Chemoproteomics

High-throughput data-independent acquisition mass spectrometry workflow for in-depth identification of protein ubiquitination using an Orbitrap Astral mass spectrometer

Kevin Yang[1], Alissa J. Nelson[2], Mukesh Kumar[2], Brett Larsen[1], Kathryn Abell[2], Florian Georgescauld[2], Anthony Possemato[2], Mathew P. Stokes[2], Sean A. Beausoleil[2], Amirmansoor Hakimi[1] and Tonya Pekar Hart[1]

[1]Thermo Fisher Scientific, San Jose, CA, USA; [2]Cell Signaling Technology, Inc., Danvers, MA, USA

Abstract

Purpose: To develop robust mass spectrometry-based proteomics workflows on the Thermo™ Scientific Orbitrap™ Astral™ mass spectrometer (MS), in conjunction with the Cell Signaling Technology PTMScan® HS Ubiquitin Remnant Motif Kit, for high-throughput investigation of ubiquitination, facilitating the development of targeted protein degradation therapeutics.

Methods: Prostate cancer cells were treated with ARCC-4 (Figure 1), a PROTAC protein degrader targeting the androgen receptor. Global proteomics was conducted on the Orbitrap Astral MS to monitor both on-target and off-target effects. Di-Gly peptides were enriched and analyzed on the Orbitrap Astral MS to confirm the degradation mechanism.

Results: Our data indicate consistent dose-dependent degradation of the androgen receptor by ARCC-4, along with correlated changes in ubiquitination sites. These results suggest that the Orbitrap Astral MS enables ultra-high-throughput and in-depth validation of targeted protein degradation compounds, such as PROTACs, with exceptional sensitivity, reproducibility, and quantitation accuracy. This makes it an invaluable tool for drug discovery.

Introduction

Targeted protein degradation (TPD) is a groundbreaking approach in drug discovery that harnesses the body's natural protein degradation systems to selectively remove harmful proteins. By inducing proximity to the ubiquitination-proteasome pathway, small molecules can trigger the degradation of disease-causing proteins, making it possible to target previously undruggable proteins with exceptional precision and efficacy. However, designing compounds that can precisely induce this proximity is challenging. Therefore, reliable quantitation of protein degradation and ubiquitination during compound screening is a critical phase in TPD drug development. The need to screen large numbers of compounds accurately makes high-throughput mass spectrometry-based workflows the optimal choice for ensuring precise lead identification.

Materials and methods

Sample Preparation

To demonstrate the utility of the combined KGG-global proteome approach for TPD studies, human prostate cancer cell line LNCaP FGC was treated with androgen receptor (AR) degrader ARCC4 or the standard-of-care inhibitor enzalutamide (Enza) for 4h. Lysates were then subjected to whole-proteome KGG profiling and global proteomics by LCMS. The KGG enrichment was performed on 2.0 mg of input peptide using the Cell Signaling Technology PTMScan HS kit #59322 (Figure 2). Enrichment reproducibility was monitored by spiking in three heavy-labeled control peptides at the beginning of the enrichment (cat. #75964).

LC-MS/MS Analysis

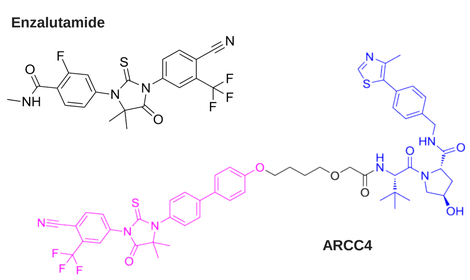
Tryptic peptides were loaded onto an IonOpticks Aurora Frontier 60x75 column and separated at a nanoflow rate of 24 samples per day (SPD) using a Thermo Scientific Vanquish™ Neo UHPLC system (Figure 3). For protein ubiquitination, di-glycine peptides were enriched using Cell Signaling Technology's PTMScan HS Ubiquitin Remnant Motif Kit. The enriched peptides were then loaded onto an IonOpticks Aurora Ultimate 25x75 column and resolved at a nanoflow rate of 50 SPD. The eluted peptides were ionized using an Easy-Spray source and analyzed on the Orbitrap Astral mass spectrometer using narrow window DIA (nDIA) analysis.

Data Analysis

Data was processed by Spectronaut™ software (Biognosys, v19) Cysteine carbamidomethylation was set as fixed modification; and protein N-terminal acetylation, methionine oxidation, and di-Gly on lysine was set as variable modifications. For lysine di-Gly analysis, PTM localization was enabled, and the probability cutoff was set as 0.75.

