Chemoproteomics

The secret life of proteins and drugs through the lens of proteome thermal stability

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Abstract

Purpose: Assess Thermo Scientific™ TMTpro™ 32plex label reagents for cellular thermal shift assay (CETSA) MS applications and screen for protein interactions with the natural product Alantolactone.

Methods: Thermal proteome profiling (TPP) and protein integral solubility assay (PISA) methods were used to prepare samples treated with Staurosporine and Alantolactone and labeled with TMTpro 32plex label reagents. Samples were acquired using real-time search with SPS MS3 on a Thermo Scientific™ Orbitrap™ Ascend MultiOmics Tribrid™ Mass Spectrometer.

Results: New protein targets for Alantolactone were found. TMTpro 32plex label reagents enabled expanded experimental design options and additional conditions to be explored in a single experiment.

Introduction

CETSA, or TPP, is the method of choice for characterizing protein (off)targets and mechanism of action of small molecule drugs inside living cells and other complex sample matrices. Over the years throughput of the method has been dramatically optimized by either applying chemical multiplexing (TMT) or utilizing *in vitro* integration of protein melting curves (proteome integral stability assay, PISA). In this work we show further method advancements using the new TMTpro 32plex label reagents with real time search SPS MS3 to further improve both throughput and quality of thermal proteome profiling for characterizing protein targets of natural product Alantolactone.

Methods

Alantolactone was profiled in human immortalized myelogenous leukemia cells (K562), and cell lysate using TPP/CETSA. Samples were incubated with different concentrations of the compound, heated to different temperatures and lysed. Thermally denatured and aggregated proteins were removed using centrifugation, and remaining soluble proteins were digested with trypsin. Proteome melting profiles were generated by labeling individual samples with TMTpro 32plex label reagents prior to combining. For PISA experiments, samples were pooled together, then labeled with TMTpro 32plex label reagents. Combined TMTpro labeled samples were fractionated using high-pH chromatography and analyzed by nanoLC-MS using a Thermo Scientific™ Vanquish™ Neo UHPLC System and Orbitrap Ascend MultiOmics mass spectrometer. Real-time search with SPS MS3 method was used to identify and quantify peptides, and data was processed with Thermo Scientific™ Proteome Discoverer™ 3.2 Software.

Figure 1. TMTpro 32plex label reagent reporter ions and suggested experimental design including normalization channels

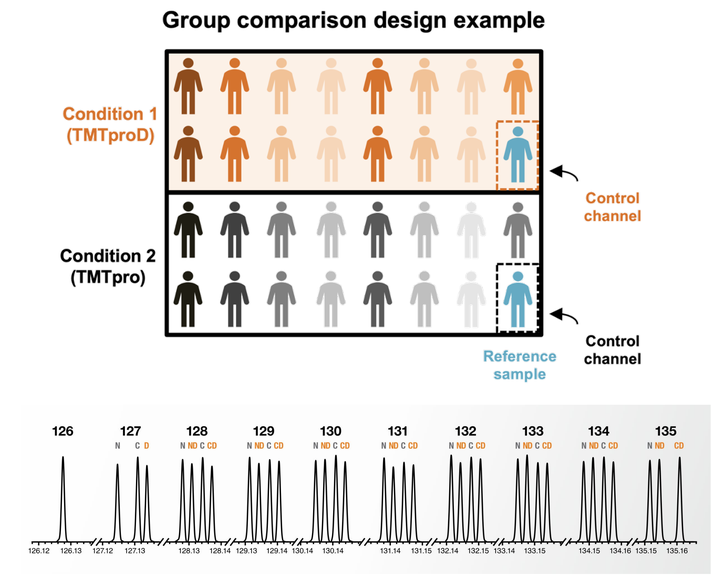


Figure 2. Data acquisition method on the Orbitrap Ascend MultiOmics mass spectrometer using real-time search to select peptides for SPS MS3 quantitation using TurboTMT 45k resolution Orbitrap scans.

