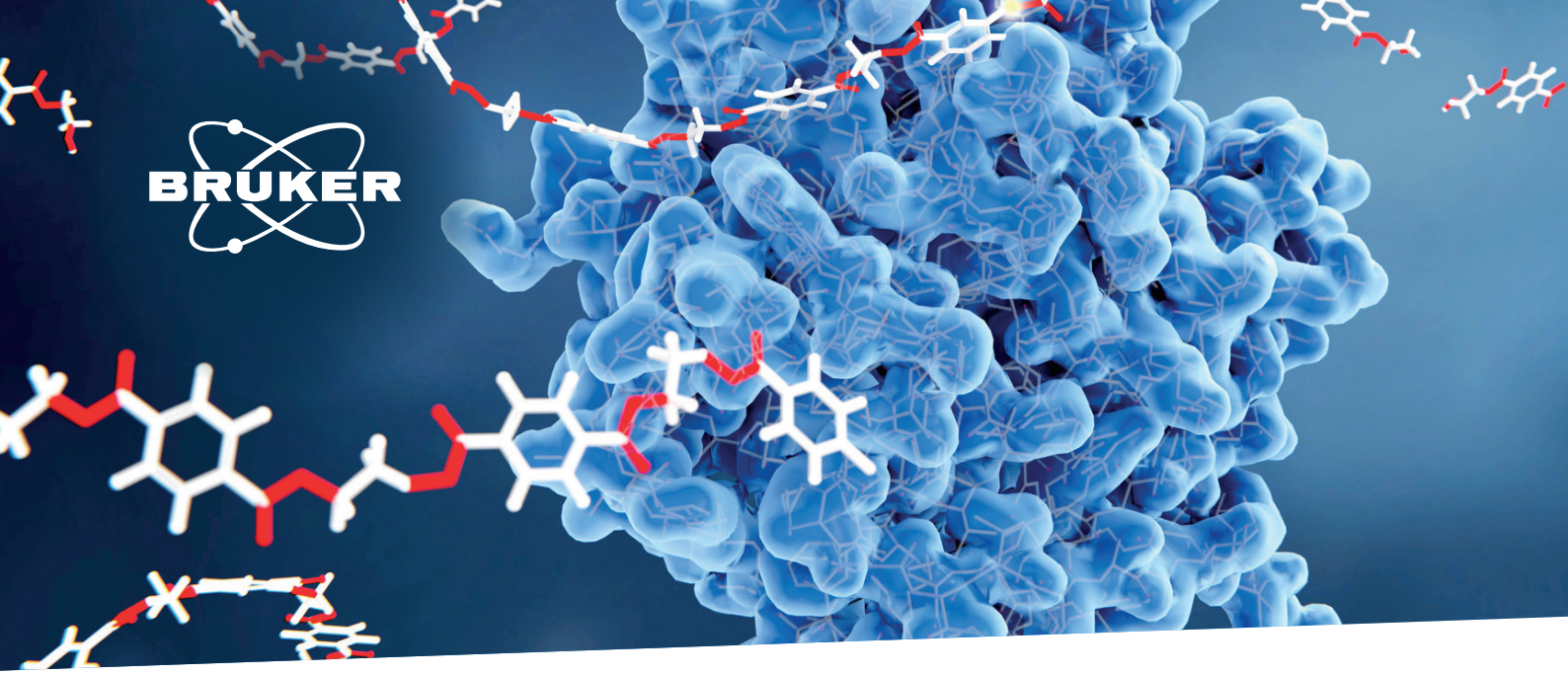

Improving off-target liability assessments with a dia-PASEF workflow for competitive Activity-Based Proteomics Profiling (cABPP)

Identifying targets and off-targets is crucial in drug development to avoid side effects and enhance efficacy. Bruker's timsTOF platform with dia-PASEF® offers a reliable solution.

Abstract

Addressing off-target effects is crucial in drug development. We present a concise methodology employing competitive activity-based proteomic profiling (cABPP) with serine hydrolase-targeting probes to discern the targets and off-targets of lipase inhibitors (inhibitors 1-3). Primary hepatocytes from wild-type mice were treated with inhibitors, followed by exposure to an activity-based probe, lysate preparation, and enrichment of probe-bound proteins. Mass spectrometry analysis with dia-PASEF unveiled both known and novel (off)-targets of the tested inhibitors, underscoring the method's sensitivity, utilizing a timsTOF Pro. The workflow, outlined in Figure 1, integrates ABP technology with advanced mass spectrometry for comprehensive drug target profiling.

