Engaged but not degraded

Target engagement profiling of degrader compounds with CETSA

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INTRODUCTION

CETSA® (Cellular Thermal Shift Assay) is a label-free method for studying small molecule-protein interactions and is especially useful in targeted protein degradation (TPD) to complement degradation profiling.

Antibody-based detection with AlphaLISA was used to investigate degradation kinetics for three target proteins: CDK4, CDK9, and BRD4. CETSA combined with mass spectrometry readouts enables selectivity profiling of full PROTACs and corresponding warheads. While degradation assays measure outcome, CETSA also detects target engagement, revealing that not all PROTAC-bound proteins are degraded. Thus, PROTACs may drive cellular effects through both degradation and conventional ligand interactions, including potential off-target effects like those of small molecule drugs.

DEGRADATION KINETICS

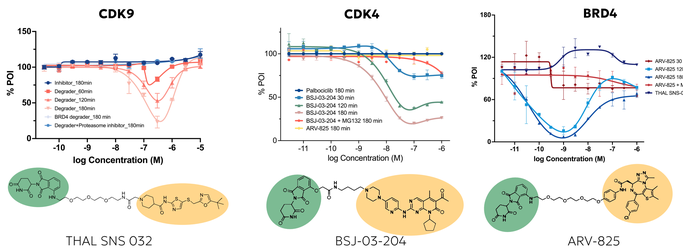


Figure 1. Degradation profiles of CDK9, CDK4, and BRD4 in K562 cells treated with indicated compounds. Compound structures depicted below.

All tested PROTACs showed a response within one hour, with maximal degradation responses observed at three hours, making them suitable for a standard 1-hour in-cell CETSA experiment (Fig. 1).

SELECTIVITY OF PROTACS AND WARHEADS

CDK4/6 DEGRADER

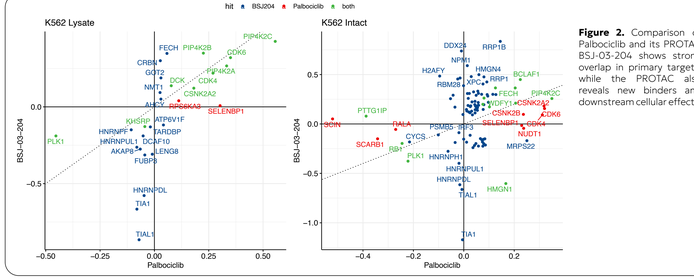


Figure 2. Comparison of Palbociclib and its PROTAC BSJ-03-204 shows strong overlap in primary targets, while the PROTAC also reveals new binders and downstream cellular effects.

Both the degrader (BSJ-03-204) and the kinase-binding inhibitor (Palbociclib) showed correlated kinase engagement in lysate, including Palbociclib's primary targets CDK4/6 (Fig. 2). The degrader showed more non-kinase off-targets in both cells and lysate. The thalidomide-binding protein Cereblon (CRBN) was thermally stabilized only by the degrader in both matrices. The degrader showed fewer kinase hits in cells than in lysate, whereas the inhibitor showed greater overlap between matrices. In cells, CDK4/6 appeared as less prominent stabilization hits with the degrader, likely due to opposing effects on protein levels, namely thermal stabilization and degradation. Both the inhibitor and degrader induced destabilization of RB1, a CDK4/6 substrate, indicating that regardless of the mode of perturbation, the same molecular pathway is affected: cell cycle regulation.

CDK9 DEGRADER

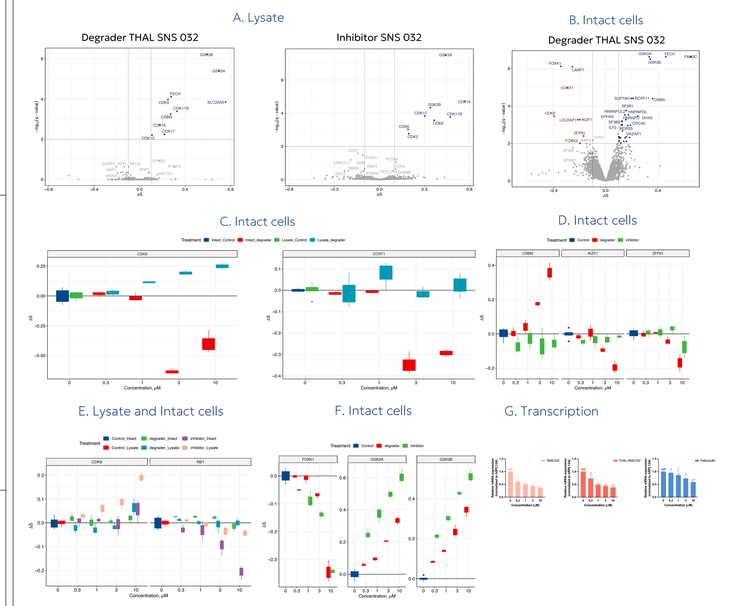
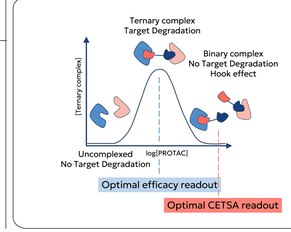