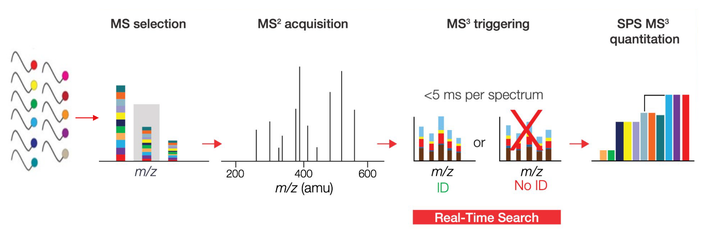
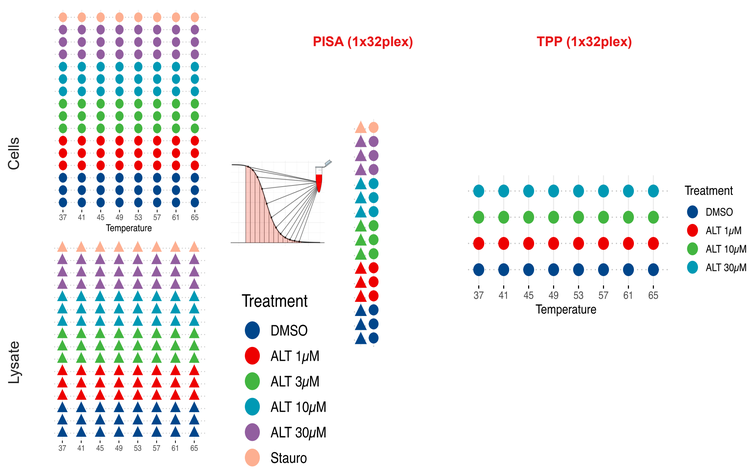


Figure 3. The experimental design for PISA samples with TMTproD reagents used for in-cell treated samples and TMTpro reagents used for lysate samples. TPP samples used 32 channels for 4 melting curves in a single sample



Results

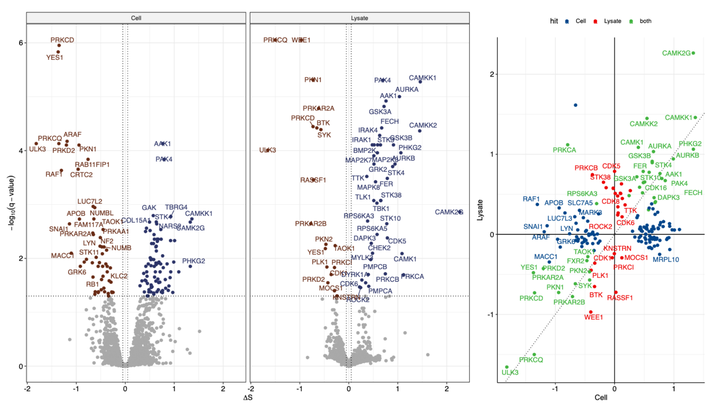
Alantolactone (ALT), a natural compound from Inula helenium L., shows promise in treating various cancers due to its anti-tumor and anti-inflammatory properties. Previously, AKR1C1 was identified as the specific cellular target of ALT in human non-small-cell lung cancer cell line using PISA approach. Herein we have used higher multiplexing capacity of newly developed 32plex TMTpro reagent to increase the depth of the ALT profiling, as well as understanding of immediate effect of ALT treatment on cellular proteome.

Table 1. Protein and peptide coverage for TPP and PISA experiments and median protein CV from PISA experiments. Data for shorter gradient methods included analysis of ½ of the fraction samples (every other fraction) and 30 minute gradients vs. the standard 45 minute gradient elution method.

