APPLICATION NOTE

Cyclin dependent kinase signaling in oncology: Assessing the effectiveness of a CDK9-targeting protac molecule using no-wash immunoassays.

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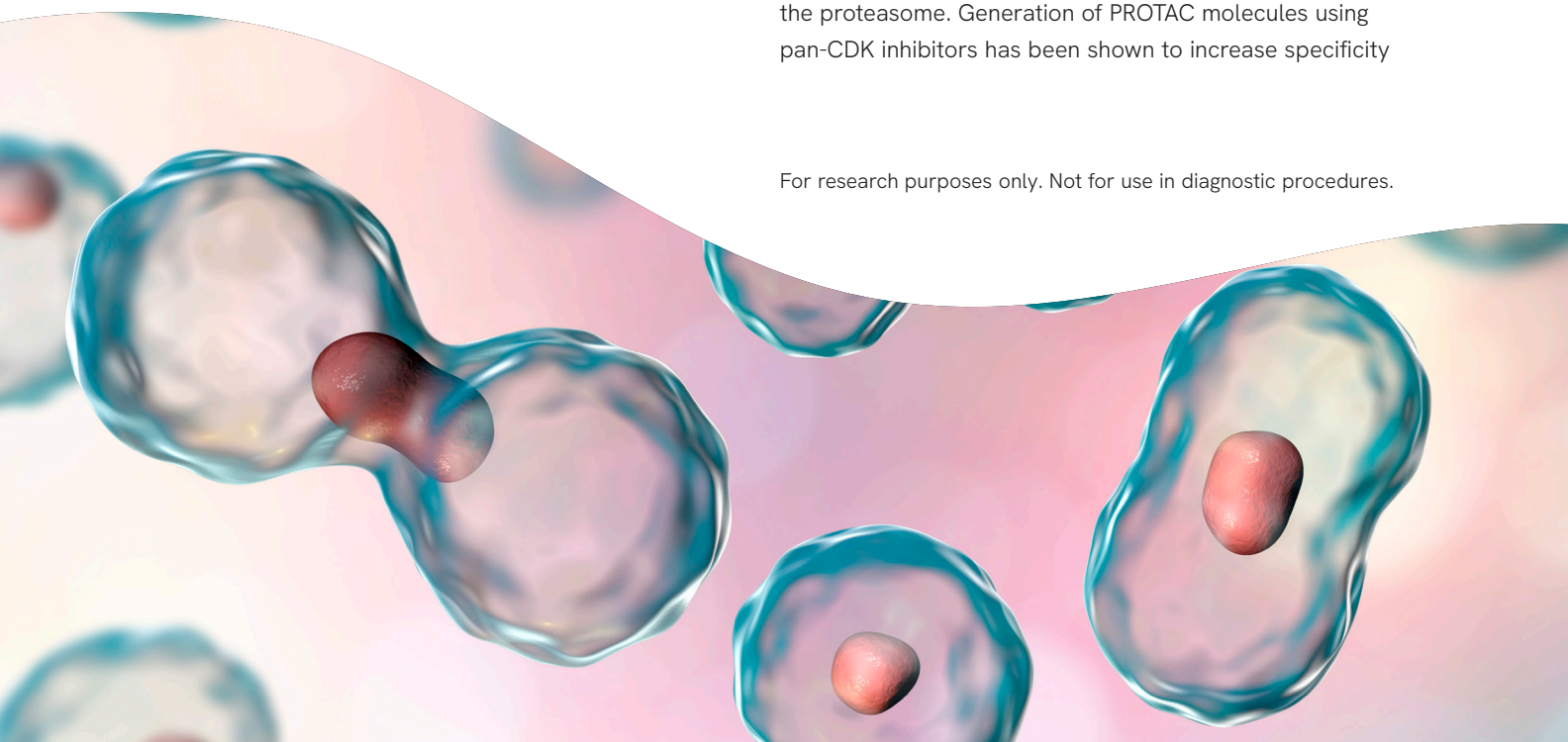
Introduction

