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Activity-based proteomics of the endocannabinoid system

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Citation

Rooden, E. J. van. (2018, September 11). *Activity-based proteomics of the endocannabinoid system*. Retrieved from <https://hdl.handle.net/1887/65174>

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Title: Activity-based proteomics of the endocannabinoid system

Issue Date: 2018-09-11

Chapter 4

Mapping *in vivo* target interaction profiles of covalent inhibitors using chemical proteomics with label-free quantification¹

Introduction

Determining target protein engagement and off-target activities of small molecules is an essential step in the drug discovery process. Information on target engagement and off-target profile at a certain concentration will help to select the best compound as a drug candidate (in terms of activity and selectivity) and may guide the dose selection by providing information on full target engagement, while minimizing the risk for untoward off-target interactions by preventing overexposure. Information on target engagement in cellular and animal models, as well as in man can be obtained through a variety of experimental techniques, including direct quantification of substrates and/or products of enzymatic reactions, ligand binding studies using radioactive or fluorescent tracers, cellular thermal shift assays and positron emission tomography.²⁻⁵

Recently, activity-based protein profiling (ABPP) has emerged as a powerful chemical proteomics technology to map the interactions between small molecules and proteins on a global scale in living systems, including cells, animals and humans.⁶⁻⁸

Activity-based Protein Profiling. ABPP is a technique pioneered by the Cravatt laboratory,⁶ and that relies on active site-directed chemical probes that react, in a mechanism-based fashion, with the catalytic nucleophile of target proteins in their native biological environment. As a result, a covalent and irreversible bond is formed between the chemical probe and the active site of the target protein. Because this process requires a catalytically active protein, these chemical probes report on the abundance of active enzymes. ABPP enables the possibility to study on-target and off-target activities of drug candidates (and metabolites) in their native physiological context, thereby greatly enhancing the therapeutic relevance of the observed target interaction profile. Generally, an activity-based probe (ABP) consists of an electrophile, a reporter group (a biotin or fluorophore), a linker between the electrophile and reporter group and (in

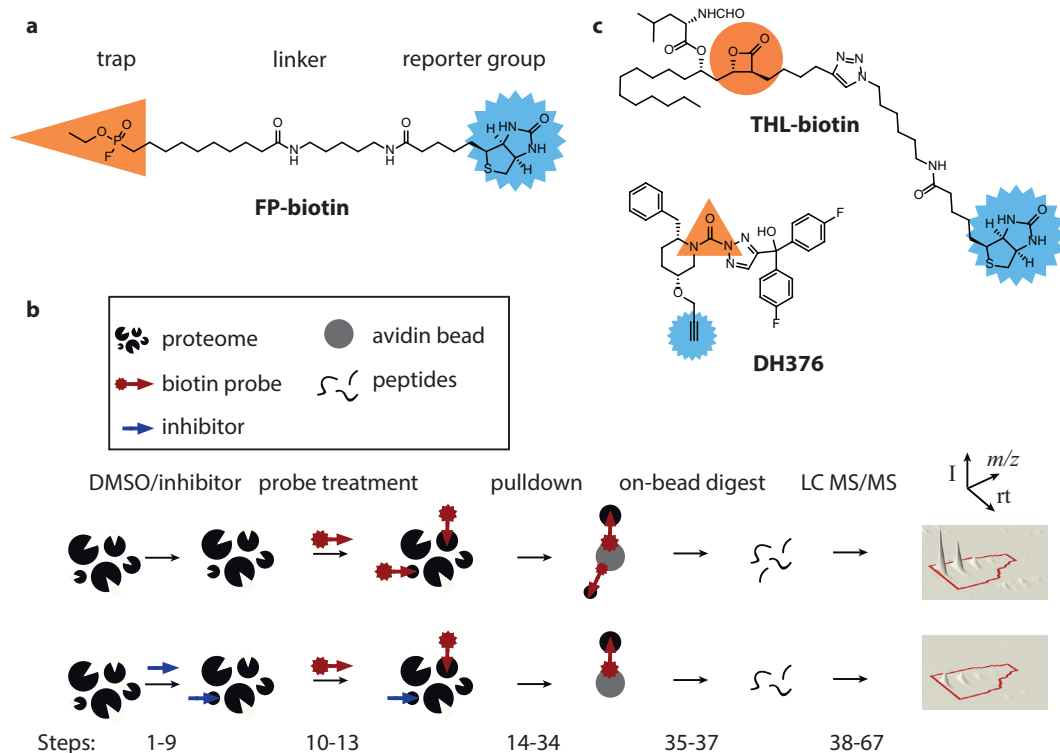


Figure 1 | Chemical proteomics workflow with inhibitor and probes used in this study. (a) General activity-based probe (ABP) design, illustrated with FP-biotin. Orange: trap (fluorophosphonate). Blue: reporter group (biotin). (b) Workflow of competitive ABPP followed by chemical proteomics: after treatment with inhibitor (or DMSO control), a proteome is labeled with a biotin-containing ABP, enriched using avidin (pulldown), followed by on-bead digestion. The resulting peptides are measured, identified and quantified by LC-MS/MS. Corresponding steps of the Procedure are indicated. (c) Structures of the ABP THL-biotin and the inhibitor DH376. Orange: trap. Blue: reporter group.

most cases) a recognition element which targets the probe to a certain enzyme (family). This general design is illustrated with the probe FP-biotin in **Figure 1a**: the fluorophosphonate (FP) is the electrophilic trap and a biotin acts as the reporter group.⁶

