PROductive **TAC**tics: Streamlined, multifaceted approaches to unlocking PROTAC® screening insights

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EXCELLENCE IN MEDICINES DEVELOPMENT

Introduction

PROteolysis **TA**rgeting **C**himeras (PROTACs®) are rapidly transforming drug discovery, offering an exciting alternative to traditional small molecules when tackling disease causing proteins. Targeted protein degradation has huge potential, however, with new innovations come new challenges. Developing effective PROTACs® demands a tailored workflow that can navigate these unique challenges across the design-make-test-analyse cycle.

In this study, we present a targeted, efficient screening cascade for CDK4/6 degradation using a commercially available PROTAC®. Our workflow begins with a high-throughput flow cytometry assay that is amenable to direct-to-biology screening with dual readouts for degradation and phenotype. This is complemented by downstream assays that monitor cytotoxicity, validate protein degradation via automated immunoblotting, and explore gene expression changes, providing a holistic view of degrader impact.

PROTAC® screening – multiparametric flow cytometry and downstream cell cycle analysis

CDK4 and CDK6 form complexes with D-type cyclins to promote G1-S transition. Thus, degradation/inhibition of CDK4/6 causes G1 cell cycle arrest. When exploiting this to tackle cancer, selectivity of CDK4 over CDK6 can be important, as some cancers (such as HR+ breast cancer) rely primarily on CDK4 rather than CDK6, whilst dual inhibition of CDK4/6 exhibits toxicity to hematologic cells.

As a primary CDK4 PROTAC® screen, flow cytometry was chosen as a powerful tool that allows multiplexing of multiplexed readouts in plate-based formats. Here, it was used to quantify degradation of CDK4 and the off-target CDK6 (hence a dual degrader was chosen as a tool for assay development), alongside quantification of cells in different stages of the cell cycle.

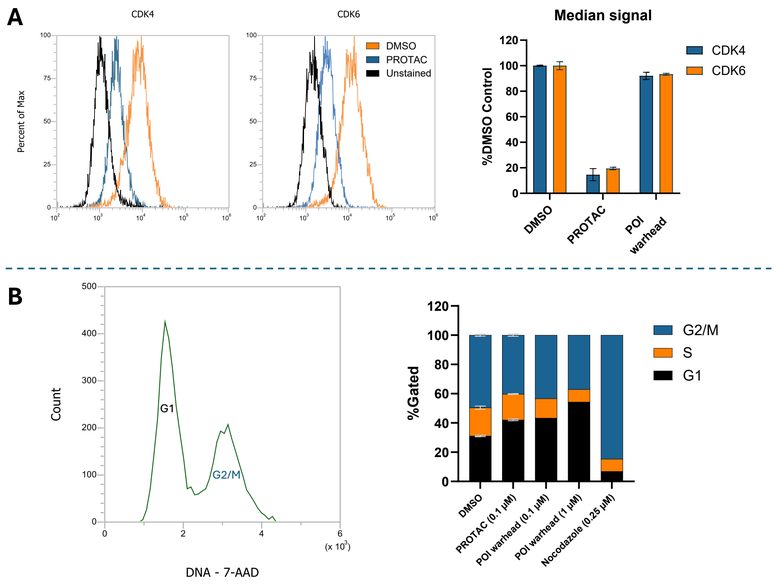


Figure 1. Treatment of Jurkat cells with the PROTAC® BSJ-03-204 causes CDK4/6 degradation and cell cycle arrest in G1. **A** Flow cytometry data demonstrating CDK4/6 signal detected under different conditions. Signal for both targets drops upon treatment with the PROTAC® but does not upon treatment with the CDK4/6 warhead (Palbociclib) on its own. **B** The proportion of cells detected in each stage of the cell cycle (through measurement of DNA content) after 24-hour treatments with PROTAC®, CDK4/6 warhead, or Nocodazole (a control for G2/M arrest). The CDK4/6 warhead is an inhibitor that causes comparable G1 arrest to the PROTAC® when used at the same concentration.

Flow cytometry was complimented by RT-qPCR for further downstream analysis of the impact of treatments on specific genes and pathways relevant to the POI. Samples can be extracted directly from the same plate, prior to flow cytometry, allowing RT-qPCR analysis once hits have been identified by flow.

Cyclin B1 regulates mitosis and is primarily expressed in G2/M. Therefore, cell cycle arrest in G1, leads to reduction in Cyclin B1 expression. Jurkat cells were treated with PROTAC® or CDK4/6 warhead before performing RT-qPCR for the detection of Cyclin B1 and 18S rRNA (internal control).