Cyclins are regulatory subunits that bind to a cyclin dependent kinase (CDK), thus activating the kinase and controlling its activity. Most CDKs form a complex with a particular cyclin and are involved in the regulation of either cell cycle or transcription. CDKs 1, 2, 4, and 6 are involved in the regulation of the cell cycle while CDKs 7, 8, 9, and 11 are key players in transcription regulation and RNA processing (Figure 1).[1] Dysregulation of CDKs has been found to be a primary driver in tumorigenesis.[2] CDK9 is an attractive target for cancer therapeutics due to its crucial role in transcription regulation, particularly of short-lived anti-apoptotic proteins such as MCL-1 and XIAP which are critical to the survival of cancer cells.[3] Inhibition of CDK9 kinase activity or degradation of the protein itself are strategies being pursued in current cancer research. One approach for specifically degrading CDK9 is using a proteolysis targeting chimera (PROTAC).

PROTACs are small molecules that induce intracellular protein degradation by harnessing the power of the ubiquitin-proteosome system (UPS) by pairing an E3 ligase ligand with a traditional small molecule ligand via a linker.[4] In the PROTAC molecule (Thal-SNS-032) used in this study, one ligand (SNS-032), also called the warhead, binds to the target protein CDK9 while the other ligand (thalidomide) simultaneously binds to the E3 ligase cereblon. Formation of this "ternary" complex brings the E3 ligase into the protein complex resulting in ubiquitination of CDK9 with subsequent degradation of the protein by the proteasome. Generation of PROTAC molecules using pan-CDK inhibitors has been shown to increase specificity



of CDK binding in the PROTAC format and improve on-target effects.[5] Inducing protein degradation can have a prolonged effect compared to kinase inhibition and provides the ability to inhibit proteins that were previously thought to be undruggable due to a lack of known catalytic activity.[6]

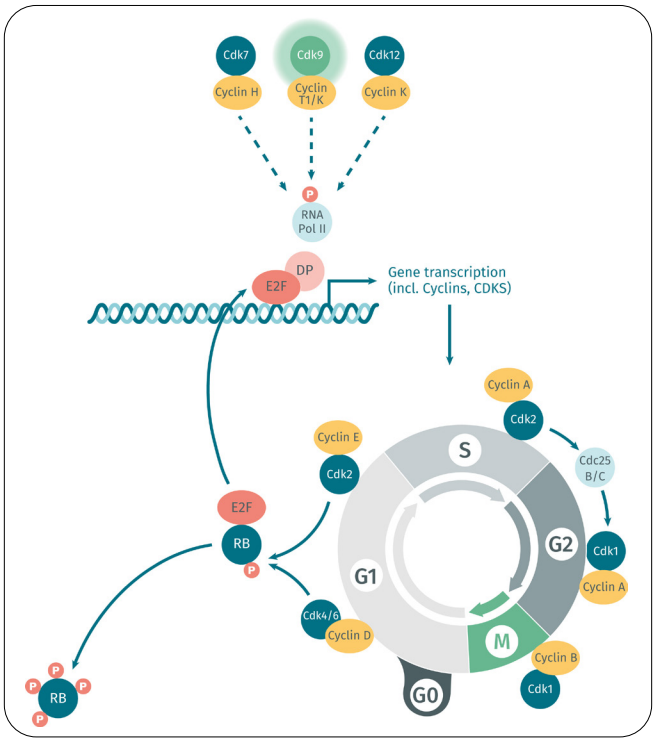


Figure 1: Role of cyclin-CDK complexes in the cell cycle.

This application note highlights the rapid and strong effect of CDK9 PROTAC molecule Thal-SNS-032 in two different cell types and compares it to the individual components of the PROTAC molecule to demonstrate specificity in CDK9 degradation. Two immunoassay methods were used to measure protein levels in the cells, HTRF™ and AlphaLISA™ *SureFire® Ultra™,* with each showcasing the ability of PROTACs to rapidly degrade a targeted protein making it a unique tool in the fight against cancer. Additionally, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an internal control for potential changes to global protein levels with drug treatment.

