ENHANCING DEGRADER RESEARCH WITH CETSA

TOMAS FRIMAN, MERVE KACAL, SÖREN BRUHN, BRINTON SEASHORE-LUDLOW[1], ALEXEY CHERNOBROVKIN AND DANIEL MARTINEZ MOLINA

PELAGO BIOSCIENCE AB SWEDEN | [1]DEPARTMENT OF ONCOLOGY-PATHOLOGY, SCILIFELAB AND KAROLINSKA INSTITUTET, STOCKHOLM SWEDEN

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INTRODUCTION

- CETSA® (Cellular Thermal Shift Assay) is a label-free method to study small molecule–protein interactions, especially useful in targeted protein degradation (TPD) to complement degradation profiling.
- Using mass spectrometry readouts, CETSA enables selective profiling of warheads and different PROTAC scaffolds. 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 via both degradation and conventional ligand interactions, including potential off-targets similar to small molecule drugs.

SELECTIVITY IN INTACT CELLS AND LYSATE

- CETSA enables compound selectivity profiling by assessing protein stability shifts via mass spectrometry, identifying direct binders and downstream signaling effects.
- Profiling of Palbociclib, a CDK4/6 inhibitor, shows a selective signature in lysates (Figure 1, Y-axis). In intact cells, the overall profile overlaps, but additional binders emerge—indicating proteins that require cellular context for binding.
- The use of CETSA on intact cells also allows identification of (downstream) cellular response. For instance, RB1, a CDK4/6 substrate, is hypophosphorylated and less stable following Palbociclib treatment compared to control.

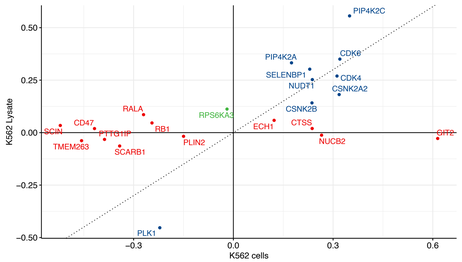


Figure 1. CETSA profiling of Palbociclib reveals strong overlap of direct targets in lysate and intact cells (blue labels). Additional stability changes in intact cells (red, X-axis) reflect cellular context-dependent effects.

FROM DRUG TO PROTAC – EFFECTS ON SELECTIVITY

CETSA is typically performed within one hour of compound treatment, reducing variability from protein expression changes. In degrader studies, including a non-heated control normalizes absolute protein levels, allowing parallel assessment of degradation and target engagement using CETSA.

Figure 2 compares Palbociclib and its PROTAC derivative BSJ-03-204. Their lysate binding profiles overlap (proteins in green), but BSJ-03-204 also reveals new binders, such as Cereblon (CRBN) via its pomalidomide moiety (proteins in blue). Notably, Ferrochelatase (FECH) is not bound by Palbociclib alone, but is engaged when Palbociclib is incorporated into the PROTAC backbone.

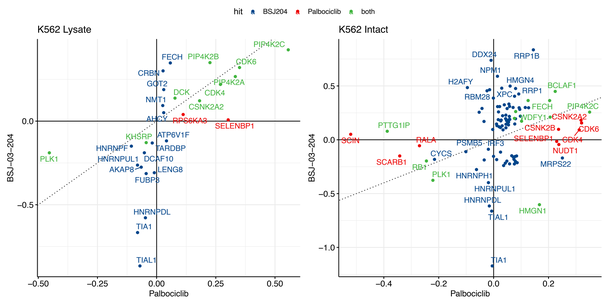
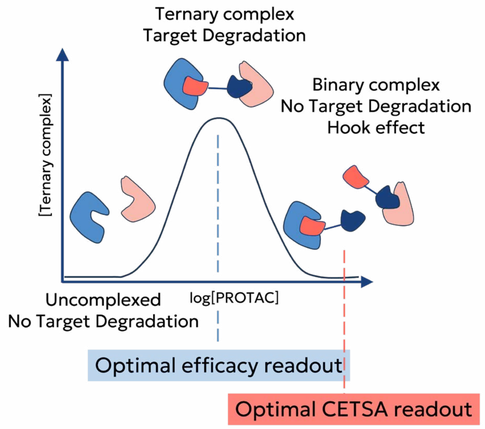


Figure 2. Comparison of Palbociclib and its PROTAC BSJ-03-204 shows strong overlap in primary targets, while the PROTAC also reveals new binders and downstream cellular effects.



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

ENGAGING BUT NOT DEGRADING

Figures 3 and 4 show how combining CETSA with degradation data reveals deeper insights into PROTAC mechanisms.

In Figure 3, two BRD-targeting PROTACs based on the JQ1 warhead display similar binding profiles. However, MZ1 (VHL-based) induces stronger BRD4 degradation than dBet1 (IMiD-based). While JQ1 does not stabilize the testis-specific BRDT, MZ1 stabilizes and degrades it—dBet1 does not.

HPCAL1 binds both PROTACs but is not degraded. The biological consequences of engaging this protein remains unknown.