Keywords:
activity-based proteomic profiling, timsTOF Pro, Evosep One, small molecule inhibitors, chemoproteomics, drug discovery, inhibitors

Introduction

The unintended inhibition of off-target proteins remains a significant challenge in drug development, potentially leading to unexpected side effects and decreased therapeutic efficacy. Competitive activity-based proteomic profiling (cABPP) is a valuable tool for the identification of potential off-target liabilities.

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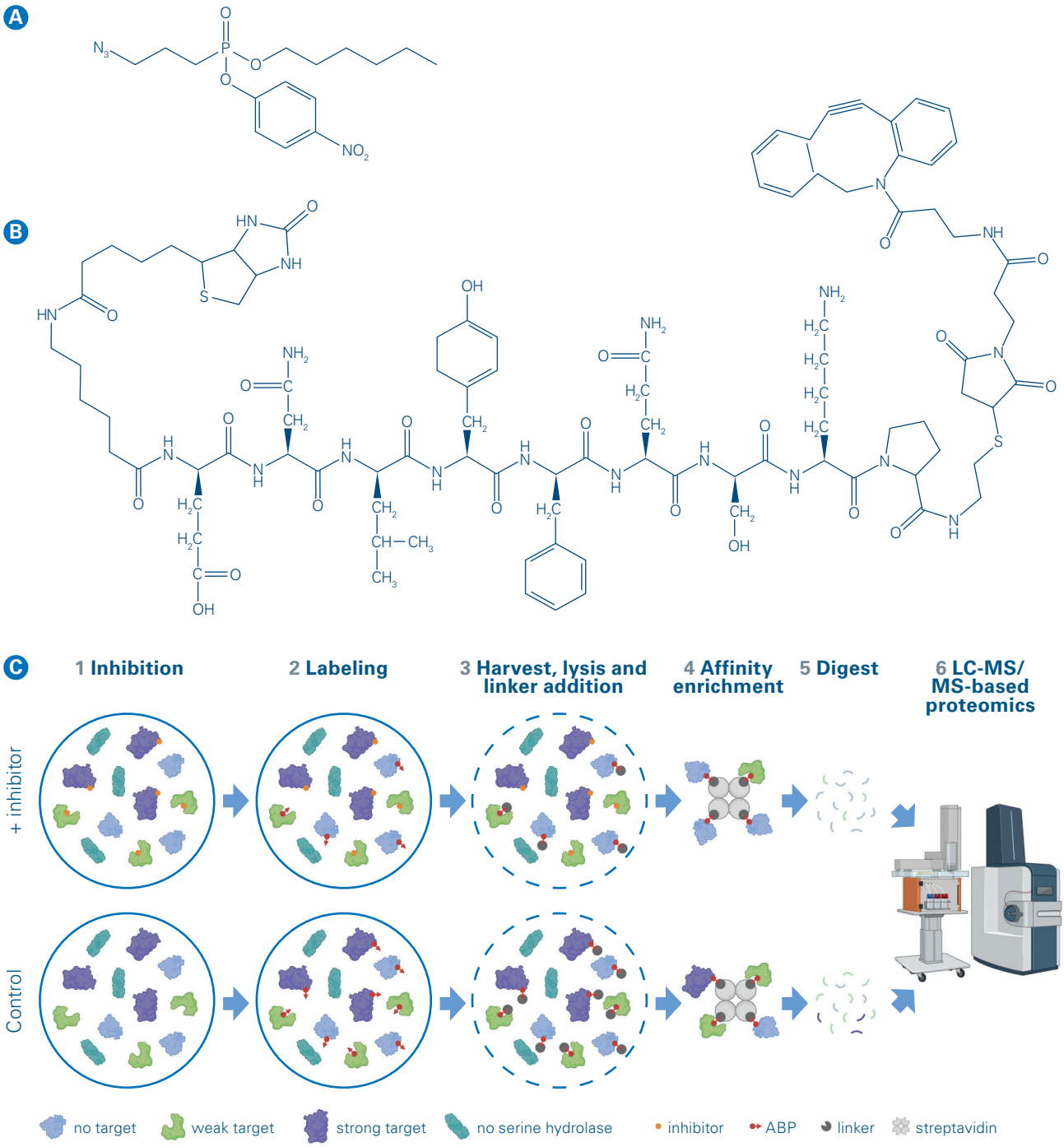

Figure 1

Competitive Activity-Based Proteomic Profiling (cABPP).