Materials and methods

Cell-based materials and compounds

HeLa cells (ATCC, CCL-2), MCF-7 cells (ATCC, HTB-22), Eagle's Minimum Essential Medium (EMEM) (ATCC, 30-2003), Fetal Bovine Serum (FBS) heat inactivated (Thermo Fisher, 10082147), recombinant human insulin (Santa Cruz, sc-360248), Thal-SNS-032 (MedChemExpress, HY-123937), SNS-032 (MedChemExpress, HY-10008), and Thalidomide (Tocris, 0652).

HTRF assay principle

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, 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 resulting in specific long-lived acceptor fluorescence (Figure 2). In the HTRF CDK9 assay (Revvity, 64CDK9TPEG) an anti CDK9 antibody is labeled with donor Europium and another anti-CDK9 antibody is labeled with the d2 acceptor. The assay plates (HTRF 96-well low volume plate, Revvity, 66PL96025) were read on an EnVision™ 2105 Multimode Plate Reader using laser excitation with APC 665 nm and Cy5 620 nm 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 shown in the results was calculated as (665 nm/620 nm) x 10^4.

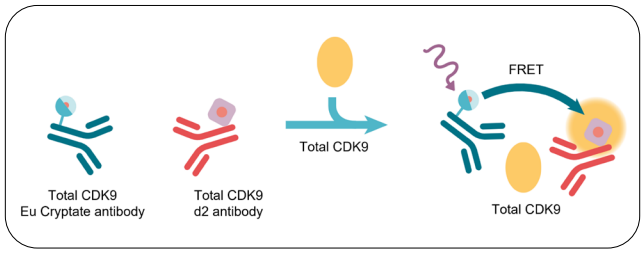


Figure 2: HTRF assay schematic. The schematic shows the detection of total CDK9 in a sandwich assay format using two labeled antibodies, an eu cryptate donor and a d2 acceptor. FRET signaling only occurs if both antibodies are bound to CDK9 and in proximity to one another. The HTRF signal is directly proportional to the amount of CDK9 present in the sample.

HTRF experimental conditions

25,000 cells/well were plated in 96-well assay plates and allowed to adhere overnight. Drug dilutions were prepared in culture media and cells were treated for 4 hours. The standard 2-plate assay format for adherent cells was followed for HTRF wherein the stimulation media was removed from the assay plate. Cells were lysed in 50 µL of

1X supplemented lysis buffer for 30 minutes with shaking at room temperature. Next, 16 µL of lysate sample was added to a separate HTRF assay plate and total CDK9 was measured using the HTRF CDK9 kit (Revvity, 64CDK9TPEG). Due to the abundance of GAPDH found in cells, only 4 µL of lysate sample was required for the GAPDH signal using the HTRF GAPDH Kit (Revvity, 64GAPDHPEG).

AlphaLISA *SureFire Ultra* assay principle

AlphaLISA is a bead-based homogeneous immunoassay platform with a simple, streamlined workflow to detect and quantitate biomolecules in both simple and complex sample types. Alpha assays require two bead types: donor beads and acceptor beads. The donor beads generate singlet oxygen upon illumination at 680 nm. The singlet oxygen can diffuse approximately 200 nm in solution. If an acceptor bead is within that distance, energy is transferred to the acceptor bead, resulting in light production. If an acceptor bead is not in proximity of a donor bead, little to no signal is produced over background (Figure 3).

The assay plates (Alphaplate-384, Revvity, 6005350) were read on an EnVision 2105 Multimode Plate Reader with the stock AlphaLISA settings.