A variety of ABPs have been described for different enzyme classes (see **Table 1**).^{9,10} An ABP can be specific for one enzyme (tailored probe)¹¹ or target a group of enzymes sharing recognition or reactivity (broad-spectrum probe).¹² Broad-spectrum probes can be used for competitive ABPP (**Fig. 1b**) to determine target engagement and the selectivity profile of inhibitors. In gel-based competitive ABPP, a fluorescent ABP is incubated with a proteome and the sample resolved and visualized by SDS-PAGE and in-gel fluorescence scanning. Pre-incubation of the proteome with an inhibitor will reduce the

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ABP labeling of proteins targeted by the inhibitor.¹³ When coupled to a biotin, ABPs enable affinity enrichment using (strept)avidin-beads, proteolytic digestion and identification of the targeted enzymes by high resolution, quantitative liquid chromatography mass spectrometry (LC-MS)-based methods.¹⁴ Compared to gel-based assays, mass spectrometry has the advantage of higher resolution (no band overlap for proteins of similar size), higher dynamic range (proteins of different abundance can be analyzed in parallel) and direct identification of the enriched proteins (bands on a gel have to be validated with genetic knock-out or well-characterized inhibitors). The disadvantage of mass spectrometry based ABPP is the more elaborate sample preparation (typically, a gel-based ABPP experiment can be performed in 3 hours, while a pulldown assay takes 2 days) and the advanced instrumentation required.¹⁵

Table 1 | Commercially available ABPs for pulldown experiments.

Probe	Enzyme targets	Supplier	Cat. no.
FP-biotin	Serine hydrolases	Santa Cruz Biotechnology	sc-215056A
Desthiobiotin-ATP	Kinases	Thermo Fisher	88311
Desthiobiotin-GTP	GTPases	Thermo Fisher	88315
Biotin-Ahx-SUMO2-VME	SUMO proteases	UbiQ	UbiQ-156
Biotin-ANP-Ub-PA	Deubiquitylating enzymes (DUBs)	UbiQ	UbiQ-077
Biotin-Ahx-Ub-Dha	Ub E1, E2 and E3 ligases	UbiQ	UbiQ-102

Label-free quantification. Quantification of the relative abundance of active proteins using MS-based methods is usually achieved by chemical or metabolic labeling of the proteins by stable heavy isotopes.^{16, 17} Recently, label-free quantification approaches have gained interest as a suitable alternative, because they allow for a more simplistic experimental set up avoiding expensive and time consuming labeling steps and do not require analysis of complex mass spectra.^{16,18–20} There is no restriction for the number of samples that are to be compared and it is easier to adapt the experimental design. In addition, label-free methods do not require any mixing of samples and, therefore, higher proteome coverage can be achieved. Disadvantages of label-free quantification are the dependency on very stable liquid chromatography separation and spray conditions and the need of technical replicates. Furthermore, data processing time is increased by the requirement of aligning the runs. Label-free quantification has been used extensively in shotgun proteomics, and several examples are reported in the literature of its use in combination with ABPP or affinity-based chemoproteomics.^{12,21,22}

Whereas quantification of protein activity in the previous Protocol was performed by dimethyl labeling,¹³ now a label-free quantification protocol¹⁸ is described with the use of data-independent

acquisition (DIA) and ion mobility separation (IMS), based on the report of Distler *et al.*²⁰ In data-dependent acquisition (DDA), peptides above a certain signal intensity threshold are selected for fragmentation. This inherent sampling of high-intensity signals makes it difficult to reproducibly quantify low abundant peptides.²³ DIA is an unbiased method, fragmenting all precursor ions in a certain mass range. However, this approach makes the resulting fragmentation spectra highly complex. In ion mobility separation (IMS), ions are separated in the mass spectrometer according to their mobility in a buffer gas, thereby providing an additional dimension of separation after liquid chromatography.²⁴ Additionally, precursor ions can be coupled to their fragments on the basis of their drift time in IMS, increasing the number of identified peptides drastically.²⁵

Overview of the procedure. The ABPP protocol is demonstrated by identification of the *in vivo* targets of the diacylglycerol lipase inhibitor DH376 in four mouse tissues (brain, kidney, liver and testis) using two biotinylated probes (FP-biotin and THL-biotin (MB108)). In a nutshell, tissue lysates of mice treated with DH376 or vehicle are compared by competitive ABPP (heat-inactivated vehicle samples are used as a control) (**Fig. 1b**). Following tissue lysis (Steps **1-9**), enzymes are labeled by incubation with a cocktail of the two biotinylated ABPs (Steps **10-13**) and enriched using affinity chromatography (avidin-agarose pulldown, Steps **15-34**), and digested with trypsin (Steps **35-37**). The resulting tryptic peptides are measured using LC-IMS-MS (liquid chromatography-ion mobility separation-mass spectrometry, Steps **38-44**). Label-free quantification is used to compare the different conditions (vehicle vs. heat-inactivated, vehicle vs. inhibitor and the relative enzyme activity across the different tissues) (Steps **45-67**). The comparison of these different conditions would not have been possible with dimethyl labeling as a quantification method, due to the lack of multiplicity.