(A) Activity-based probe (ABP): Hexyl 4-nitrophenyl (3-azidopropyl) phosphonate; (B) Linker: DBCO-TEV-Biotin strain promoted click linker; (C) Schematic representation of a cABPP workflow (Created with BioRender.com).

This method leverages the power of activity-based probes (ABPs), which usually mimic a naturally occurring substrate, and exhibit selectivity towards an enzyme family. ABPs engage in competition with endogenous substrates for the enzyme's active site, displacing the substrate and forming a covalent bond that terminally blocks the active site.

In competitive ABPP, inhibitors are co-incubated with the probe. Inhibitors that target a particular enzyme impede the probe's access to the active site, and a reduction in probe binding efficiency correlates inversely with an inhibitor's affinity for a specific enzyme. The

extent of probe-binding to an enzyme can be determined through various reporter tags on the probe. This could be a fluorophore for visualization or a tag for affinity enrichment (e.g., biotin). Affinity enrichment of probed proteins coupled with in-depth mass spectrometry enables the identification of novel off-targets.

In this study, we demonstrate the effectiveness of a label-free timsTOF-based cABPP workflow in identifying the target and off-targets of several lipase inhibitors, here named inhibitors 1-3. Our results showcase the sensitivity of cABPP in detecting both known and novel off-targets.

Material and Methods

Competitive Activity-Based Proteomic Profiling workflow

Primary hepatocytes were prepared from wild-type mice according to Charni-Natan and Goldstein (1). The cells were plated in 6-well plates at a density to reach around 80-90% confluency overnight. The next day, the medium was exchanged to 1 mL of FBS-free medium containing the desired concentration of inhibitors (here termed inhibitors 1-3) or DMSO (Control) for 2 hours at 37°C. To a subset of cells ("Probe" group) we then added 30 µM of a serine hydrolase-targeting activity-based probe (ABP): (Hexyl 4-nitrophenyl (3-azidopropyl) phosphonate)) (Figure 1A) for 30 min at 37°C, while DMSO was added to the other set of cells ("No Probe" group). The cells were subsequently washed with PBS and lysed by brief sonication in ABPP lysis buffer (100 mM Tris-HCl pH 8.5, 1% SDS, 30 mM NEM, 10 mM TCEP) prior to incubation at 95°C for 10 min. Following protein estimation, 500 µg protein per sample was precipitated and dissolved in a re-solubilization buffer (100 mM Tris-HCl pH 8.5, 1% SDS). The samples were then incubated with a click linker containing biotin as well as a strained alkyne group (Figure 1B) that readily reacts with the functional azide on the ABP, enabling a fast covalent link between the probe-bound proteins and biotin. The excess linker was removed by filtration through 3 kDa centrifugation cut-off filters (#UFC5003, Merck), and probe-bound proteins were enriched on streptavidin-agarose beads (#29347, Thermo Fisher) for 4 hours at RT. The beads were washed thoroughly and subsequently digested with sequencing-grade modified trypsin (#V5111, Promega). 500 ng of the sample was then loaded onto Evotips according to the sample loading protocol for Evotips. Briefly, Evotips were washed with 20 µL Solvent B (Acetonitrile with 0.1% formic acid) and placed in 1-propanol for 10 seconds prior to pre-conditioning with 20 µL Solvent A (H_2O with 0.1% formic acid). Finally, samples were diluted to contain 500 ng / 20 µL in solvent A and were loaded onto the Evotips. Chromatographic separation was then carried out on a reversed-phase UHPLC Aurora Elite column (150 mm x 75 µm, 1.7 µm, IonOpticks) in the Evosep One chromatography platform (2). The Evosep Whisper 40 samples per day (SPD) gradient was employed at a flow of 100 nL/min and the eluted peptides were introduced to a timsTOF Pro mass spectrometer by electrospray ionization (ESI) through a CaptiveSpray ion source. The timsTOF was operated in data-independent acquisition dia-PASEF mode. For protein identification and label-free quantitation, we employed DIA-NN (3). Library generation was enabled using a UniProt mus musculus FASTA file containing common contaminants. DIA-NN default settings were used apart from adding NEMylation on cystein as fixed modification. Statistical tests were performed using Perseus (version 1.6.15.0) (4) and R (version 4.3.0). The cABPP workflow is schematically shown in Figure 1C.