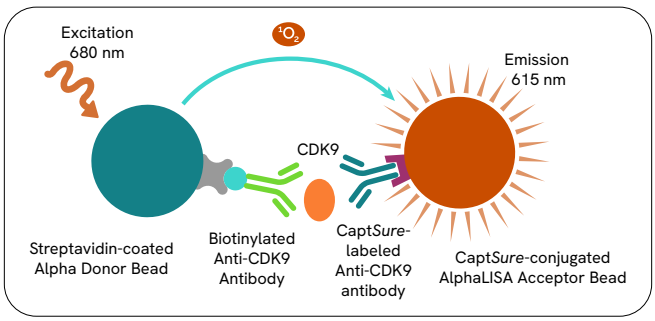


Figure 3: AlphaLISA *SureFire Ultra* Assay Schematic. The schematic shows the detection of CDK9 protein whereby the biotinylated antibody recognizes CDK9 and a second CaptSure-labeled antibody recognizes a distinct epitope on CDK9. Each antibody is bound by the appropriate donor (streptavidin-coated Alpha donor bead), or acceptor (CaptSure-conjugated AlphaLISA acceptor bead). The Alpha signal is directly proportional to the amount of CDK9 present in the sample.

AlphaLISA *SureFire Ultra* cell-based experimental conditions

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. HeLa cells were plated in EMEM + 10% FBS, while MCF-7 cells were plated in EMEM + 10% FBS + 0.01 mg/mL recombinant human insulin. Drug treatments were prepared at 4X final concentration in EMEM media and 25 µL of drug or vehicle (DMSO) was added to each assay plate for 4 hours. Cells were lysed in 50 µL final volume of AlphaLISA *SureFire Ultra* Lysis buffer following the recommended procedure in the AlphaLISA *SureFire Ultra* manual. Media was aspirated from adherent cells and 50 µL of 1X lysis buffer was added to each well. Assay plates were placed on an orbital shaker at 350 rpm for 10 minutes. After visual inspection to confirm lysis was complete, assay plates were sealed and stored at -80 °C until processing of each sample was completed. Assay plates were thawed at room temperature and 10 µL of lysate was used from each sample. Biological replicates were tested for Total CDK9 (Revvity, ALSU-TCDK9-A500) or GAPDH (Revvity, ALSU-TGAPD-A500) following the AlphaLISA *SureFire Ultra* manual. For the GAPDH samples, the cell lysate was first diluted 1:20 in 1X *SureFire Ultra* Lysis Buffer to remain within the linear detection range of the assay due to the high level of GAPDH present in most cell types.

Results and discussion

CDK9 PROTAC molecule thal-SNS-032 rapidly degrades CDK9

HeLa cells were treated with 1 µM of the CDK9 PROTAC molecule Thal-SNS-032 consisting of the CDK9 targeting molecule SNS-032 and the E3 ubiquitin ligase cereblon-targeting molecule thalidomide joined together by a polyethylene glycol (PEG) linker. Thal-SNS-032 binds CDK9 in cells and brings it into a complex with cereblon which then ubiquitinates CDK9 and targets it for proteasomal degradation. Control compounds were tested in parallel to gauge their effect on CDK9 levels. The SNS-032 warhead was used to inhibit CDK9 and thalidomide was used to bind to cereblon. In Figure 4 we see a strong effect of the PROTAC molecule Thal-SNS-032 on CDK9 levels in HeLa cells at 4 hours, resulting in an approximate 80% reduction in the HTRF signal (HTRF Ratio). The CDK9 inhibitor SNS-032 or the cereblon binder thalidomide alone show little to no change on the protein level of CDK9 when measured by HTRF. To highlight the specificity of CDK9 degradation, GAPDH was

measured as an internal protein control. Figure 4 also shows there is no change in the GAPDH signal. This suggests that the loss of CDK9 signal caused by Thal-SNS-032 is due to targeted degradation of CDK9 and not a change in global protein levels in the HeLa cell lysate.