Applications of competitive ABPP. An ABPP protocol is presented that can be used to determine target engagement and selectivity of inhibitors and drug candidates in native proteomes. An earlier version of this protocol has been applied to identify the off-targets of the fatty acid amide inhibitor BIA 10-2474.²⁶ Competitive ABPP studies are performed with two different activity-based probes: the broad-spectrum serine hydrolase-directed probe fluorophosphonate-biotin (FP-biotin, **Fig. 1a**) and the tailored probe MB108 (THL-biotin, **Fig. 1c**).^{27,28} The latter probe preferentially reacts with endocannabinoid hydrolases diacylglycerol lipase α (DAGL α), ABHD6, and ABHD12, as well as with several other enzymes. Together, both probes enabled target engagement assays for FAAH (fatty acid amide hydrolase) and a broad array (>50) of other brain serine hydrolases.²⁵⁻³¹ This competitive ABPP assay confirmed that BIA 10-2474 interacted with FAAH and FAAH2 in human cells.²⁶ Furthermore, it was discovered that BIA 10-2474 is

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not a selective experimental drug, because it inhibits several other lipases, including ABHD6, PNPLA6 and Ces2, which are not targeted by a clinically safe FAAH inhibitor.²⁶

Using an adapted version of the protocol, LEI105 (an α -ketoheterocycle)²⁸ was previously discovered and optimized as a selective and reversible inhibitor for DAGL, an enzyme which catalyzes the conversion of diacylglycerol to the endocannabinoid 2-arachidonoylglycerol (2-AG).²⁹ Comparative ABPP was used to map the activity of different endocannabinoid hydrolases in various brain regions, both in cannabinoid type 1 receptor knockout and wildtype brain tissue.³⁰ In addition, competitive ABPP was crucial to identify the first brain active DAGL inhibitor DH376^{31,32} (**Fig. 1c**). Acute pharmacological blockade of DAGL by DH376 resulted in a rapid and dramatic reorganization of the lipid signaling pathways in the brain under normal and neuroinflammatory conditions.³⁰ Target engagement and selectivity profiling by competitive ABPP using mouse brain proteomes confirmed that DH376 was a selective DAGL inhibitor that only cross-reacted with ABHD6, CES1c and Lipe.³¹ ABPP guided the optimal dose selection for further animal studies, showing that DAGLs are involved in modulation of pro-inflammatory prostaglandins and cytokines, lipopolysaccharide-induced anapyrexia and fasting-induced food intake.^{31,32}

Limitations and comparison to alternative approaches. For this approach, ABPs are required. For the serine hydrolase (e.g., FP-biotin) and kinase enzyme families, commercial ABPs are available (**Table 1**). THL-biotin and other probes synthesized by our laboratory are available upon request. However, it remains a current limitation of ABPP that organic synthetic expertise is required to synthesize/develop new ABPs.

Competitive ABPP only allows the determination of inhibitor selectivity for the proteins targeted by the ABP. In this protocol, a cocktail of two probes is used to be able to profile more enzymes in parallel. It should, however, be kept in mind that the inhibitor may interact with targets that belong to other protein families. Furthermore, false positive and negative hits are possible. It is, therefore, recommended to confirm off-target activity by orthogonal techniques *in vitro* using recombinant proteins.

There are several alternative methods to study the target interaction profile of covalent inhibitors in living organisms.^{2,4} One possibility is to turn the inhibitor of interest into an ABP by attaching a reporter group.³ The advantage of this approach is that all possible targets may be profiled. It is however important to realize that modification of the inhibitor could influence its activity and the activity of the modified inhibitor should be confirmed in an *in vitro* assay. Several complementary approaches have been developed that rely on the observation that inhibitor binding stabilizes the target protein. The cellular thermal shift assay (CETSA) relies on thermal stabilization of the target proteins by inhibitor binding.⁵ Drug affinity responsive target stability (DARTS) is an approach which relies on the assumption that inhibitor targets

are more resistant against proteolysis.³³ Since there is no enrichment step of target proteins in these approaches, detection of low abundance proteins is challenging.

A gel-based approach with fluorescence visualization can also be used for competitive ABPP. This approach has been described in detail in the previous protocol.¹³ The main advantage of gel-based ABPP compared to LC-MS methods is the higher throughput of samples. However, LC-MS based ABPP is unrivalled in its depth of analysis, resolution and sensitivity.

Ion mobility separation is a powerful method to increase the analytical depth of the proteomic analysis. Unfortunately, open source software is not yet available to process raw ion mobility data. In this protocol, the vendor software Progenesis is used for data processing. Open source software is published for the label-free data analysis, but this workflow still depends on the vendor software PLGS for raw data processing.³⁴ For label-free quantification, a very stable liquid chromatography system is required. For the acquisition and processing of high-resolution mass spectrometry data a certain level of expertise is needed.

Since chemical proteomics is a multidisciplinary field in which chemistry, biology and mass spectrometry expertise is needed, this protocol can hopefully serve as a guideline to avoid certain pitfalls.

Experimental design. In the experiment described in this protocol, DH376 is administered to mice and different tissues are collected and lysed. The lysate is separated into membrane and cytosol fractions by centrifugation. The fractionation helps to identify low abundance membrane proteins. Depending on the abundance and distribution of proteins of interest, fractionation can be omitted or elaborated. For example, an elusive calcium-dependent N-acyltransferase was recently reported to be a target of FP-biotin, but only identified by using sucrose gradient fractionation of mouse brain membrane.³⁵ Comparison of samples to controls is necessary to distinguish specific binders from contaminants and background. In this protocol, heat-inactivated vehicle-treated controls are used to determine if a protein is identified in an activity-based manner. To determine if an enzyme target is enriched compared to the heat-inactivated control, the cut-off values of ANOVA ($p < 0.05$ and ratio > 2) are applied. Furthermore, a cocktail of FP-biotin and THL-biotin is used to simultaneously detect multiple serine hydrolases of the endocannabinoid system. This principle of a probe cocktail can also be applied to different ABPs to study enzymatic activities of interest in parallel, thereby minimizing the number of samples. The most cumbersome part of this protocol (which can lead to the highest sample variation) is the methanol-chloroform precipitation (Steps **14-21**).³⁶ This step is necessary to remove excess probe prior to avidin enrichment. This step should be practiced (on any 1.0 mg/mL protein solution), before performing this on valuable samples. The first day of this protocol (up until overnight trypsinolysis in Step **37**) is time-demanding, however there is one optional pause point (step **23**).

