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Stability Proteomic Methods To Detect Novel Drug Targets

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Abstract

The investigation of p53 mutations, prevalent in osteosarcoma (OS), has led to the identification of potential anti-cancer compounds—KU0171032, KU-D2, and KU-D2-F. These compounds exhibit cytotoxic effects in p53-deficient OS cells. Previous targeted experiments including Cellular Thermal Shift Assays (CETSA) suggest a potential interaction between KU0171032 and NBS1, which is crucial for DNA repair. Recently, Stability Proteomics Methods (SPMs) have been used to identify the protein targets of small molecules. SPMs implemented include Thermal Proteome Profiling (TPP), Limited Proteolysis (LiP), and Solvent Proteome Integral Solubility Alteration (Solvent-PISA). These SPMs quantify compound stabilization/destabilization of protein targets from thermal denaturation, proteolysis, and solvent-induced precipitation. The pilot studies involve treating cell lysates with staurosporine, a pan-kinase inhibitor, and analyzing samples by quantitative liquid chromatography tandem mass spectrometry (LC-MS/MS), revealing thermodynamically stabilized proteins. OnePot TPP, LiP, and Solvent-PISA workflows successfully identified kinase targets of staurosporine binding. We quantified over 2800 proteins by each method and identified 12 (TPP), 12 (Lip), and 16 (Solvent-PISA) staurosporine targets using these different methods. Thus far we've shown that OnePot TPP, LiP, and Solvent-PISA are feasible approaches to identify small molecule-protein interactions in cells. Analysis of KU-D2-F treated p53-deficient U2OS cell lysates using OnePot TPP and Solvent-PISA workflows displayed good protein coverage and interesting putative interactions. Ongoing work is necessary to reproduce findings and will be aided by reducing batch effects. This work presents a systematic approach, using staurosporine as a benchmark in piloting SPMs. This allows for the assessment of technique feasibility, emphasizing the potential of OnePot TPP, Solvent-PISA, and LiP methodologies in uncovering novel drug targets. Future directions include further experiments to identify protein binders and better understand the mechanism of the KU0171032 compound series.

Methods

p53-deficient U2OS cell lysates were prepared via detergent lysis or sonicated in PBS. Cell lysates were incubated with staurosporine or KU-D2-F for 30 minutes at 25°C. Following drug incubation, the replicates were processed via the One-pot TPP, Solvent-PISA, or LiP workflows.

TPP and Solvent-PISA: Each replicate was divided into 10 aliquots and exposed to either a gradient of temperature or of a solvent blend composed of acetone, ethanol, and acetic acid. Within each sample, the aliquots were recombined and centrifuged to remove denatured protein. The supernatants of each sample were then digested and labeled with TMTpro 18plex reagent and combined to enable multiplexing. The labeled and pooled samples were then desalted and fractionated by high-pH reversed phase chromatography prior to liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

LiP: Each replicate was digested with Proteinase K for 5 minutes at 25°C. The reaction was quenched at 99°C for 5 minutes. The partially digested protein was then subjected to reduction, alkylation, trypsin digestion and desalting.

Mass Spectrometry: All samples were analyzed on our Thermo Orbitrap Exploris 480 mass spectrometer coupled to an Easy-nLC 1200 system with Proteome Discoverer (One-pot TPP and Solvent-PISA) or DIA-NN (LiP) for data analysis.

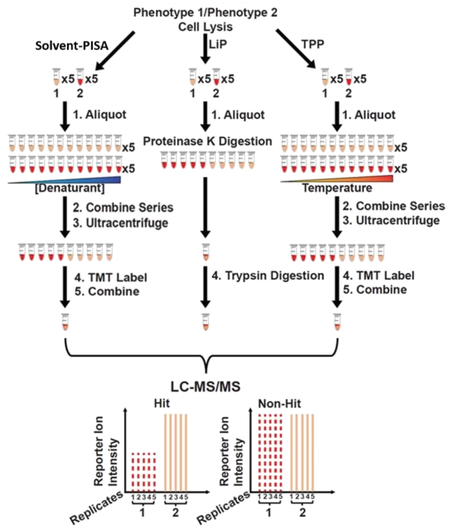

Fig 1: Schematic representation of the Solvent-PISA, LiP, and One-pot TPP workflows employed in this work to characterize changes to protein stability due to compound binding on a proteome-wide scale in p53-deficient U2OS lysates.

Results

One-pot TPP, LiP, and Solvent-PISA workflows successfully identified kinase targets of staurosporine binding

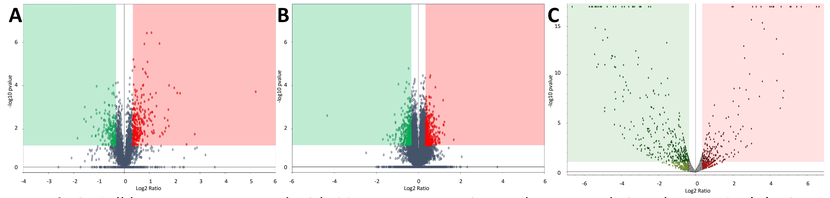

Fig 2: Cell lysates were treated with 20µM staurosporine and processed via solvent-PISA (A), LiP (B), and One-pot TPP (C). Data are presented as a volcano plot to highlight significant changes in abundance. Significant changes were determined by setting $p<0.1$ and log2 fold change$\geq$0.35 and are indicated with red or green dots.

