On Target Driven Efficacy Assessment of CDK2 Inhibitors in the Right Biological Context

Target Engagement and Selectivity Profiling in K562 and MCF7 Cells

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INTRODUCTION

CETSA® (Cellular Thermal Shift Assay) is a label-free method for assessing target engagement of small molecule-protein interactions. Target engagement determination is especially important in oncology, where cell death is often used as the efficacy readout. A key caveat is that the observed cell death must be driven by the intended target rather than by off-target effects, which could represent a future liability. In this study, we assessed the selectivity profiles of three different CDK2 inhibitor probes in both our standard cell line, K562, and an indication-specific cell line, MCF-7.

Using mass spectrometry readouts, CETSA enables selective proteome-wide profiling of small molecules in both intact cells and lysate. Two experimental setups were used. Initial selectivity profiling was performed using a high-throughput protocol that enables multi-concentration mass spectrometry analysis of multiple compounds via data-independent acquisition (DIA). The in-depth follow-up proteome-wide experiments used a protocol with more temperatures and peptides for mass spectrometry analysis based on data-dependent acquisition (DDA) and Tandem Mass Tag (TMT 16) labeling, which increases proteome coverage and the resolution of small thermal shifts.

IS EFFICACY DRIVEN BY INTENDED TARGETS OR POLY-PHARMACOLOGY?

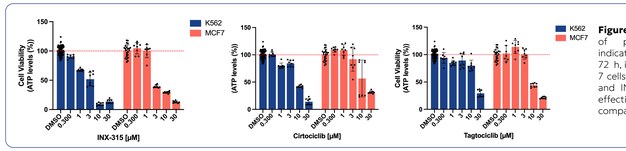

Figure 1. Inhibition of proliferation by indicated compounds at 72 h, in K562 and MCF-7 cells. Both Tagtociclib and INX-315 are more effective in MCF-7 compared to K562 cells.

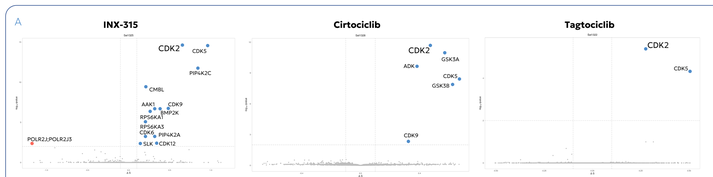

Figure2. Initial selectivity profiling with CETSA MS (DIA) in lysate derived from K562 and MCF-7 cells. A) Significantly thermally shifted proteins in K562 lysate treated with 3 µM of indicated compound. Note that all compounds showed target engagement of CDK2. B) Number of significantly shifted proteins were similar between K562 and MCF-7 cells with good overlap of protein hits. Note the high number of hits with INX-315.

Viability data suggest that INX-315 was the most potent inhibitor of viability in both cell lines (Figure 1), but the initial selectivity assessment indicated that it was also the least selective of the three compounds (Figure 2A). Tagtociclib showed a stronger effect on viability in the indication-specific MCF-7 cell line (Figure 1), suggesting a more disease-relevant mechanism of action compared with the other compounds. A similar selectivity profile was observed in both cell lines, with INX-315 appearing more promiscuous than the other compounds. Because of this apparent polypharmacology, INX-315 was omitted from further investigation. To better understand the relationships among selectivity, potency, and efficacy, Cirtociclib and Tagtociclib were further investigated using in-depth proteome-wide CETSA in K562 and MCF-7 cells.