Table 1 MS acquisition parameters

MS	timsTOF Pro	
Source	CaptiveSpray Interface (CSI)	
	Capillary	1600 V
	Dry Gas	3.0 L/min
	Dry Temp	180°C
Ionization	Positive Mode	
Acquisition Mode	TIMS-MS	
	Ramp time	100 ms
	Mobility range	0.7 – 1.35 $1/K_0$
	Mass range	100 – 1700 *m/z*
Transfer parameters	Deflection delta	70 V
	Funnel 1 RF	300 Vpp
	Funnel 2 RF	200 Vpp
	Multipole RF	500 Vpp
	Collision Energy	10 eV
	Collision RF	1500 Vpp
	Quadrupole Low Mass	200 *m/z*
	Transfer time	60 µs
	Pre Pulse Storage Time	12 µs
Calibration	Instrument mass calibration using sodium formate; enhanced quadratic calibration mode. Instrument mobility calibration using Tuning Mix ES-TOF CCS compendium (ESI)	

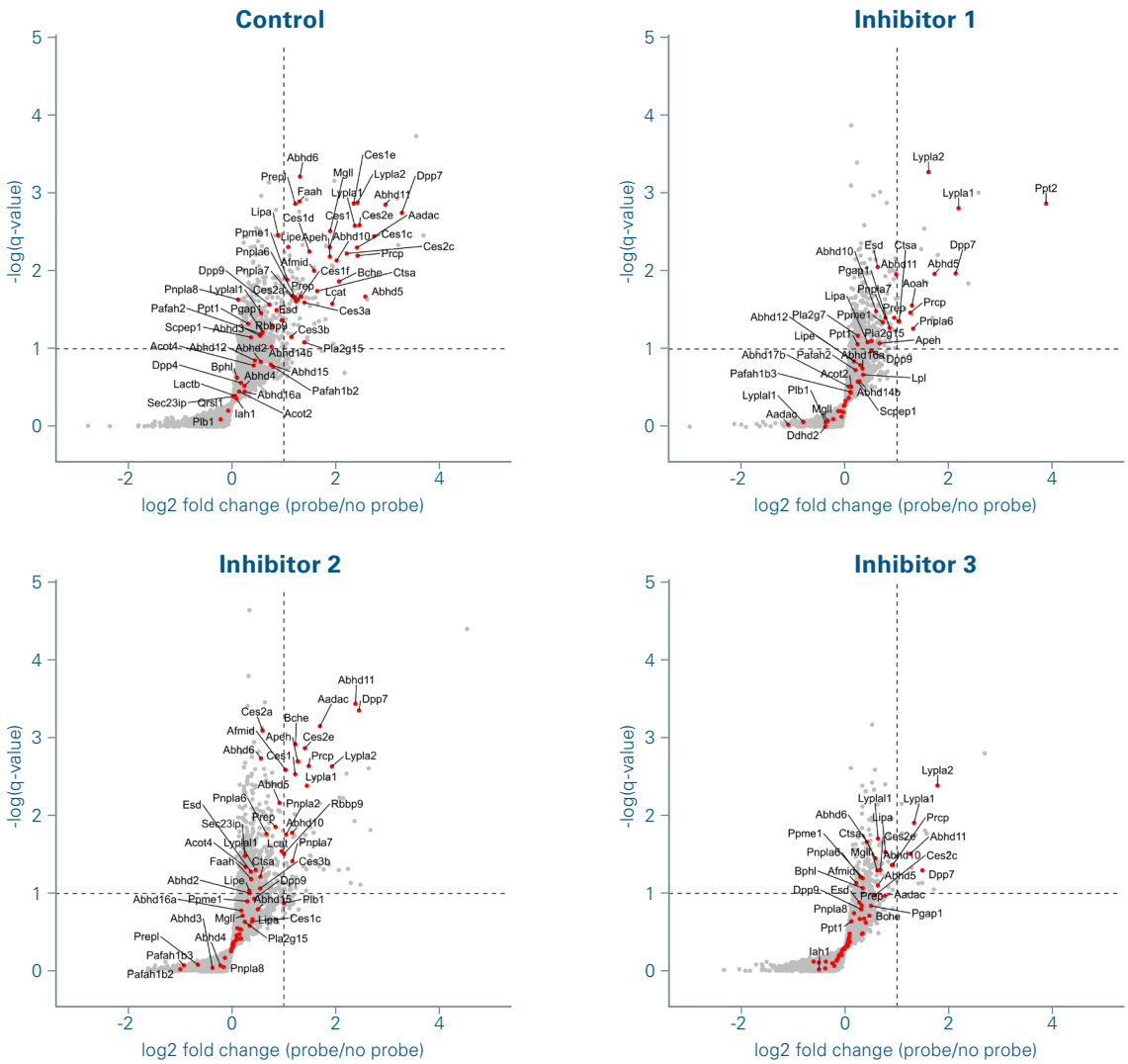

Figure 2

Enrichment efficiency of the ABPP workflow employing the serine hydrolase-targeted ABP, a click chemistry-enabled linker, and streptavidin enrichment.

One-sided volcano plots of probe-treated samples versus non-probe-treated samples among each group (Control or Inhibitor-treated), where red dots represent serine hydrolases.

Results and Discussion

To test our cABPP workflow, murine primary hepatocytes were exposed to three distinct inhibitors targeting different intracellular lipases. Given that most lipases show serine hydrolase activity, we subsequently employed a serine hydrolase-targeting ABPP workflow. We first checked for the enrichment of all serine hydrolases based on treatment with our ABP. To this end, t-tests were conducted to compare "Probe" samples with "No Probe" samples within each group (Control, Inhibitor 1, Inhibitor 2, and Inhibitor 3). One-sided volcano plots of these two-sided multi-testing corrected t-tests are shown in Figure 2, where serine hydrolases (marked in red) proved to be more abundant in "Probe" samples, highlighting successful serine hydrolase enrichment based on the here described ABPP approach. The extent of enrichment was partially influenced by the treatment with inhibitors, presumably due to the occupancy of the active sites of certain serine hydrolases, as illustrated in Figure 1C. This impact on enrichment is evident when comparing the different volcano plots in Figure 2.

To assess the impact of the different inhibitors on individual serine hydrolases, we compared the enriched ("Probe") samples treated with inhibitors to the corresponding enriched ("Probe") control samples. The interaction between an inhibitor and the active site of an enzyme leads