Acquired data was searched against FASTA files for human proteome were downloaded from Uniprot™. Peptide-spectrum match (PSM) and protein identifications were filtered for 1% FDR, and a Q-value cutoff of 1% was used for the DIA analysis. The resulting candidate tables were exported to tabular data formats, which were then processed with Python™ for downstream data analysis and visualization.

Figure 1. Androgen receptor degrader drug and control.



ARCC4 is a commercially available degrader designed to recruit the VHL E3-ligase to specifically degrade androgen receptor, a key clinical target in prostate cancer. Enzalutamide, which is an AR inhibitor, is the standard of care for prostate cancer and does not induce protein degradation.

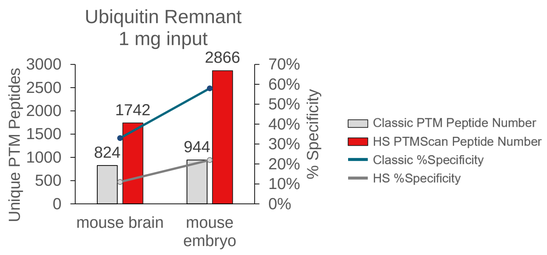
Results

LFQ-DIA workflow on Orbitrap Astral MS allows for in-depth and high-throughput PROTAC compound screening.

Prostate cancer cells were treated with varying concentrations of ARCC-4, a known androgen receptor (AR) degrader with high specificity, or enzalutamide, the AR-binding moiety of ARCC-4, as a negative control (Figure 4). The tryptic peptides were analyzed on an Orbitrap Astral MS for label-free quantitation. We developed an LFQ-DIA workflow capable of processing 24 samples per day (SPD), enabling deep proteome coverage for monitoring the on-target and off-target effects in TPD compound screening. Approximately 10,000 protein groups were identified and quantified.

Notably, we observed decreased androgen receptor expression with increasing ARCC-4 concentrations, while enzalutamide had no such effect (Figure 5). Moreover, the vast majority of the proteome remained unaffected (Figure 6), which is consistent with the specificity of ARCC-4 Androgen Receptor degrader. Our results highlights the suitability of the Orbitrap Astral MS for PROTAC compound screening in the TPD discovery pipeline, offering excellent proteome coverage and quantitation.

Figure 2. PTMScan HS enrichment protocol improves upon classic method.

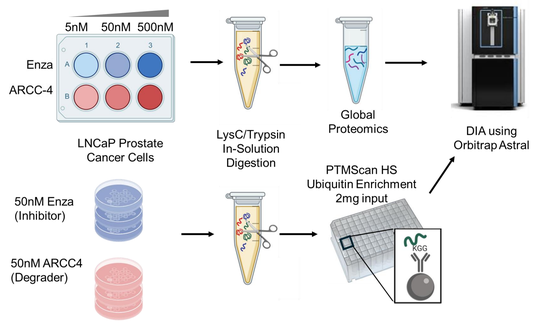


The HS kits were validated against the Classic versions with matched peptide input in multiple tissue types (brain and embryo shown). Unique modified peptide numbers are shown as bars and the percent specificity (number of PTM peptides/number of PTM + unmodified peptides) is shown as a line plot. Classic performance is in gray, and HS is in red.

PTMScan coupled with Orbitrap Astral MS enables in-depth ubiquitination analysis.

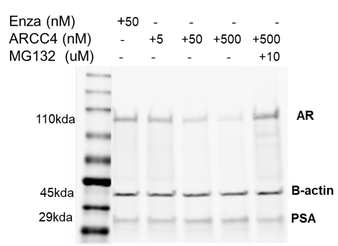
To confirm that androgen receptor was cleared through ubiquitination-dependent proteasomal degradation, cells were treated with 50 nM of ARCC-4 or enzalutamide, followed by enrichment of di-glycine peptides using Cell Signaling Technology's PTMScan HS Ubiquitin Remnant Motif Kit. We identified 65,445 lysine di-Gly sites, underscoring the sensitivity of the Orbitrap Astral MS in studying low-input enriched di-glycine peptides (Figure 7). Additionally, the specificity of the di-glycine peptides was over 40%, demonstrating the efficiency of the PTMScan HS enrichment kit.

Figure 3. Proteome and ubiquitinome profiling of AR degrader-treated cells.