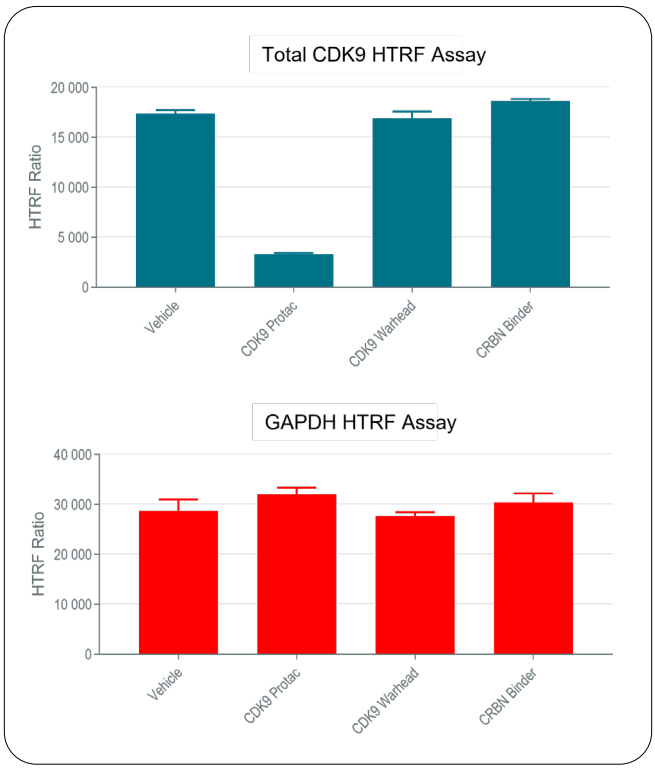


Figure 4: Total CDK9 and GAPDH HTRF Assays. HeLa cells were treated with 1 µM of each compound for 4 hours. CDK9 levels were measured in cell lysates. Cells treated with the CDK9 PROTAC molecule Thal-SNS-032 showed a dramatic reduction in CDK9 levels. However, little to no change in CDK9 was observed when cells were treated with the control compounds. There was no change in the GAPDH housekeeping protein with any compound tested at 4 hours in HeLa cells, suggesting that the global protein pool is unaffected.

The same experiment was performed independently in HeLa cells assessing the effect of the CDK9 PROTAC molecule Thal-SNS-032 compared to the control compounds and the cell lysate was assayed using AlphaLISA *SureFire Ultra* technology. Figure 5 shows an approximate 95% loss in Alpha signal with Thal-SNS-032 in HeLa cells at 4 hours. Interestingly there was an ~15% loss of CDK9 Alpha signal when cells were treated with the CDK9 inhibitor SNS-032 and measured by AlphaLISA *SureFire Ultra*. This could be due to the downregulation of the CDK9 protein caused by inhibition on the target seen in this experiment. The GAPDH levels are again not affected by compound treatments suggesting that changes in CDK9 levels are targeted and not due to changes in the global protein concentration at 4 hours in HeLa cells.

