Enhancing PROTAC research: CETSA as a tool for assessing selectivity of warheads and PROTACS and for detecting binders that remain intact

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INTRODUCTION

CETSA® (Cellular Thermal Shift Assay) is an effective method for studying interactions between small molecules and their protein targets, particularly useful in targeted protein degradation (TPD) as it improves insights from degradation profiles. CETSA can be conducted in cell lysates and intact cells without modifying compounds, proteins, or cellular environments.

By using mass spectrometric readouts, CETSA assesses binding selectivity for warheads targeting specific proteins and evaluates selectivity across various PROTAC scaffolds. The impact of effective PROTAC interactions can be monitored through protein degradation assays. However, parallel assessment of target engagement by CETSA, shows that not all proteins that bind the PROTAC warhead undergo degradation. Thus, cellular effects from PROTACs may arise from both primary target degradation and traditional protein-ligand interactions, potentially leading to off-target effects similar like those seen in traditional small molecule drug development projects.

SELECTIVITY IN INTACT CELLS AND LYSATE

The selectivity of a compound can be effectively evaluated using the Cellular Thermal Shift Assay (CETSA), which assesses stability changes in proteins suitable for mass spectrometric analysis, allowing for the identification of direct binders and downstream signalling events. For example, profiling Palbociclib, a selective CDK4/6 inhibitor, using CETSA reveals a selective profile in lysates (Figure 1, Y-axis). When performing the experiments in intact cells, we see a good overlap with the lysate profile, but the cellular environment also helps identify proteins requiring a cellular context for Palbociclib binding. In addition, the intact cell format also offers information on cellular responses, for example is the RB1 protein, a CDK4/6 phosphorylation substrate, which after Palbociclib treatment is left hypophosphorylated and less stable compared to the control.

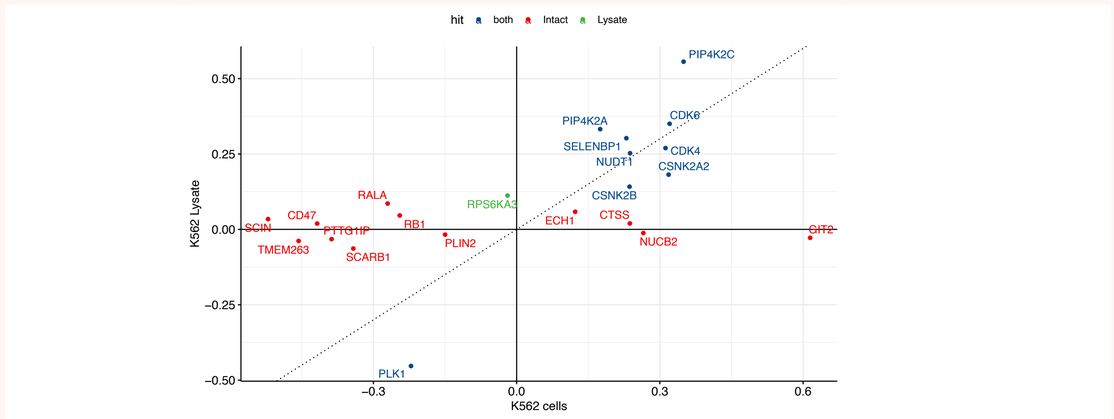

Figure 1. The CDK4/6 inhibitor Palbociclib shows a good correlation of direct targets identified in both lysate and intact cells (blue protein names), a number of additional stability changes can be identified in the intact cell (highlighted in red, along the X-axis) pertaining from the biological activity of the intact cell.

FROM DRUG TO PROTAC – EFFECTS ON SELECTIVITY

CETSA is usually performed within one hour of adding the test compound, minimizing the impact of varying protein expression patterns. When studying degraders like PROTACs and molecular glues, including a non-heated control normalizes absolute protein levels. This approach allows for the parallel assessment of degrader-dependent protein degradation and identification of target engagement levels through CETSA.

