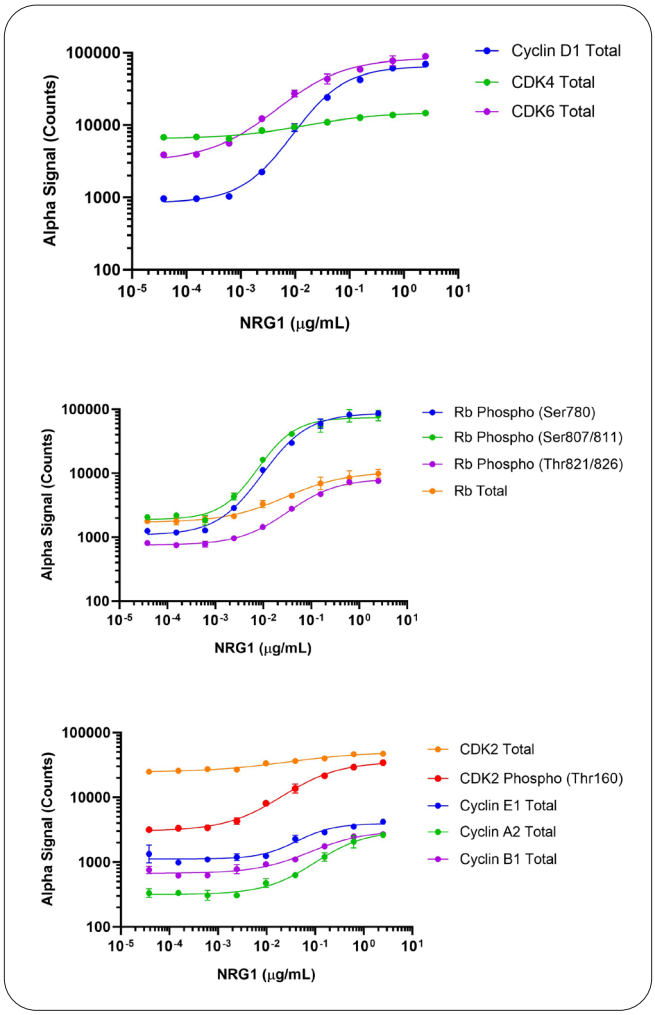


Figure 4: Dose-response curve of cell cycle regulatory proteins measured in MCF7 cell lysates after treatment with NRG1.

Conclusion

This study utilized AlphaLISA *SureFire Ultra* immunoassay technology to provide valuable insights into the dynamic regulation of the cell cycle in response to PROTAC and NRG1 treatments in MCF7 cells. The results not only highlight the versatility, precision, and superior sensitivity of ALSU immunoassay but also demonstrate how this technology can offer a deeper understanding of the molecular responses associated with PROTAC and growth factor treatments. These insights into cell cycle regulation contribute to the ongoing efforts to develop targeted therapeutic strategies for diseases characterized by dysregulated cell cycle progression, including cancer. Overall, our study provides a valuable foundation for the utilization of ALSU technology in further investigations into the specific mechanisms governing cell cycle dynamics and potential therapeutic interventions.

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