Experiment	Matrix	Protein	Peptide	Median CV
PISA	Lysate	7600	87014	5.7%
PISA (shorter gradient)	Lysate	6196	44192	4.7%
PISA	Intact Cells	7607	87014	8.7%
PISA (shorter gradient)	Intact Cells	6197	44190	5.7%
TPP	Intact Cells	7644	86960	-

Staurosporine was used as a positive control for the PISA experiment, and to confirm the data quality with TMTpro 32plex label reagents. Results in figure 4 show all significant protein hits were kinases in the lysate sample, with intact treated cells showing additional non-kinase proteins hits. Several forms of protein kinase C (alpha and beta) are significantly stabilized by Staurosporine in lysate, but de-stabilized in cells. Similarly, Staurosporine-induced stabilization of CDK5, CDK8, or STK38 was only observed in lysate, with no changes in thermal stability detected in intact cells. No thermal stability changes was seen in lysate for ARAF, RAF1, LUC7L2 or LYN, while they were significantly de-stabilized in intact cells. Observed trends highlight the advantage of including intact cell and lysate treated samples in CETSA experiments to fully characterize drug-protein interactions.

Figure 4. Kinase hits from 10 uM Staurosporine treatment in protein lysate and intact cell samples. A combined plot shows kinase hits common to both sample types.



This allowed us to identify 12 more potential ALT targets and distinguish between thermal stability changes caused by direct binding events and those induced by downstream signaling events. Several members of the aldo-keto reductase (AKR) superfamily show changes in thermal stability.

Results from PISA profiling of ALT in both intact and lysed K562 cells is shown in figure 5. TMT 32plex label reagents enabled compound-induced proteome thermal stability changes at four different compound concentrations, in two matrices (intact and lysed cells) simultaneously.

Figure 5. Protein thermal stability shifts from PISA analysis of cells and lysate after Alantolactone treatment

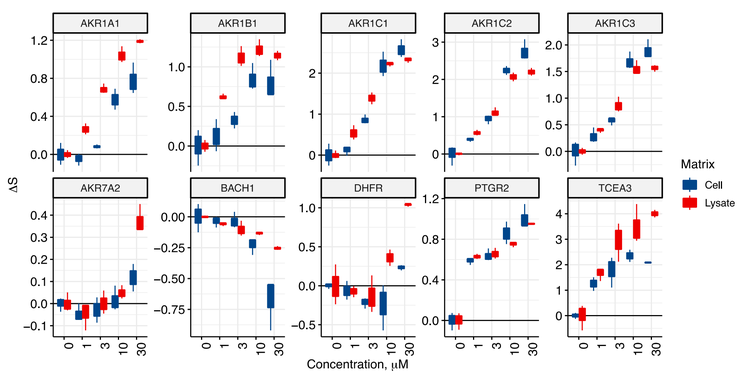
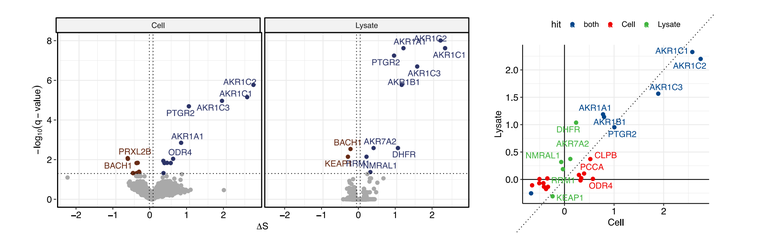
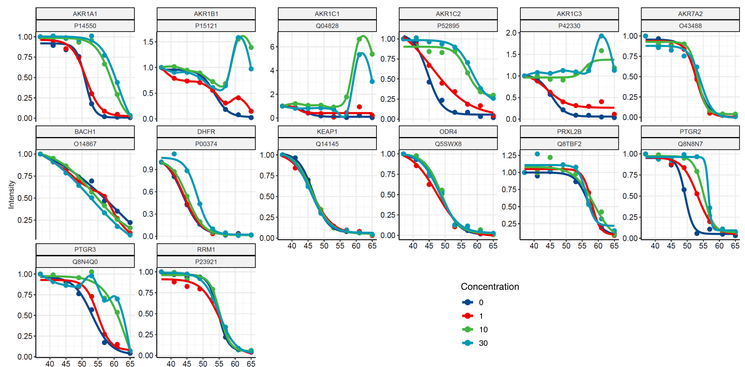