Figure 2 shows the correlation between Palbociclib and the Palbociclib based PROTAC BSJ-03-204. The binding profiles in lysates reveal similarities (left panel, green protein names), while the PROTAC also identifies novel binding partners. BSJ-03-204, featuring the E3 ligase recruiter Pomalidomide, engages with the classical IMID target, Cereblon (CRBN). Interestingly, Ferrochelatase (FECH) does not bind to Palbociclib alone but is engaged when Palbociclib is part of the PROTAC backbone.

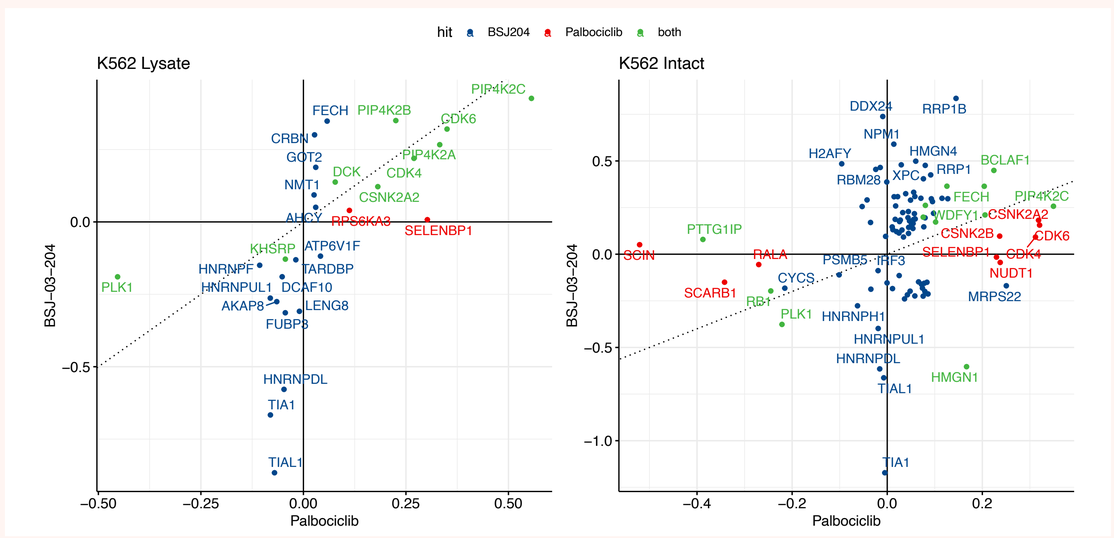

Figure 2. Comparing the Palbociclib drug and the corresponding PROTAC shows good agreement between primary targets but also highlights gained interactions and downstream cellular events for the degrader.

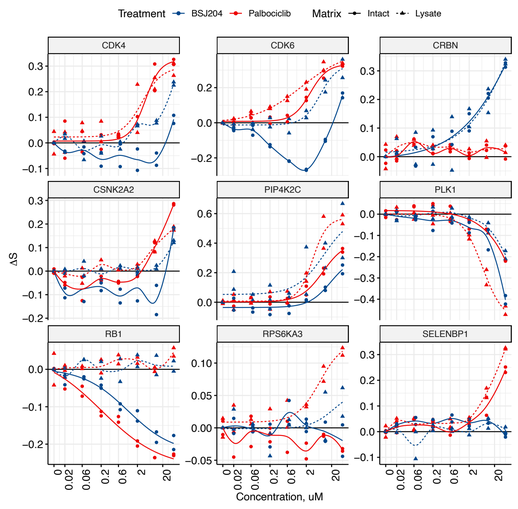

Figure 3.

Figure 3 exemplifies what the so called degradation hook (attributed to the formation of two separate PROTAC binary complexes) will look like when combining degradation data and CETSA data in the same experiment. The primary targets of BSJ-03-204, CDK4 and, notably, CDK6 exhibit a pronounced hook effect. At compound concentrations exceeding 2 µM, no further increase in the degradation rate is observed; instead, the addition of more PROTAC results in a clear stabilization from binding, as evidenced by CETSA. In contrast, secondary effects, such as the destabilization of RB1, do not exhibit a hook effect, since the observed hypophosphorylation occurs regardless if the function of CDK4/6 is abolished by means of degradation or inhibition.