IN-DEPTH PROTEOME-WIDE CETSA IN LYSATE REVEALS DIFFERENCES IN TARGET ENGAGEMENT POTENCY

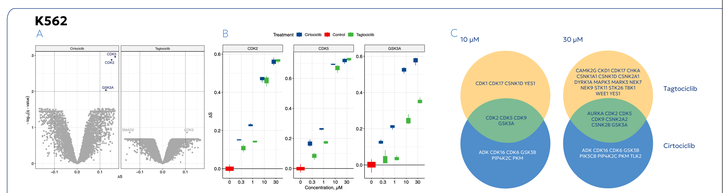

Figure 3. A) Volcano plots showing target engagement profiles for indicated compounds at 0.3 µM in K562 lysate. B) Concentration responses for hits in A. C) Venn diagrams of kinase selectivity profiles in K562 lysate at 10 and 30 µM.

Cirtociclib was more potent than Tagtociclib at inducing a thermal shift in CDK2, but it was also less selective at 10 µM (Figure 3). Cirtociclib's higher potency may help explain why it was more potent in the K562 viability assay. At 30 µM, a clear oversaturation response was observed with Tagtociclib and, to a lesser extent, with Cirtociclib (Figure 3C). Both compounds engaged several CDKs, including typical CDK inhibitor off-targets such as GSK3A and CDK5/9. Cirtociclib also affected non-protein kinases, including PKM, ADK, and PIP4K2C (Figure 3).

AFFECTED MOLECULAR PATHWAYS WERE IDENTIFIED WITH IN-CELL CETSA MS

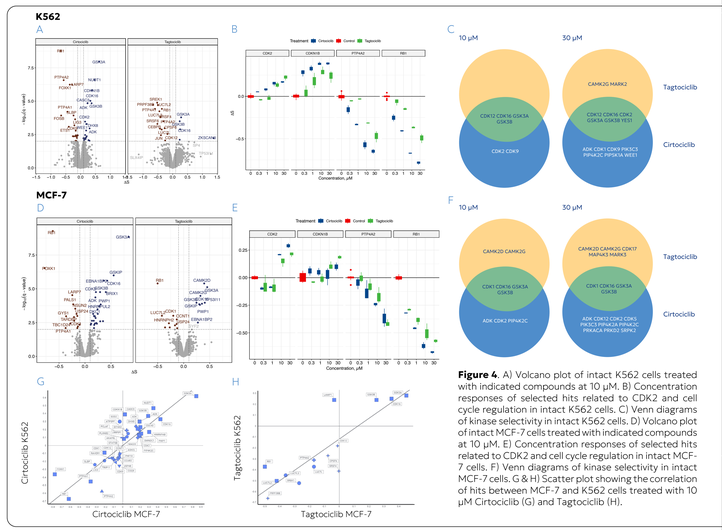

Figure 4. A) Volcano plot of intact K562 cells treated with indicated compounds at 10 µM. B) Concentration responses of selected hits related to CDK2 and cell cycle regulation in intact K562 cells. C) Venn diagrams of kinase selectivity in intact K562 cells. D) Volcano plot of intact MCF-7 cells treated with indicated compounds at 10 µM. E) Concentration responses of selected hits related to CDK2 and cell cycle regulation in intact MCF-7 cells. F) Venn diagrams of kinase selectivity in intact MCF-7 cells. G & H) Scatter plot showing the correlation of hits between MCF-7 and K562 cells treated with 10 µM Cirtociclib (G) and Tagtociclib (H).

In-depth proteome-wide CETSA in intact K562 and MCF-7 cells showed overlapping, but not identical, kinase engagement profiles (Figure 4C & F). Downstream effector proteins associated with cell cycle regulation, including RB1 and PTP4A2, were significantly shifted in both cell lines (Figure 4B & E). However, the thermal shift of RB1 was significant from the lowest concentration tested, 0.3 µM, in MCF-7 cells, compared with 10 µM in K562 cells. This may indicate that the RB1 pathway is more active in MCF-7 cells. It may also help explain why Tagtociclib was more potent in reducing proliferation in MCF-7 than in K562 cells, particularly since Tagtociclib was more selective than Cirtociclib at lower concentrations. Although not all protein hits met the significance threshold in both cell lines, the hits still correlated between cell lines treated with the same compound (Figure 4G & H).