We show that with Solvent-PISA, LiP, and OnePot TPP workflows we could achieve good protein coverage (Fig 3) and observed statistically significant, known kinase targets of staurosporine (Fig 2, Table 1). Thus far we've shown that OnePot TPP, LiP, and Solvent-PISA are feasible approaches to identify small molecule-protein interactions in cell lysates.

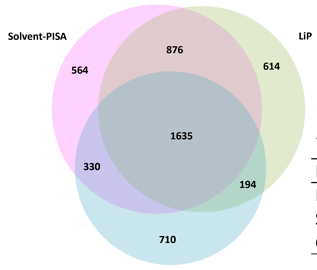

Fig 3: Venn diagram showing the protein coverage of staurosporine treated p53-deficient U2OS cell lysate processed via different SPMs.

Table 1: Summary of Solvent-PISA, LiP, and TPP Results.

Experiment	Total Proteins	Total Kinases	Hits	Kinase Hits
LiP	3319	13	724	12
Solvent-PISA	3406	67	463	16
One-Pot TPP	2869	75	720	11

Analysis of KU-D2-F treated p53-deficient U2OS cell lysates using One-pot TPP and Solvent-PISA workflows displayed good protein coverage and interesting putative interactions.

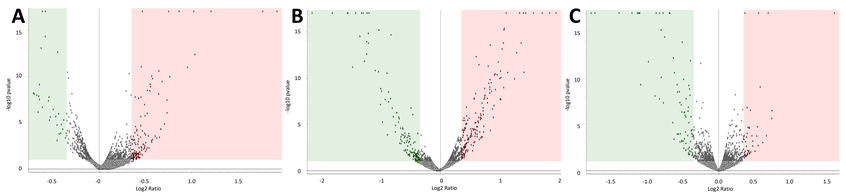

Fig 4: U2OS cell lysates were treated with 100µM KU-D2-F and analyzed by (A) One-pot TPP 03/06/2024, (B) One-pot TPP 02/15/2024 and (C) Solvent-PISA 04/15/2024. Data are presented as a volcano plot to highlight significant changes in abundance. Significant changes were determined by setting $p<0.1$ and log2 fold change$\geq$0.35 and are indicated with red or green dots.

Results

Initial results from One-Pot TPP show changes in protein folding stability with KU-D2-F treatment indicating novel drug targets

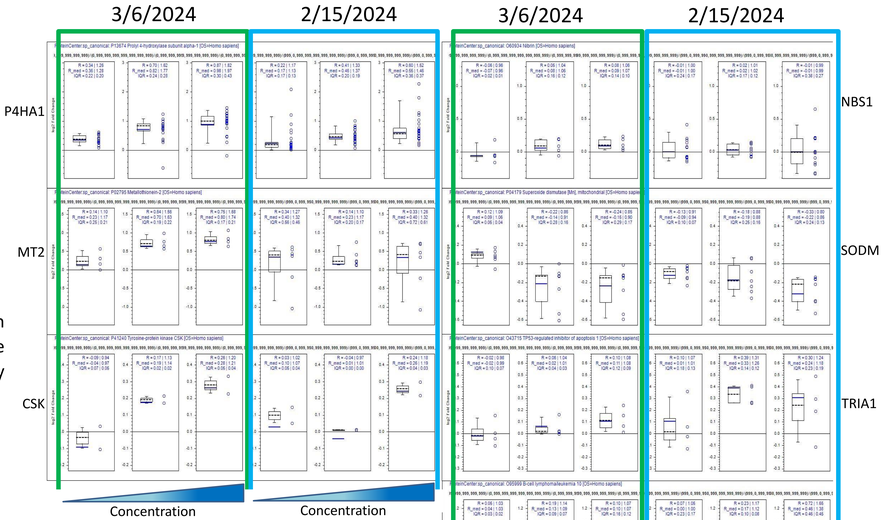

Fig 5: Box and whisker plots displaying changes in protein folding stability in p53-deficient U2OS cell lysates when treated with 10uM, 30uM, and 100uM KU-D2-F and processed via the TPP workflow for notable protein targets. For the box and whisker plots, the boxes represent the interquartile range, and the whiskers represent the full range of observed values. The doted midline denotes the median value.

Conclusions

- Assessment of technique feasibility was conducted by piloting each SPM workflow after treatment with staurosporine. Each successful SPM workflow displayed good protein coverage and protein folding stability of relevant staurosporine kinase targets.
- KU-D2-F-treated p53-deficient U2OS cell lysate processed via the One-pot TPP and Solvent-PISA workflows maintained good protein coverage and One-pot TPP revealed novel putative drug targets.
- Future directions include conducting at least three replicates of each SPM workflow, including LiP, to better understand the mechanism of the KU0171032 compound series.
- This work shows that SPM workflows can be used to orthogonally determine small molecule-protein interactions of novel drugs.
- Initial results show that SPM workflows were able to identify protein targets of KU-D2-F in p53-deficient U2OS cells which could allow us to understand the mechanism of these potential cancer drugs.

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