ENGAGING BUT NOT DEGRADING

Figures 4 and 5 illustrate how parallel monitoring of degradation and target engagement improves our understanding of PROTAC mechanisms. In Figure 4, two BRD-targeting PROTACs based on the JQ1 warhead show similar interaction patterns with JQ1. Volcano plots indicate that dBet1, an IMiD-based PROTAC, and MZ1, a VHL-based counterpart, differ in efficacy, with MZ1 causing significantly greater degradation of BRD4 than dBet1. While the JQ1 warhead does not stabilize the testis-specific BRD protein (BRDT), MZ1 stabilizes and degrades it, dBet1 shows no such activity on this protein. Additionally, the protein HPCAL1 binds to both PROTAC but is not degraded. The cellular implications of engaging this protein remain unexplored in the literature.

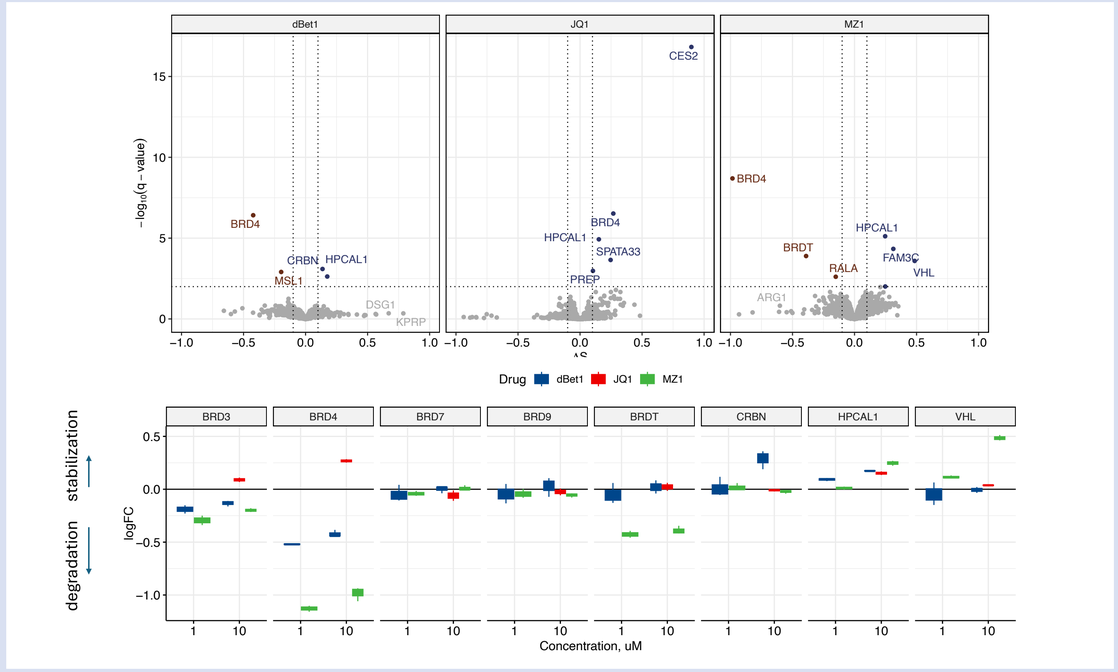

Figure 4. Volcano plots showing the protein targets for JQ1 and two counterpart PROTACS. The boxplot shows the selectivity between BRD family members as well as highlights E3 ligase stabilization and examples of protein off-targets that are not degraded by PROTAC binding

The second example involves a CDK9 inhibitor that stabilizes its target protein, while its degrader counterpart robustly degrades CDK9, as shown in Figure 5. Cyclin T1 (CCNT1), a complex partner, is co-degraded with CDK9, but CETSA data indicate it is not stabilized when CDK9 binds the warhead alone. The off-target interaction profile shows that both the CDK9 inhibitor and the degrader stabilize GSK3A/B without causing degradation. In contrast to the CDK9/CCNT1 complex, the GSK Interacting Protein (GSKIP) is stabilized alongside GSK3A/B upon ligand binding. The transcription factor FOXK1, a substrate for mTOR and GSK3-mediated phosphorylation, is hypophosphorylated by GSK3 inhibition and as such less stable. FOXK1 regulates the expression of proteins involved in key metabolic and autophagy processes. Thus, the cellular effects of this specific degrader may include off-target interactions that contribute additional effects beyond protein degradation, functioning through more conventional small-molecule inhibitor mechanisms.