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This protocol describes the use of LC-IMS-MS with a Synapt G2-Si instrument. The data processing is performed with the commercial software Progenesis, and Top3 quantification is used.³⁷ Top3 quantification only gives reliable quantification data when low-scoring identified peptides are filtered out. The Benjamini-Hochberg procedure to correct for multiple comparisons, using an FDR of 10% is applied.³⁸

Materials

Reagents

- Acetonitrile, ULC/MS grade (Biosolve, cat. no. 012041) **! CAUTION** Acetonitrile is flammable and harmful if inhaled, swallowed or in contact with skin or eyes. Handle in a fume hood and wear labcoat and safety glasses.
- Activity-based probe of interest: In this example FP-Biotin (Santa Cruz Biotechnology, cat. no. sc-215056A) and THL-Biotin (MB108; synthesized as described²⁷ and available upon request). For an overview of commercially available ABPs, see **Table 1**.
- Ammonium bicarbonate (NH_4HCO_3 ; Fluka, cat. no. 09830).
- Avidin-Agarose from egg white (Sigma, cat. no. A9207).
- Benzonase Nuclease (Santa Cruz Biotechnology, cat. no. sc-202391).
- Bovine Serum Albumin (BSA; Sigma, cat. no. A9647).
- Bradford reagent (Bio-Rad, cat. no. 500-0006).
- Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$; Merck Millipore, cat. no. 102382).
- Chloroform (Sigma, cat. no. 32211-M) **! CAUTION** Chloroform is toxic if inhaled and a suspected carcinogen, handle in a fume hood and wear a labcoat and safety glasses.
- DMSO (Sigma, cat. no. 34943-M).
- Dithiothreitol (DTT; BioChemica, cat. no A1101) **! CAUTION** DTT is an eye and skin irritant.
- Empore™ C18 47 mm Extraction Disc (3M Purification Inc., Model 2215).
- Formic acid, LC-MS grade (Actu-all Chemicals, art. nr. 8060128A1) **! CAUTION** Can cause severe burns, handle in a fume hood, wear a labcoat and safety glasses
- [Glu¹]-fibrinopeptide B (GluFib; Waters, product no. 700004729).
- Glycerol 85% (Merck Millipore, cat. no. 104092).
- Hydrochloric acid (HCl; Sigma, cat. no. 30721-M) **! CAUTION** Can cause severe burns, handle in a fume hood, wear a labcoat and safety glasses.
- HEPES, free acid (Millipore, cat. no. 391340).
- Inhibitor of interest: In this example the diacylglycerol lipase inhibitor DH376 (synthesized as

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described³¹ and available upon request).

- Iodoacetamide (IAA; Sigma, cat. no. I6125) **! CAUTION** IAA is toxic if swallowed and may cause an allergic reaction.
- Leucine Enkephalin (LeuEnk; Waters, product no. 186006013).
- Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$; Acros Organics, cat. no. 413415000).
- Methanol, reagent grade (Sigma, cat. no. 32213-M) or ULC/MS grade (Biosolve, cat. no. 136841) **! CAUTION** Methanol is flammable and toxic. Handle in a fume hood and wear safety glasses.
- Mice: In the example described in this Protocol, four tissues (brain, kidney, liver and testis) from mice (C57BL/6 mice, Charles River Mice) treated with vehicle or DH376 were used (see Reagent Setup). **! CAUTION** Any experiments involving live mice must conform to relevant Institutional and National regulations. The animal experiments described in this Protocol were conducted in accordance with the ethical committee of Leiden University (DEC#14137).
- Sodium dodecyl sulfate (SDS; MP Biomedicals cat. no. 811032) **! CAUTION** SDS is toxic.
- Sodium chloride (NaCl ; Chem-Lab, art. no. CL00.1423).
- Sodium hydroxide (NaOH ; Acros Organics, cat. no. 134070010) **! CAUTION** NaOH can cause severe burns, wear a labcoat and safety glasses.
- Tris(hydroxymethyl)aminomethane (Tris; Acros Organics cat. no. 16762).
- Trypsin, sequencing grade (Promega, cat. no. V5111).
- Urea (Sigma, cat. no. 33247).
- Water, ULC/MS grade (Biosolve, cat. no. 232141) and Milli-Q water (see Equipment). **▲ CRITICAL** Avoid using autoclaved water, because it may contain high chemical background.
- Yeast enolase (Waters, product no. 186002325. SwissProt P00924).

Equipment

- Analytical column (HSS-T3 C18 1.8 μM , 75 μM x 250 mm, Waters).
- Bio-Spin columns (Bio-Rad, cat. no. 7326204).
- Centrifuge for 15 mL tubes, 2500 g required. (Heraeus Megafuge 1.0R).
- Centrifuge for 1.5-2 mL tubes, 18,400 g required (Eppendorf, 5415D).
- Dounce homogenizer (Wheaton, supplier no. 357422).
- Eppendorf ThermoMixer C.
- Example datasets: The mass spectrometry proteomics data used in this Protocol have been deposited to the ProteomeXchange Consortium via the PRIDE³⁹ partner repository with the dataset identifier PXD007965.