Figure 3. A) CETSA MS volcano plots of lysate treated with degrader (THAL SNS 032) and inhibitor SNS 032, respectively. B) Volcano plot of cells treated with degrader. C) Concentration dependent destabilization/degradation of CDK9 and CCNT1. D) Concentration dependent effects on thermal stability of CRBN and its neo substrates ZFP91 and IKZF1. E) Concentration dependent effects on thermal stability of CDK6 in lysate and RB1 in cells. F) Concentration dependent effects on thermal stability of FOXK1 and GSK3A/B in cells. HIF1A mRNA levels after 6 h treatment with indicated compounds.

The degrader (THAL SNS 032) and inhibitor (SNS 032) showed overlapping kinase engagement profiles, but with important differences, for example CDK2/6. The lack of CDK2/6 engagement by the degrader coincided with the lack of thermal destabilization of their substrate RB1 (Fig. 3E). Only the degrader showed apparent thermal destabilization of CDK9 and its associated cyclin, CCNT1 (Fig. 3C), most likely due to degradation. The degrader also showed thermal stabilization of CRBN and apparent destabilization or degradation of the neosubstrates ZFP91 and IKZF1 (Fig. 3D). Both the inhibitor and degrader showed target engagement of GSK3A/B and thermal destabilization of their substrate FOXK1 (Fig. 3F). HIF1A mRNA levels are regulated by FOXK1, and both the inhibitor and degrader showed a concentration-dependent effect on its expression(Fig. 3G). In contrast, Palbociclib did not shift GSK3A/B or FOXK1(Fig. 2) and did not affect HIF1A expression to the same extent.



- CETSA in lysate shows primary and off-target engagement
- CETSA in intact cells shows degradation of POI and target engagement

BRD4 DEGRADER
CETSA in Intact Cells

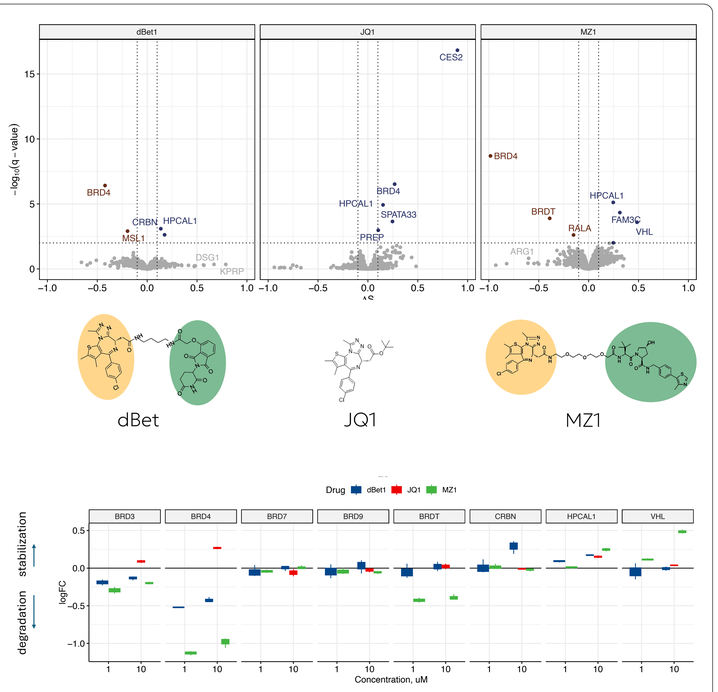


Figure 4. Volcano plots show target engagement by JQ1 and its PROTACs (dBet1, MZ1). The boxplot highlights BRD selectivity, E3 ligase stabilization, and engaged off-targets that are not not degraded.

Selectivity assessment by CETSA is not confined to CRBN-based ligands or kinases, as demonstrated here with the chromatin reader protein BRD4 and VHL as the substrate recognition module of the von Hippel-Lindau ubiquitination complex (Fig. 4). Again, the BRD4-binding warhead JQ1 stabilizes the target protein, whereas the degraders induce apparent destabilization or degradation. The selectivity of JQ1 among bromodomain proteins is maintained in the degrader versions, except for MZ1, which also affects BRDT.

CONCLUSION

- Targeted protein degradation is a growing therapeutic strategy, and PROTACs are increasingly used as chemical probes for target validation.
- CETSA enables the quantification of cellular potency and validation of target engagement by combining degradation (protein levels) with with changes in thermal stability.
- Unbiased proteome-wide CETSA profiling can identify off-targets - both degraded and engaged-only proteins. Due to an unmodified cellular environment, CETSA also reveals downstream signaling effects from both binary and ternary complexes.