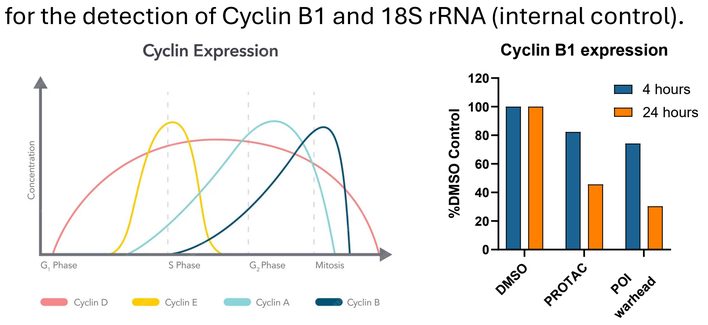
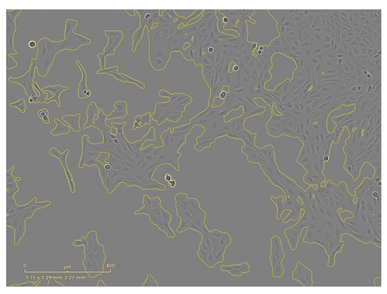


Figure 2. Treatment of Jurkat cells with BSJ-03-204 (PROTAC®) causes a reduction in Cyclin B1 expression. Left graph shows representation of expression of the cyclins across the cell cycle. Right graph shows Cyclin B1 transcript levels measured by RT-qPCR in Jurkat cells following treatment.

Cell health and Cytotoxicity evaluation

An important step in any drug discovery project is assessing the impact of compounds on cell health and cytotoxicity – particularly when studying the cell cycle. At CatSci, we use live-cell imaging capabilities (Incucyte® SX5; Sartorius) to quantify cell proliferation and determine morphology changes, along side multimodal plate readers to quantify cellular metabolism through luminescence-based assays.



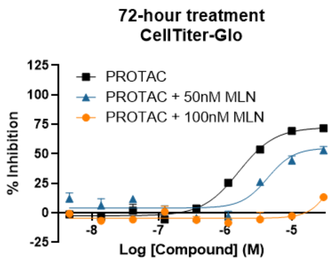


Figure 3. Measurement of cell proliferation and cytotoxicity. Left shows image of U-2 OS cells taken on the Incucyte® SX5 live-cell imager. Yellow lines indicated analysis mask, identifying cells for growth analysis. Right graph shows CellTiter-Glo® data of Jurkats treated with CDK4/6 PROTAC® +/- the Neddylation inhibitor MLN4924 (inhibiting PROTAC® mediated degradation).

Targeted degradation and mechanistic validation – Immunoblotting; Simple Western™ Jess

Here, we demonstrate validation of CDK4/6 targeted degradation and the mechanism of degradation using automated capillary-based Immunoblotting (Simple Western™ Jess; Bio-Techne), enabling quantification and detailed analysis of PROTAC® impact.

Jurkat cells were treated with a decreasing concentration of a PROTAC® for 4 hours before lysing and detecting CDK4 or CDK6. The resultant signal decreased with increasing PROTAC® concentrations, followed by the increase in signal at high concentration (also known as the "hook effect") is characteristic of targeted protein degradation via a POI-E3 ligase ternary complex.