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- Insulin syringe (Terumo, Myjector U-100).
- Microplate, 96-well clear flat bottom (greiner bio-one, cat. no. 655191).
- Milli-Q advantage A10 water purification system (Merck Millipore).
- NanoACQUITY UPLC System (Waters).
- Overhead shaker (Heidolph, Reax 2).
- Pipette, 10 mL (Sarstedt, order no. 86.1254.001).
- Pipette tip, blue (Sarstedt, order no. 70.762.100).
- Pipette tip, yellow (Sarstedt, order no. 70.760.502).
- Pipette tip, grey (Sarstedt, order no. 70.1130.600).
- Probe sonicator (Branson, Digital Sonifier).
- Software: Progenesis (v3.0: <http://www.nonlinear.com/progenesis/qi-for-proteomics/>), Excel (Microsoft 2010), KNIME (3.2.1: www.knime.com).
- SpeedVac (Eppendorf, concentrator 5301).
- Suction pump (Meyvis B.V.).
- SYNAPT G2-Si high definition mass spectrometer (Waters).
- Tecan GENios Microplate Reader.
- Trap column (C18 100 Å, 5 µM, 180 µM x 20 mm, Waters, P/N 186006527).
- Tube, 15 mL (Sarstedt, order no. 62.554.502).
- Tube, 2 mL (Sarstedt, order no. 72.691).
- Tube, clear 1.5 mL (Sarstedt, order no. 72.690.550).
- Tube, protein low binding (Sarstedt, order no. 72.706.600).
- Ultracentrifuge (Beckman Coulter, optima L-90K).
- Vials, LC-MS (Waters, part no. 600000671CV).
- Vortex mixer.

Reagent setup

All reagents are made with 18.2 MΩ MilliQ water unless indicated otherwise.

▲ CRITICAL

Ammonium bicarbonate buffer Dissolve 198 mg NH_4HCO_3 in water to a final volume of 10 mL for a 250 mM solution. ▲ CRITICAL Ammonium bicarbonate is thermally unstable. Always prepare this buffer directly before use.

Benzonase stock Prepare a 10 U/µL solution in storage buffer (50% (vol/vol) glycerol, 20 mM Tris pH 8.0, 2 mM MgCl_2 , 20 mM NaCl). Aliquots of this solution can be stored at -20 °C for at least 6 months.

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CaCl₂ stock Dissolve 147 mg calcium chloride dehydrate in 1 mL water to prepare a 1 M CaCl₂ solution. This solution can be stored at room temperature (18-24 °C) for up to one month.

DTT stock Dissolve 1.54 g DTT in water to a final volume of 10 mL for a 1 M solution. Aliquots can be stored at -20 °C for up to 3 months. Discard after thawing.

FP-Biotin stock Dissolve FP-Biotin in DMSO to a final concentration of 1 mM. Aliquots of this solution can be stored at -20 °C for at least one year.

GFP stock Dissolve 0.1 mg of GluFib in 1 mL water (64 pmol/μL final concentration). Aliquots of this solution can be stored at -20 °C for at least one year.

HCl stock Prepare a 1 mM HCl solution by diluting 37% (wt/vol) HCl (~12 M) into water. Check if pH is 3. This solution can be stored at room temperature for up to one month.

HEPES stock Prepare 1 M HEPES in water and adjust to pH 7.2 with NaOH. This solution can be stored at room temperature for up to one month.

HEPES/DTT buffer For 30 mL buffer, combine 29.4 mL water, 0.6 mL HEPES stock and 60 μL DTT stock (final concentrations 20 mM HEPES, 2 mM DTT). Always prepare this buffer directly before use.

IAA stock Dissolve 92 mg iodoacetamide in 1 mL water for a final concentration of 0.5 M.

▲ CRITICAL IAA is light sensitive. Always prepare this solution directly before use.

LC-MS sample solution For 2 mL, combine 1900 μL ULC/MS grade water, 60 μL acetonitrile, 2 μL formic acid and 40 μL yeast enolase stock (final concentrations 3% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid and 20 fmol/μL enolase). Prepare this solution directly before use.

LeuEnk stock Dissolve 3 mg of LeuEnk in 3 mL water. Aliquots of this solution can be stored at -20 °C for up to one year.

Lockmass solution Prepare 30 mL of a 1:1 (vol:vol) solution of acetonitrile and ULC/MS grade water containing 0.1% (vol/vol) formic acid, add 47 μL GFP stock solution and 6 μL LeuEnk stock solution (final concentrations 200 pg/μL LeuEnk and 100 fmol/μL GFP). This solution can be stored at room temperature for up to one month.

Lysis buffer For lysing 12 tissues, prepare 30 mL lysis buffer by combining 29.2 mL water, 0.6 mL HEPES stock, 60 μL DTT stock, 30 μL MgCl₂ stock and 75 μL benzonase (final concentrations 20 mM HEPES, 2 mM DTT, 1 mM MgCl₂, 25 U/mL benzonase). Always prepare this buffer fresh before use and keep on ice.

▲ CRITICAL Do not add protease inhibitor to the lysis buffer as this might inhibit several of the probe targets.

MgCl₂ stock Dissolve 2.0 g of MgCl₂·6 H₂O in water to a final volume of 10 mL for a 1 M solution. This solution can be stored at room temperature for up to one month.

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Mobile phase A/weak wash Prepare a 0.1% (vol/vol) formic acid solution in ULC/MS grade water. This solution can be stored at room temperature for up to one month.

Mobile phase B/strong wash Prepare a 0.1% (vol/vol) formic acid solution in ULC/MS grade acetonitrile. This solution can be stored at room temperature for up to one month.

Mouse tissues Mice were injected with 30 mg/kg DH376 (or vehicle) i.p. in 18:1:1 (vol:vol:vol) solution of saline:ethanol:PEG40 ethoxylated castor oil (10 μ L/g body weight of mouse). After 2 h, mice were euthanized and tissues were collected. Tissues can be stored at -80 °C for at least two years.

▲ CRITICAL The vehicle and inhibitor treated tissues to be compared should be prepared under the same conditions to prevent changes in enzyme activity (arising from different amounts of freeze-thaw cycles for example).