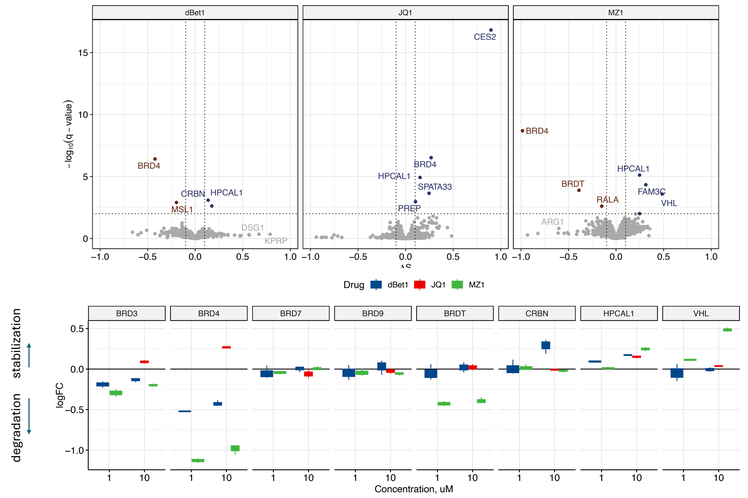


Figure 3. Volcano plots show target engagement by JQ1 and its PROTACs (dBet1, MZ1). The boxplot highlights BRD selectivity, E3 ligase stabilization, and engaged off-targets that are not not degraded.

Figure 4A highlights that the CDK9-targeting degrader THAL-SNS-032 and the SNS-032 inhibitor both stabilize CDK9 in lysate at 10 µM. However, in intact cells treated with the degrader (but not the inhibitor), CDK9 and its partner CCNT1 are robustly degraded (Figure 4B).

Figure 4C shows a hook effect: CDK9 and CCNT1 levels drop at 3 µM and rebound at 10 µM, suggesting oversaturation and binary complex formation. Off-target profiling shows engagement of GSK3A/B without degradation (Figure 4D), while FOXK1, their downstream substrate, is hypophosphorylated and destabilized. FOXK1, which regulates autophagy via HIF1A, whose mRNA levels is similarly affected by both degrader and inhibitor, unlike Palbociclib that primarily affecting a different pathway (Figure 4E). These effects may reflect non-degradative off-target interactions.

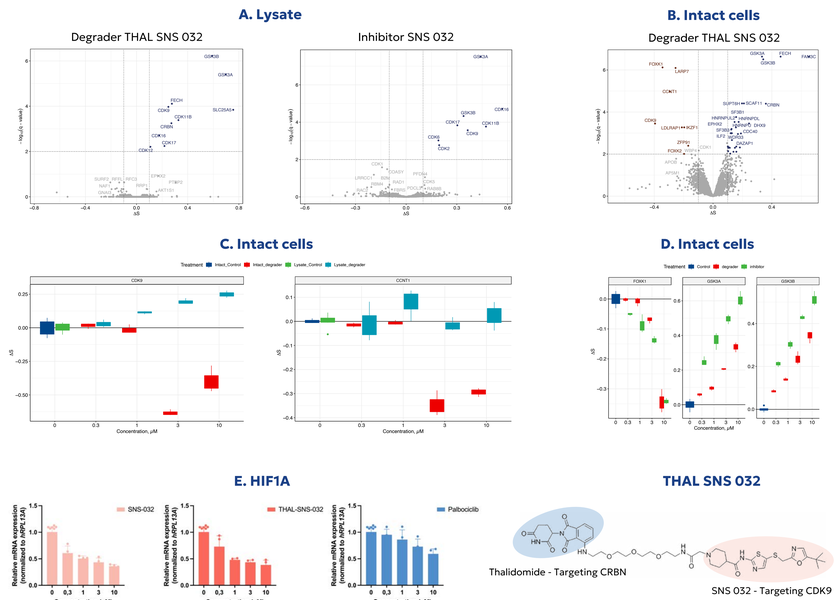


Figure 4. A: CETSA volcano plots in K562 lysate for degrader (THAL SNS 032) and inhibitor (SNS 032).
B: CETSA profile of 10 µM degrader in intact cells.
C: CDK9 and CCNT1 show a hook effect, with degradation peaking at 3 µM.
D: GSK3A/B are stabilized, and downstream FOXK1 is destabilized.
E: HIF1A mRNA levels are reduced by both degrader and inhibitor after 6 h.

CONCLUSIONS

- 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 stability shifts (engagement).
- Using unbiased proteome-wide CETSA profiling, we can identify off-targets—both degraded and engaged-only proteins. Thanks to the unmodified cellular environment, CETSA also reveals downstream signaling effects from both binary and ternary complexes.

References

1. Martinez Molina, D. et al. Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay. Science 341, 84-87 (2013)
2. Chernobrovkin, A. et al. A Tale of Two Tails: Efficient Profiling of Protein Degraders by Specific Functional and Target Engagement Readouts. SLAS Discovery 26 534–546 (2021)