GSK3 OFF-TARGET PATHWAY IDENTIFIED WITH CIRTOCICLIB IN BOTH CELL LINES

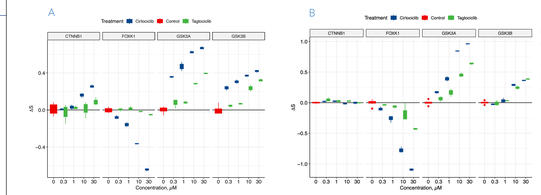

Figure 5. A & B) Concentration responses of GSK3A/B and two of their substrates: beta-catenin (CTNNB1) and FOXK1 in intact K562 (A) and MCF-7 (B) cells, respectively.

Cirtociclib had a more prominent effect on GSK3A/B and their substrates in both cell lines (Figure 5), which is also supported by the lysate data (Figure 3). The GSK3-FOXK1 axis may represent an off-target pathway that contributes to the viability phenotype induced by Cirtociclib.

A MOLECULAR PHENOTYPE COUPLED TO TRANSCRIPTION AND RNA MODIFICATION DOMINATED THE CETSA PROFILE

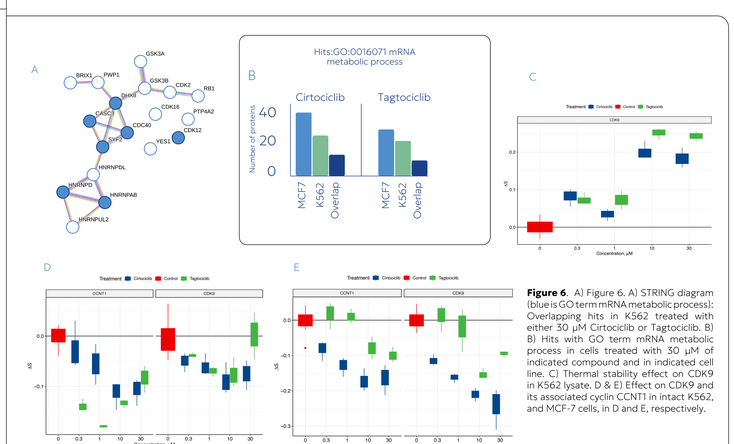

Figure 6. A) Figure 6. A) STRING diagram (blue is GO term mRNA metabolic process): Overlapping hits in K562 treated with either 30 µM Cirtociclib or Tagtociclib. B) B) Hits with GO term mRNA metabolic process in cells treated with 30 µM of indicated compound and in indicated cell line. C) Thermal stability effect on CDK9 in K562 lysate. D & E) Effect on CDK9 and its associated cyclin CCNT1 in intact K562, and MCF-7 cells, in D and E, respectively.

Both compounds induced thermal shifts in proteins associated with mRNA metabolism and splicing (Figure 6A & B). However, only a subset of hits was shared between cell lines and compounds (Figure 6A). Hits related to mRNA processing and transcription could reflect CDK9 inhibition through effects on RNA polymerase II.

CONCLUSION

- High-throughput CETSA-MS is a practical approach for comparing hit candidates. Lysate profiling captures direct interactions across accessible proteins, while in-depth analysis adds resolution on both lysate interactions and molecular phenotypes in intact cells.
- Cell line choice matters, but when the target protein or pathway is expressed and active, a generic line such as K562 can provide information similar to that from an indication-specific line such as MCF-7.
- The three CDK2 inhibitors differed in both potency and selectivity. INX-315 was the least selective and the most potent in viability assays. Cirtociclib showed greater CDK2 engagement potency but was also more promiscuous. Unlike Tagtociclib, its viability phenotype was not enhanced in MCF-7 cells.
- Tagtociclib and Cirtociclib produced broadly similar molecular phenotypes across cell lines, affecting cell cycle mediators and proteins involved in mRNA metabolism and splicing, most likely downstream of CDK9 and potentially CDK12.