NaCl stock Dissolve 0.58 g of NaCl in water to a final volume of 10 mL for a 1 M solution. This solution can be stored at room temperature for up to one month.

On Bead-Digestion buffer (OB-Dig) For 24 samples, combine 4668 μ L water, 600 μ L Tris stock, 600 μ L NaCl stock, 6 μ L CaCl_2 stock and 120 μ L acetonitrile (final concentrations 100 mM Tris, 100 mM NaCl, 1 mM CaCl_2 and 2% (vol/vol) acetonitrile). This buffer should be prepared fresh.

PBS 10x stock Dissolve 68.05 g KH_2PO_4 in 500 mL water (heat to 40 °C dissolve), dissolve 261.23 g K_2HPO_4 in 1500 mL water, mix and add 877 g NaCl, add water to a final volume of 10 L, filter over 0.22 μ M filter (final concentrations 150 mM KH_2PO_4 , 50 mM KH_2PO_4 and 1.5 M NaCl). This solution can be stored at room temperature for up to three months.

PBS Dilute PBS 10x stock ten times in Milli-Q water (pH should be 7.5). This solution can be stored at room temperature for up to one month.

PBS/SDS Add 50 mL SDS stock to 950 mL PBS (final concentration 0.5% (wt/vol) SDS). This solution can be stored at room temperature for up to one year.

Probe cocktail Mix equal volumetric amounts of FP-Biotin stock and THL-Biotin stock. Aliquots of this solution can be stored at -20 °C for at least one year.

SDS stock Prepare a 10% (wt/vol) SDS solution in water. This solution can be stored at room temperature for up to one year.

Seal wash Prepare a 10% (vol/vol) acetonitrile solution in water (both ULC/MS grade).

StageTip solution A Prepare an 0.5% (vol/vol) formic acid solution in water. This solution can be stored at room temperature for up to one month.

StageTip solution B Prepare an 80% (vol/vol) acetonitrile, 0.5% (vol/vol) formic acid solution in water. This solution can be stored at room temperature for up to one month.

StageTips See equipment setup.

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THL-Biotin stock Dissolve THL-Biotin in DMSO to a final concentration of 1 mM. Aliquots of this solution can be stored at -20 °C for at least one year.

Tris stock Prepare 1 M Tris in water and adjust to pH 8 with HCl. This solution can be stored at room temperature for up to one month.

Trypsin solution Dissolve 20 µg of trypsin in 40 µL 1 mM HCl stock (final concentration 0.5 µg/µL). Store this solution at -20 °C for up to one month and avoid freeze-thaw cycles.

Urea buffer Add 1 mL ammonium bicarbonate buffer (250 mM) to 3.6 g urea and adjust with water to a final volume of 10 mL (final concentrations 6 M urea and 25 mM ammonium bicarbonate). This buffer should be prepared fresh.

Yeast enolase stock Dissolve 1 nmol yeast enolase in 1 mL 3% (vol/vol) acetonitrile in water. This solution can be stored at room temperature for at least one year.

Equipment setup

StageTips StageTips are used as a final step in sample preparation. Their preparation and use is described in Nature Protocols by Rappsilber *et al.*⁴⁰ Empore™ C18 47 mm Extraction Discs (see Reagents) are used to make the StageTips. Typically, two discs are stacked on top of each other to make StageTips with two layers of column material, inserted in a yellow pipette tip. It is recommended to make all the StageTips required for one experiment at once to achieve a more consistent back-pressure. The C18 material should be pressed into the pipette tips with as little pressure as possible.

NanoUPLC The LC-MS method is based on the approach described by Distler *et al.*²⁰ A summary of changes in approach is given here. DMSO is not added to the LC solvents. Therefore, a lower source temperature is used (80 °C instead of 100 °C). Because the affinity chromatography step (pulldown) makes the samples less complex, this gradient is shorter. A trap-elute protocol is used, where the digest is loaded on a trap column followed by elution and separation on the analytical column. The sample is brought onto this column at a flow rate of 10 µL/min with 99.5% solvent A for 2 min before switching to the analytical column. Peptide separation is achieved using a multistep concave gradient based on the gradients used in Distler *et al.*²⁰ The column is re-equilibrated to initial conditions after washing with 90% solvent B. The detailed protocol is specified below:

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Time (min)	Gradient composition (%B)	Flow rate (nL/min)
0.0	1.0	400
2.4	1.0	400
4.2	5.0	300
10.2	7.6	300
15.6	10.3	300
21.0	13.1	300
25.8	16.1	300
30.6	19.2	300
35.4	22.4	300
40.2	25.7	300
45.0	29.1	300
49.8	32.6	300
54.0	36.2	300
58.2	40.0	300
58.8	90.0	400
60.3	90.0	600
61.2	90.0	600
61.5	1.0	400
70.8	1.0	400

The rear seals of the pump are flushed every 30 min with 10% (vol/vol) ACN. [Glu¹]-fibrinopeptide B (GluFib) is used as a lock mass compound. The auxiliary pump of the LC system is used to deliver this peptide to the reference sprayer (0.2 μ L/min).

MS acquisition method A UDMS^c method is set up as described in Distler *et al.*²⁰ Briefly, the mass range is set from 50 to 2,000 Da with a scan time of 0.6 seconds in positive, resolution mode. The collision energy is set to 4 V in the trap cell for low-energy MS mode. For the elevated energy scan, the transfer cell collision energy is ramped using drift-time specific collision energies.³⁴ The lock mass is sampled every 30 seconds.

Procedure

Tissue lysis ● **TIMING** ~4 h for 12 tissues

▲ **CRITICAL** Steps 1-9 are performed on ice to prevent protease activity. Make sure to cool centrifuges (4 °C) and dounce homogenizer (on ice).