Human prostate cancer cell line LNCaP FGC was grown in triplicate dishes per condition. ARCC4 (degrader) was dosed at 5nM, 50nM, or 500nM for 4h; control plates were treated with non-degrading Enza at matching concentrations. Lysates were prepared for proteomics analysis by in-solution digestion in urea. DiGly peptides were enriched using PTMScan HS #59322 on a Kingfisher robotic system. All samples were acquired in DIA mode on an Orbitrap Astral MS.

Figure 4. Western blots confirm ARCC4 activity.

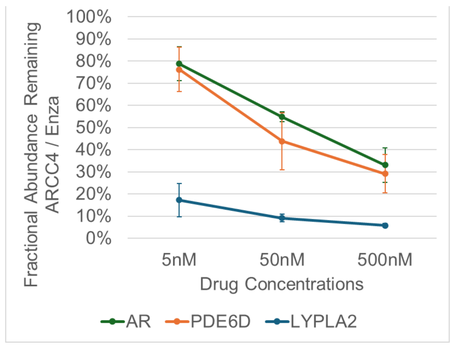


ARCC4 induces dose-dependent degradation of androgen receptor. Proteasome inhibition with MG132 reverses the degradation. Antibodies used: Androgen Receptor cat# 5153; B-actin, cat# 6497: PSA/KLK3, cat#5365.

Ubiquitination of Androgen Receptor

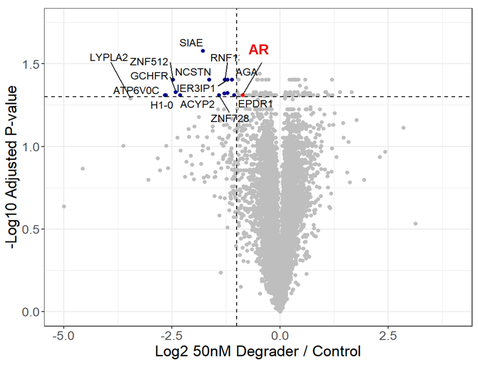
Correlating changes in ubiquitination sites with protein expression levels enabled differentiation between drug-induced off-target effects and putative substrate proteins (Figure 8 and 9). The presence of elevated ubiquitination sites supports a VHL-mediated degradation pathway for specific substrates. Furthermore, mapping ubiquitination sites onto the three-dimensional structure of substrate proteins may elucidate potential E3 ligase binding regions (Figure 10).

Figure 5. AR and off-target substrates exhibit dose-dependent response to ARCC4.



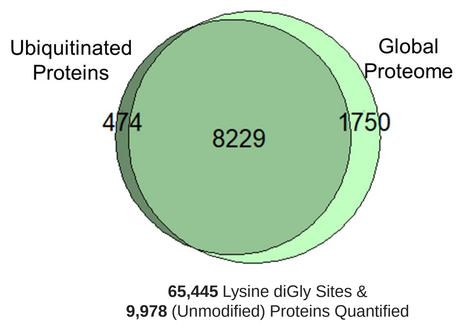
In the global proteome samples, proteins that decreased in abundance between Enza and ARCC4 conditions and continued trending downwards with increasing dosage were identified. At 500nM ARCC4, AR decreased to 33% of its abundance with 500nM Enza. PDE6D and LYPLA2 also were degraded in a dose-dependent manner. Error bars indicate 1 sd.

Figure 6. Multiple proteins degraded by ARCC4.



Dotted lines indicate where the adjusted p-value=0.05 (y-axis) and where protein abundance decreased by 50% (x-axis). AR is indicated with a red point. Proteins indicated by blue points represent off-target activity of the drug. In the 5nM and 500nM comparisons, no proteins reached significance, though many of the same proteins decreased in abundance.

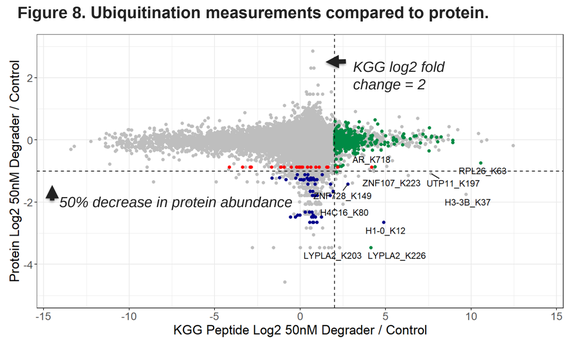
Figure 7. Deep proteome coverage enables high overlap between ubiquitin and protein measurements.