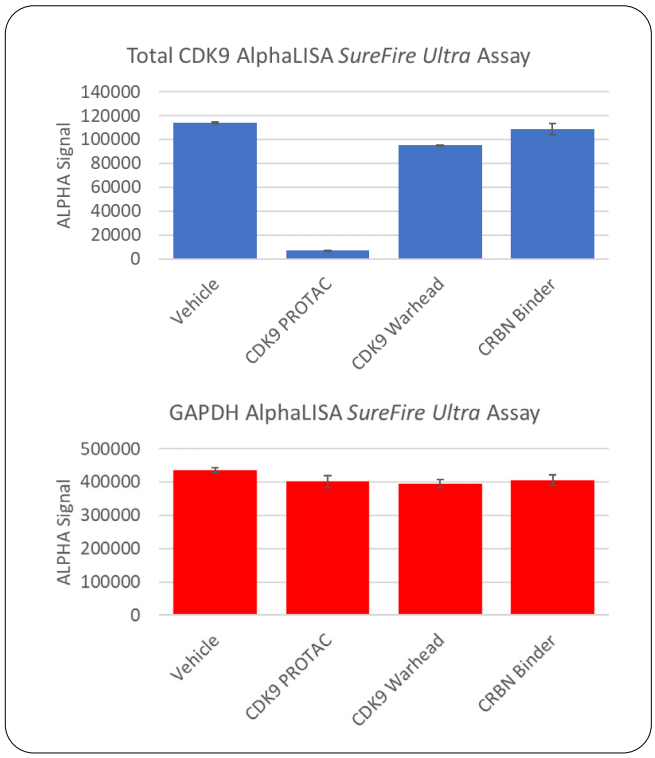


Figure 5: Total CDK9 and GAPDH AlphaLISA *SureFire Ultra* Assays. HeLa cells were treated with 1 µM of each compound for 4 hours. CDK9 levels were measured in cell lysates. Cells treated with the CDK9 PROTAC molecule Thal-SNS-032 showed a strong decrease in CDK9. Cells treated with the CDK9 inhibitor SNS-032 showed a small, albeit notable reduction in CDK9 levels. GAPDH housekeeping protein did not change suggesting that the global protein pool in the HeLa cells is stable when treated with these compounds at 4 hours.

CDK9 PROTAC molecule thal-SNS-032 degrades CDK9 at nanomolar doses in hela and MCF-7 cell

To further evaluate the potency of Thal-SNS-032 we performed a dose-response test at 4 hours of treatment in HeLa and MCF-7 cells. CDK9 and GAPDH levels were measured by HTRF and AlphaLISA *SureFire Ultra* technologies in two independent experiments. Figure 6 shows the HTRF results with half-maximal degradation values (DC50) calculated from the signal degradation curve. Thal-SNS-032 degraded CDK9 with DC50 values of 166 nM (HeLa) and 60 nM (MCF-7) with no apparent change to GAPDH levels.

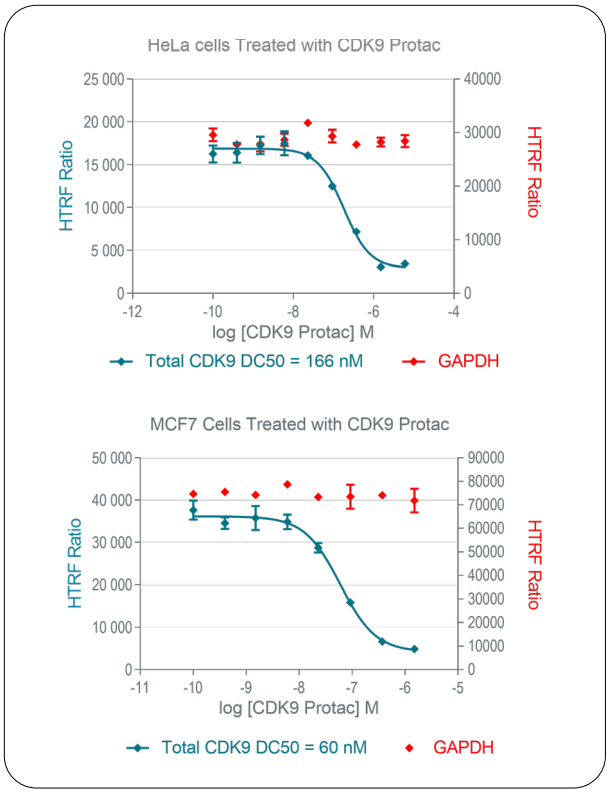
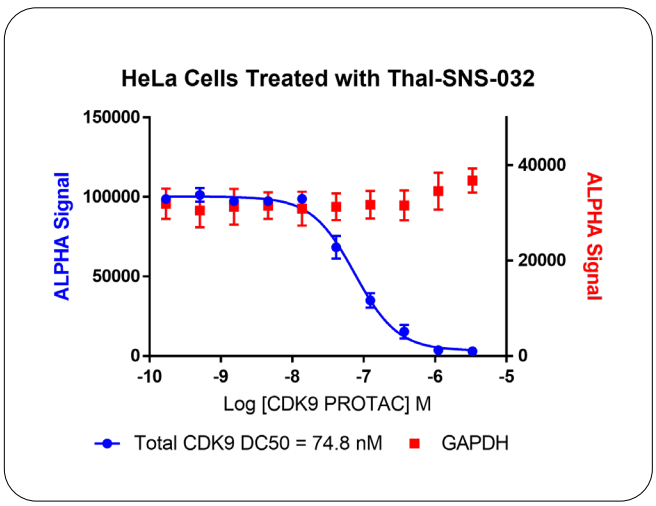