- 1| Thaw the tissue on ice (~0.5 h) and cool the lysis buffer on ice.
▲ **CRITICAL STEP** The vehicle and inhibitor treated tissues to be compared should be prepared under the same conditions to prevent changes in enzyme activity (arising from different amounts of freeze-thaw cycles for example).
- 2| Manually lyse the tissue in 2 mL pre-cooled lysis buffer using a dounce homogenizer. The amount of strokes required depends on both the type of tissue and the size. Typically, for brain (soft tissue) and heart (tough tissue) 5 and 25 strokes are used for complete homogenization. Transfer the lysed tissue to a 2 mL tube.
- 3| Incubate the homogenized tissue on ice for 15 min.
- 4| Pellet cell debris by centrifugation (3 min, 2500g, 4 °C) and transfer the supernatant to an ultracentrifuge tube. Balance pairs of samples using an analytical balance and if necessary, adjust the weight by adding lysis buffer.
- 5| Separate the lysate into membrane and cytosol fractions by ultracentrifugation (45 min, 100,000g, 4 °C).
▲ **CRITICAL STEP** Depending on the abundance and distribution of proteins of interest, this fractionation step can be omitted or elaborated (Experimental Design).
! **CAUTION** The tubes should be undamaged, properly balanced and sealed. The rotor should be undamaged, clean and dry.
- 6| Collect the supernatant into a tube as cytosolic fraction.
- 7| Resuspend the pellet (membrane fraction) in 1-2 mL HEPES/DTT buffer (amount depends on the size of the pellet, typically 1 mL for kidney and testis, 1.5 mL for brain and 2 mL for liver gives sufficient protein concentration) by pipetting up and down. After transferring to a 2 mL tube, use an insulin syringe to suck up the membrane fraction and push it through the needle

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once for homogenization.

- 8| According to the manufacturer's protocol (Bulletin #4110065, Bio-Rad), perform the Bradford assay to determine the protein concentration. Usually, approximately the following protein amounts are obtained:

Tissue	Protein yield cytosol (mg)	Protein yield membrane (mg)
Brain	~6	~5
Kidney	~8	~5
Liver	~30	~20
Testis	~4	~3

- 9| Dilute the samples to 1.0 mg/mL using HEPES/DTT buffer, aliquot into 0.245 mL fractions, snap freeze in liquid nitrogen and store at -80 °C.

▲ **CRITICAL STEP** To retain enzyme activity and prevent protein degradation it is important that the lysates are snap frozen and freeze-thaw cycles are avoided.

■ **PAUSE POINT** The lysates can be stored at -80 °C for at least six months. For some enzymes, freshly prepared lysate may give better probe labeling.

Probe incubation ● **TIMING** ~2 h

- 10| Thaw the lysates on ice (~1 h) and transfer 245 µL of the protein sample (1.0 mg/mL) into each clear 1.5 mL tube. Prepare one tube for each inhibitor-treated sample, two tubes for each vehicle sample.
- 11| In order to prepare heat-inactivated control samples, incubate one of the vehicle-treated samples for 5 min at 100 °C. It is advised to add 25 µL 10% (wt/vol) SDS (final concentration is 1% (wt/vol) SDS) to prevent protein precipitation in this step.
- 12| Add 5 µL of probe cocktail to each sample and vortex briefly. The same protocol can be used when the probes are tested separately, the only modification being the use of 50% less avidin beads in **Step 28**.
- 13| Incubate the samples for 30 min at 37 °C while shaking (300 rpm), followed by a short spin down.

Methanol/chloroform precipitation ● **TIMING** ~1.5 h for 24 samples

! CAUTION Perform steps **1-21** in a fume hood and discard the supernatants obtained in steps **18** and **21** into halogenated organic waste.

14| Add 250 μL water to each sample for a final volume of 500 μL .

▲ **CRITICAL STEP** The ratios between water, MeOH and CHCl_3 are important. It is therefore required to adjust the volume to 500 μL also when using a different volume of protein sample.

15| Add 666 μL MeOH and briefly vortex.

16| Add 166 μL CHCl_3 and briefly vortex.

17| Add 150 μL water and briefly vortex, this should result in a cloudy suspension (**Fig. 2-1**).