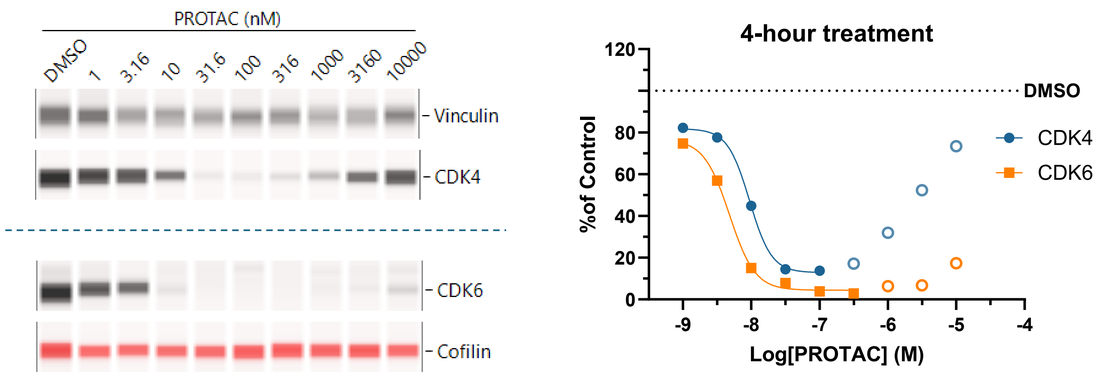


Figure 4. Validation of targeted CDK4/6 degradation by the tool PROTAC® BSJ-03-204. Top left shows an immunoblot of CDK4 multiplexed with Vinculin, and CDK6 multiplexed with Cofilin, in Jurkat cells treated with a dilution series of a BSJ-03-204. Top right shows the CDK4/6 area under the curve analysis, normalised to Vinculin or Cofilin respectively, plotted as percentage signal remaining vs the DMSO control sample. Bottom right shows a typical "Hook effect" caused by saturation of both the POI and the E3 ligase with PROTAC®, preventing formation of ternary complexes at saturating PROTAC® concentrations.

The mechanism was further validated with two methods:

- Prevention of degradation through blocking the required pathway using MLN4924 (Neddylation inhibitor) or MG-132 (proteosome inhibitor)
- Prevention of degradation through competition of the bifunctional PROTAC® with the POI warhead on its own (without the E3 ligase warhead)

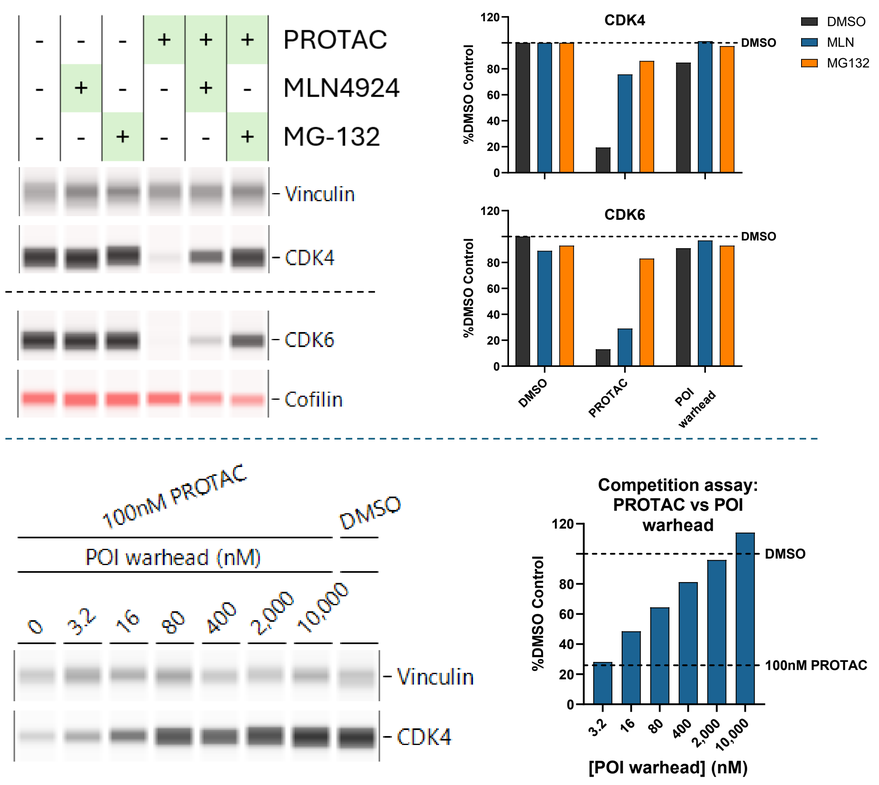


Figure 5. Confirmation of CDK4/6 degradation mechanism. Top of the figure shows an immunoblots and associated graphs of CDK4 and Vinculin, or CDK6 and Cofilin, in Jurkat cells treated with a tool PROTAC® (100nM), (or CDK4/6 warhead; blots not shown), +/- MLN4924 or MG-132. Bottom of the figure shows a competition assay in Jurkat cells treated with a PROTAC® (100nM) +/- increasing concentrations of the CDK4/6 warhead. Both graphs show CDK4 normalised area under the curve, plotted as percentage signal remaining vs the DMSO control sample.

Summary and conclusion

Here, we demonstrate a custom targeted protein degradation screening cascade successfully developed to identify CDK4 degraders using a commercially available CDK4/6 targeting PROTAC®. Initial screening assays were developed to be amenable to direct-to-biology workflows, enabling efficient screening of different PROTAC® warhead/linker combinations directly from plate-based chemistry, before triaging successful degraders into downstream assays for more detailed analyses. The technologies used are flexible and can be adapted to different relevant readouts depending on the target of interest.