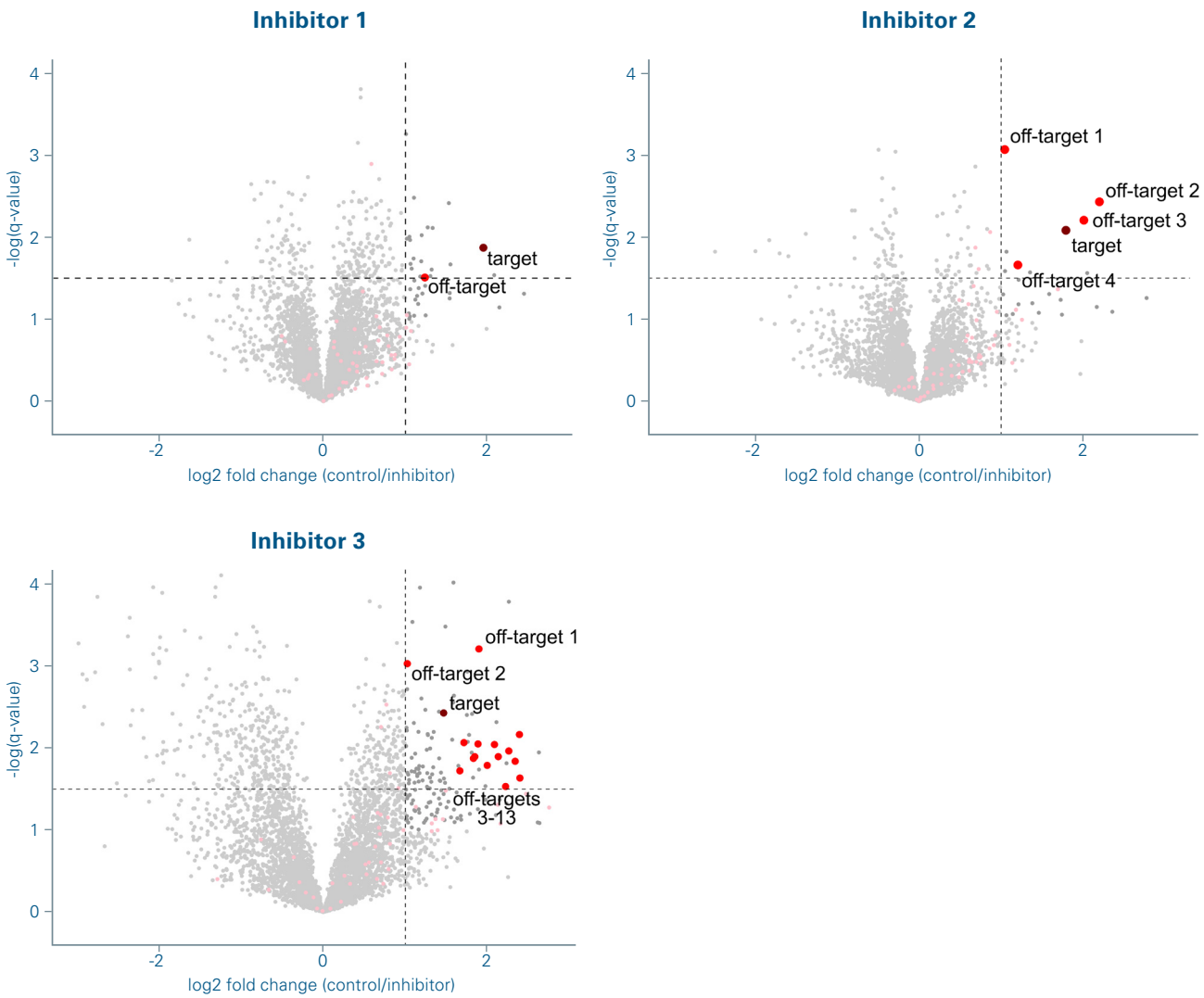

Figure 3

Comparison of enriched inhibitor-treated samples with enriched control samples validates binding to target proteins and reveals binding to several off-target proteins.

Volcano plots display two-sided multi-testing-corrected t-tests between Control and Inhibitor-treated enriched proteins. Proteins shown in the top right corners of the volcano plots represent target proteins of inhibitors.

to reduced binding of the ABP. Consequently, this diminishes both the enrichment and further the abundance levels measured by timsTOF-based proteomics. Serine hydrolases exhibiting higher levels in the control groups, in comparison to the inhibitor-treated groups, are indicative of proteins that are targeted by the corresponding inhibitor. These proteins are shown in the upper right-hand corners of the volcano plots in Figure 3. For all tested inhibitors these proteins include the respective target enzyme of the inhibitor shown in dark red, which validates the here employed cABPP workflow. While most serine hydrolases (represented as light pink dots) are spread across the whole volcano plots, there are several serine hydrolases that were less enriched due to inhibitor treatment, suggesting that these are possible off-target interactions with the inhibitors. In the case of Inhibitor 1, we identified one weak off-target protein, which showed less reduction in response to inhibitor treatment compared to the actual target. For both Inhibitor 2 and 3 there were several off-target proteins that appear to be inhibited to a higher extent than the target, which suggests that these inhibitors are not as selective as previously thought.

Table 2 dia-PASEF and MS/MS parameters.