65,445 Lysine diGly Sites &
9,978 (Unmodified) Proteins Quantified

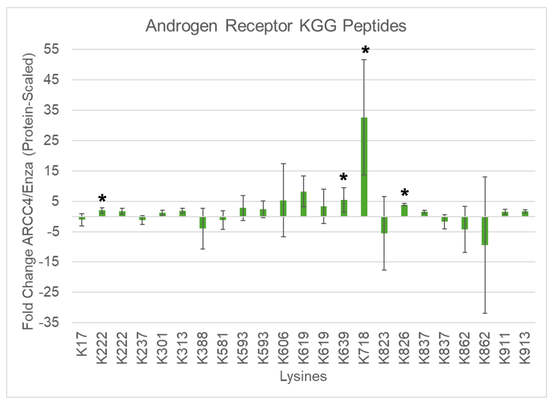
In the global proteome samples, 9978 proteins were quantified with complete profiles across all samples. PTMScan HS enriched 72,507 ubiquitinated peptides from 8704 proteins that could be quantified with up to 50% missing values imputed using background signal. DIA with the Orbitrap Astral MS enabled nearly 80% overlap at the protein level between these two datasets.

Figure 8. Ubiquitination measurements compared to protein.



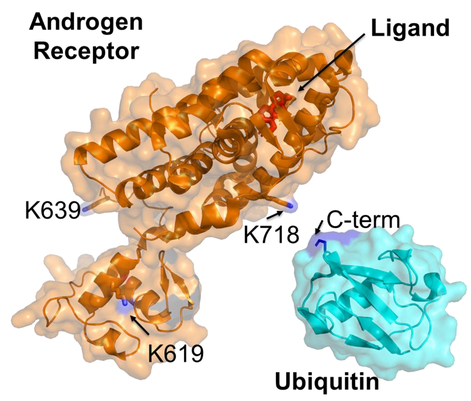
Many proteins appeared to be off-target substrates based on the global proteome measurement alone, but only a subset of those proteins also had a lysine with a substantial increase in ubiquitination. LYPA2, H1-0, and ZNF728 show the most severe off-target degradation. Dotted lines indicate where the KGG peptide abundance increased by 4-fold and where protein abundance decreased by 50% (y-axis). No features reached significant p-values in both the KGG and protein measurements.

Figure 9. Deep proteome coverage enables high overlap between ubiquitin and protein measurements.



Bars indicate linear peptide fold-changes scaled for protein abundance changes between the treatments. Some lysines are covered by more than one peptide, typically due to digestion variants. Error bars indicate one standard deviation in the ratio measurement. Stars (*) indicate peptides that had unadjusted p-values <0.05. Ubiquitination at Lys718 increased 32-fold, far above other sites. This site likely is primarily responsible for the degradation of AR by ARCC4.

Figure 10. Ubiquitinated Mapped to AR Structure.



PDB structure 1E3G covering the ligand binding domain was supplemented with an Alpha Fold model of part of the DNA-binding domain. A bound ligand molecule shown in red illustrates the buried binding pocket that ARCC4 likely uses. The structure of ubiquitin is shown for scale. Modeling the ubiquitination sites of the substrate protein provides the starting point for a structure-based optimization of the drug molecule. The model also enables structural predictions for how the PROTAC would recruit the E3 ligase complex and leave room for elongating a polyubiquitin chain.

Conclusions

- The Orbitrap Astral MS enables high-throughput compound screening and in-depth validation for targeted protein degradation.
- Cell Signaling Technology PTMScan HS ubiquitin enables in-depth ubiquitination analysis using Orbitrap Astral MS.
- The Astral DIA method enabled deep coverage of both the global and ubiquitinated proteome, detecting androgen receptor (AR) even after significant degradation, including 19 ubiquitinated lysines.

References

- Salami J, Alabi S, Willard RR, Vitale NJ, Wang J, Dong H, Jin M, McDonnell DP, Crew AP, Neklesa TK, Crews CM. Androgen receptor degradation by the proteolysis-targeting chimera ARCC-4 outperforms enzalutamide in cellular models of prostate cancer drug resistance. Commun Biol. 2018 Aug 2;1:100. doi: 10.1038/s42003-018-0105-8. PMID: 30271980; PMCID: PMC6123676.
- Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Bäsler S, Schäfer M, Egner U, Carrondo MA. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem. 2000 Aug 25;275(34):26164-71. doi: 10.1074/jbc.M004571200. PMID: 10840043. PDB 1E3G.

Trademarks/licensing

PO003797 0325

Learn more at thermofisher.com/astral