Figure 6: Total CDK9 and GAPDH HTRF Assays. Dose-response treatment of CDK9 PROTAC molecule Thal-SNS-032 in HeLa and MCF-7 cells treated for 4 hours. CDK9 half-maximal degradation values (DC50) of 166 nM and 60 nM were achieved in HeLa and MCF-7 cells, respectively. There was no observable change to the GAPDH protein level.

Similar results were obtained when detecting the CDK9 signal using the AlphaLISA *SureFire Ultra* method in an independent experiment. Figure 7 shows the AlphaLISA *SureFire Ultra* results with half-maximal degradation values (DC50) calculated from the signal degradation curve. Thal-SNS-032 degraded CDK9 equally in both cell types with DC50 values of 74.8 nM (HeLa) and 85.9 nM (MCF-7) and showed no apparent change to GAPDH levels.



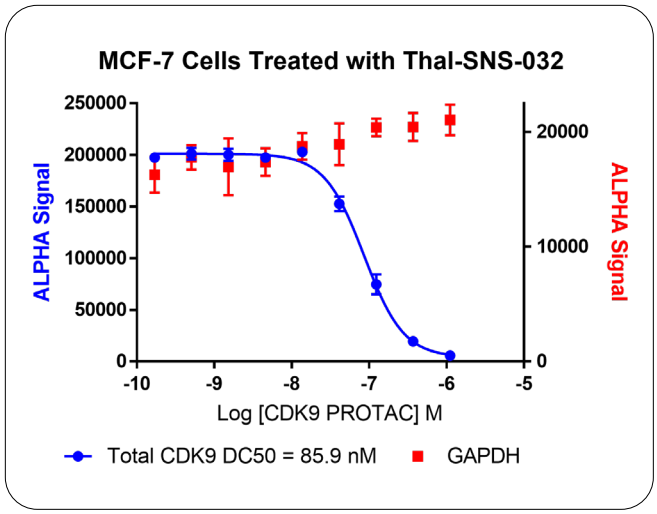


Figure 7: Total CDK9 and GAPDH AlphaLISA *SureFire Ultra* Assays. Dose-response treatment of CDK9 PROTAC molecule Thal-SNS-032 in HeLa and MCF-7 cells. CDK9 half-maximal degradation values (DC50) of 74.8 nM and 85.9 nM were achieved in HeLa and MCF-7 cells, respectively, with no decrease to the GAPDH protein level.

Summary

CDK9 is a protein target important in oncology research due to its role in transcription and its frequent dysregulation in many types of cancer. Herein we demonstrated that CDK9 can be rapidly degraded by PROTAC molecule Thal-SNS-032 in HeLa and MCF-7 cell lines with a reduction in signal of 80-95% depending on the detection method. We used GAPDH, an internal housekeeping protein, to monitor changes to total protein levels in the cells with compound treatment. We demonstrated the specificity of Thal-SNS-032 to reduce CDK9 protein levels in cells over its individual components, SNS-032 and thalidomide alone, with both HTRF and AlphaLISA *SureFire Ultra* technologies. Both HTRF and AlphaLISA *SureFire Ultra* assay technologies are homogenous, no-wash sandwich immunoassay formats that provide rapid and reliable results. We calculated relatively comparable DC50 (half-maximal degradation) values with each format demonstrating that either format is a good option for assessing efficacy. Revvity offers total CDK9 kits, part of a broader portfolio of total CDK detection kits, enabling profiling studies as well as the ability to measure cyclin dependent kinase signaling in an oncology research setting.

Reference

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