Collision Energy	$1/K_0$ [V*s/cm²]		Collision Energy [eV]		
	0.60		20.00		
	1.60		59.00		
dia-PASEF MS/MS	Mass Range		300 – 1200 Da		
	Mobility Range		0.71 – 1.31 $1/K_0$		
	Cycle Time Estimate		1.38 s		
dia-PASEF windows	Cycle ID	Start IM [$1/K_0$]	End IM [$1/K_0$]	Start Mass [*m/z*]	End Mass [*m/z*]
	1	0.90	1.35	674	694
	1	0.70	0.90	300	430
	2	0.93	1.35	694	717
	2	0.70	0.93	430	465
	3	0.95	1.35	717	741
	3	0.70	0.95	465	492
	4	0.96	1.35	741	767
	4	0.70	0.96	492	515
	5	0.97	1.35	767	794
	5	0.70	0.97	515	536
	6	0.98	1.35	794	824
	6	0.70	0.98	536	556
	7	0.99	1.35	824	857
	7	0.70	0.99	556	575
	8	1.00	1.35	857	893
	8	0.70	1.00	575	594
	9	1.01	1.35	893	936
	9	0.70	1.01	594	613
	10	1.02	1.35	936	991
	10	0.70	1.02	613	633
	11	1.03	1.35	991	1065
	11	0.70	1.03	633	653
	12	1.06	1.35	1065	1200
	12	0.70	1.06	653	674

This cABPP approach could be expanded to test several inhibitor concentrations to evaluate dose response. In a further step, these findings could also be validated through biochemical and/or cellular assays, to confirm the biological relevance of the identified off-targets.

In this case study, we harnessed the capabilities of the timsTOF Pro mass spectrometer, specifically leveraging its trapped ion mobility spectrometry (TIMS) and Parallel Accumulation Serial Fragmentation (PASEF®) technology. The overall robustness, sensitivity, selectivity, and speed of the timsTOF Pro system proved instrumental in executing a label-free competitive activity-based proteomic profiling (cABPP) workflow.

Traditionally, labeling-based methods for cABPP can be cumbersome and time-intensive. However, the timsTOF's prowess eliminates the need for such tedious workflows, offering a streamlined and efficient alternative. The TIMS technology, with its unique ability to separate ions based on size and shape, enhances sensitivity and peak capacity. This is particularly advantageous when dealing with a high-throughput application and resulting shorter gradients, where the overall complexity per time point is increased. This increased complexity is spread across the additional ion mobility dimension and causes no compromise.

Furthermore, the more recently launched timsTOF HT (2022) and timsTOF Ultra (2023) systems, equipped with advanced features such as a high-capacity TIMS XR cartridge and HDR 14-bit digitizer, continue to improve the overall performance and specifically the dynamic range and quantitative precision.

Notably, the advantage of performing label-free cABPP on the timsTOF extends beyond efficiency. It offers a comprehensive view of the proteome, eliminating potential biases introduced by labeling reagents. The timsTOF's ability to accumulate and concentrate ions with specific mass-to-charge and mobility allows to precisely target precursor ions of interest for identification and quantification with high confidence.

In summary, this study showcases the exceptional benefits of employing the timsTOF mass spectrometer for label-free cABPP. The advanced TIMS technology, coupled with PASEF and other innovative features, elevates the field of proteomic profiling. cABPP is a powerful tool in the early stages of drug discovery and development. It offers a systematic and comprehensive approach to identifying off-targets, aiding in the optimization of lead compounds, and minimizing the risk of unexpected adverse effects. By combining the strengths of activity-based probes and label-free timsTOF-based mass spectrometry, cABPP empowers researchers to make informed decisions and design safer and more effective therapeutics.

Conclusion

- The here described ABPP approach using a serine hydrolase-targeted probe combined with click chemistry-enabled protein enrichment and timsTOF-based proteomics allowed the enrichment of serine hydrolases in all tested conditions.
- Binding of an inhibitor to a target protein causes reduced probe binding, which results in reduced enrichment and is detected as decreased abundance in the inhibitor treated samples compared to the control.
- This label-free timsTOF-based cABPP method was validated by finding the known target of each inhibitor and further enabled the identification of unknown (off-)target proteins that in some cases are inhibited to a higher extent than the known target.
- All timsTOF mass spectrometers offer a robust method for proteomics profiling with uncompromised sensitivity, selectivity, and speed by leveraging the unique capabilities of TIMS and PASEF technology.

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