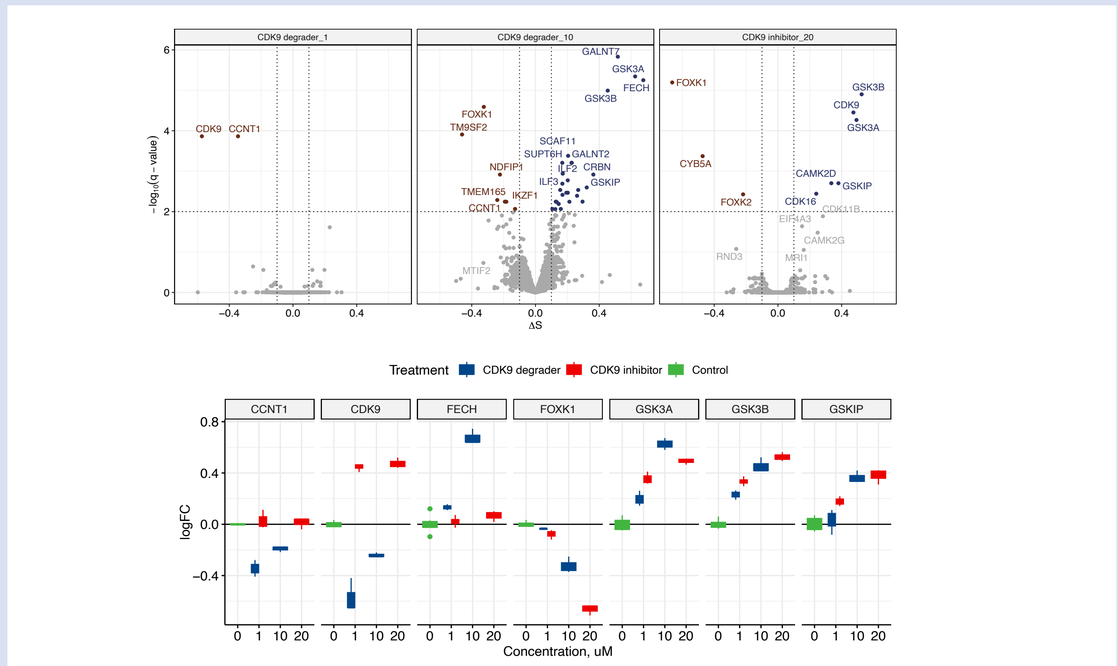

Figure 5. Volcano plots showing the CDK9 degrader at two different concentrations, highlighting the steep dose dependence . At 1 uM CDK9 and its cyclin T1 is already being degraded, whereas at 10 uM also off-targets appear, as apparent from the box plot, these off-targets are mainly binding the protac but without showing any degradation at the 1 hour timepoint.

SUMMARY

Targeted protein degradation represents a transformative approach in drug discovery, with the potential to significantly advance therapeutic development. As this field is still in its nascent stages, there is a pressing need for sophisticated tools to elucidate the connections between molecular dynamics and cellular effects. Utilizing Cellular Thermal Shift Assay (CETSA) as a platform to monitor the molecular events associated with degraders presents several opportunities:

- Identification of Additional Interacting Proteins: As research progresses from warhead development to the optimization of PROTAC backbones, CETSA can help uncover additional binding partners.
- Optimization of E3 Ligase Recruiters and Linkers: CETSA facilitates the refinement of E3 ligase recruiters and linkers to achieve optimal target engagement with the ligase.
- Distinction Between Protein Degradation Mechanisms: This assay enables the differentiation between proteins undergoing degradation via PROTAC binding and those forming traditional binary complexes.
- Correlation of Hook Effect with Target Engagement: CETSA allows for the correlation of degradation hook effects with optimal target engagement levels across various targets and off-targets.
- Screening Libraries of Ligase Recruiters: CETSA can be employed to screen libraries of potential ligase recruiters, aiding in the discovery of effective components for targeted protein degradation.

These applications underscore the value of CETSA in advancing our understanding of targeted protein degradation and its implications for drug discovery.