Figure 6. Global proteome view of proteins that show thermal stability shifts from Alantolactone treatment in cell and lysate



In the second experiment, proteome melting in intact cells was studied at three different ALT concentrations and vehicle control. With the TMT pro 32plex label reagent, four eight-point melt curves could be combined within one multiplexed sample. As a result, compound-induced changes in protein abundance, protein solubility, protein stability could be visualized and deconvoluted in unprecedented ways. ALT affects different proteins in significantly different ways. While proteins like ARK1A1 and PTGR2 appear as “normal” targets of ALT, with only change in protein thermal stability is observed, quite different effect on ARK1B1, AKR1C2 and ARK1C3 was observed, which manifest complex nature of ALT interaction with the cellular proteome.

Figure 7. Protein melting curves from thermal proteome profiling with different concentrations of Alantolactone



Aiming to evaluate the impact of a reduced instrument analysis time, PISA data was also collected by analyzing every other fraction with a 30 minute gradient instead of a 45 minute LC-MS gradient. A lower number of protein and peptides were detected with the reduced instrument time, as seen in the Staurosporine plots. However CVs and significant protein hits from Alantolactone treatment were not impacted significantly.

Figure 8. Kinase hits from 10 uM Staurosporine treatment in protein lysate and intact cell samples using . A combined plot shows kinase hits common to both sample types.

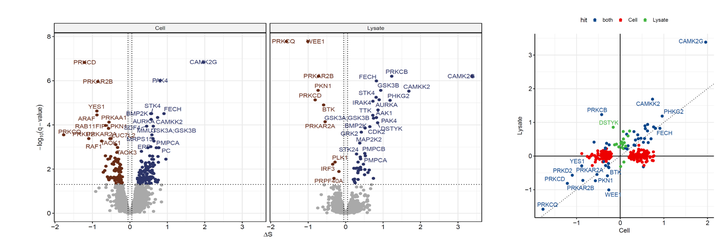
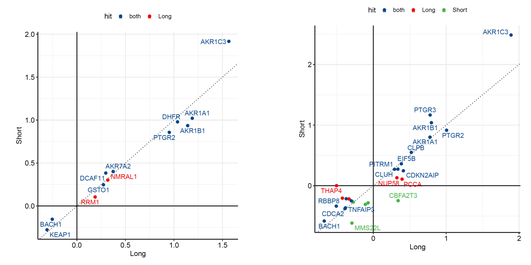


Figure 9. Comparison of protein thermal stability shift changes detected from Alantolactone treatment. Sample fractions were analyzed with 45 minute LC-MS gradients and compared to data when only ½ of fractions were analyzed with 30 minute LC-MS gradients.



Conclusions

- TMTpro 32plex label reagent expands experimental design options for CETSA MS experiments without compromising data quality
- Real-time search with SPS MS3 on the Orbitrap Ascend MultiOmics MS provides deep proteome coverage for fractionated TMTpro 32plex experiments
- Unique thermal shifts can be observed from lysate and intact cell treated samples
- Alantolactone interacts with several different proteins, including multiple proteins from the aldo-keto reductase (AKR) superfamily

References

1. Daniel Martinez Molina et al., Science 341,84-87(2013).DOI:10.1126/science.1233606
2. Mikhail M. Savitski et al. , Science 346,1255784(2014).DOI:10.1126/science.1255784
3. Fu Z, Li S, Liu J, Zhang C, Jian C, Wang L, Zhang Y, Shi C. Natural Product Alantolactone Targeting AKR1C1 Suppresses Cell Proliferation and Metastasis in Non-Small-Cell Lung Cancer. Front Pharmacol. 2022 Mar 15;13:847906. doi: 10.3389/fphar.2022.847906. PMID: 35370661; PMCID: PMC8965451.

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