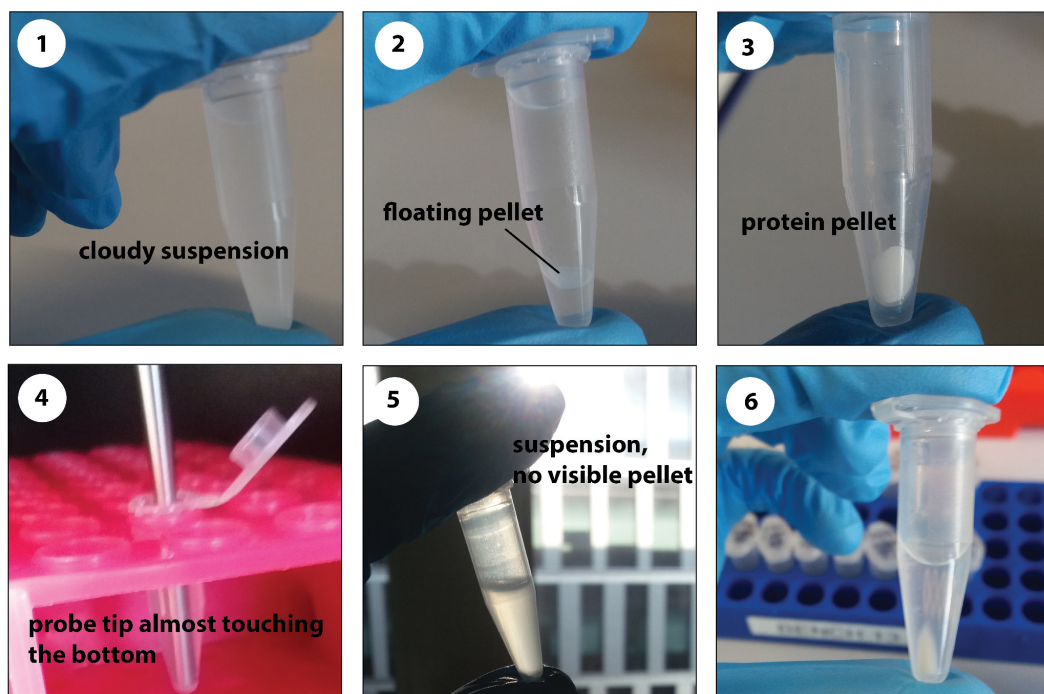


Figure 2 | Cartoon of a successfully performed methanol/chloroform precipitation (Steps **14-21**).

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- 18| Pellet the precipitated protein by centrifugation (10 min, 1500g at room temperature), this should result in a floating pellet (**Fig. 2-2**). Remove the upper and lower layer without disturbing the floating pellet (this works best by holding the tube at a 45° angle to stick the protein pellet against the side, **Fig. 2-3**).

▲ **CRITICAL STEP** Handle the samples carefully to prevent disrupting the protein pellet.

? TROUBLESHOOTING

- 19| Add 600 µL MeOH to the pellet.
- 20| Resuspend the pellet by sonication with a probe sonicator (10 sec, 30% amplitude), this should result in a suspension without any visible protein pellet (**Fig. 2-5**)

▲ **CRITICAL STEP** The tip of the probe sonicator should be positioned just above the bottom of the tube (**Fig. 2-4**).

- 21| Pellet the protein by centrifugation (5 min, 18,400g at room temperature) (**Fig. 2-6**) and remove the supernatant.

▲ **CRITICAL STEP** Close the tubes after removing the supernatant to prevent the pellet from drying out, as this makes redissolving difficult.

Reduction, alkylation and avidin enrichment ● **TIMING** ~5 h

▲ **CRITICAL** The incubation times of steps 24-27 can be used to perform steps 28-31.

- 22| Add 250 µL urea buffer to each sample.
- 23| Resuspend the pellet by thoroughly pipetting up and down with a yellow pipette tip (~10 times, pipettor set to ~200 uL)

? TROUBLESHOOTING

■ **PAUSE POINT** The solubilized protein samples can be stored at -80 °C for at least one month.

- 24| Add 2.5 µL DTT stock, vortex briefly, spin down briefly and incubate for 15 min at 65 °C while shaking (600 rpm).
- 25| Let samples cool down to room temperature (at least 5 min).
- 26| Add 20 µL IAA stock, vortex briefly and incubate for 30 min at room temperature in the dark

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(for example, in a drawer or wrapped in aluminum foil).

- 27| Add 70 μ L SDS stock, vortex briefly and incubate for 5 min at 65 °C.
 - 28| For 24 samples, remove 2.4 mL avidin beads from a 50% slurry (100 μ L slurry per sample) divide over four 15 mL tubes (600 μ L per tube). Be sure to properly homogenize the slurry before pipetting. When testing the individual probes (Step 12), 50 μ L slurry can be used per sample.
 - 29| Wash the beads three times with 10 mL PBS. Pellet the beads by centrifugation (2 min, 2500 *g*) and remove the supernatant with a suction pump.
- ▲ **CRITICAL STEP** Be careful not to suck up the beads.
- 30| Resuspend the beads in 6 mL PBS per tube.
 - 31| For 24 samples, prepare 24 tubes (15 mL) with 2 mL PBS and 1 mL beads from step 30. Add each individual sample from step 27 to one of these tubes.
 - 32| Incubate the samples while rotating at low speed using an overhead shaker for at least 3 h at room temperature.

Wash beads ● **TIMING** ~1.5 h for 24 samples

▲ **CRITICAL** This step can be performed twice as fast with two people – one adding buffer and centrifuging and the other removing the supernatant.

- 33| Pellet the beads by centrifugation (2 min, 2500 *g* at room temperature) and remove the supernatant.

▲ **CRITICAL STEP** Be careful not to suck up the beads.

- 34| Wash the beads once with 6 mL PBS/SDS, followed by three times with 6 mL PBS. Pellet beads by centrifugation (2 min, 2500 *g* at room temperature) after each washing step and remove the supernatant.

▲ **CRITICAL STEP** Be careful not to suck up the beads.

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On-bead digestion ● TIMING ~0.5 h + overnight digestion

35| Add 250 μ L OB-Dig buffer to the beads and transfer to a 1.5 mL low binding tube.

▲ **CRITICAL STEP** Make sure to transfer all the beads, pipette up and down to homogenize and do not push the pipette tip to the bottom of the tube as this will result in leaving the beads in the tube.

36| Add 1 μ L (500 ng) trypsin solution per sample. The trypsin stock can also be diluted (24 μ L in 6 mL OB-Dig buffer) to be able to pipette larger volumes (250 μ L) – this might improve consistency.

37| Digest overnight at 37 °C with vigorous shaking (950 rpm).

Sample preparation ● TIMING ~3 h

38| Spin down briefly and add 12.5 μ L formic acid, briefly vortex and spin down.

39| Remove the beads by filtering the sample through a biospin column by centrifugation (2 min, 600 g at room temperature) and collect the flow-through in a 2 mL tube.

40| Condition the StageTips (see Equipment Setup), load the sample and wash the sample following the scheme below. The flow-through from conditioning, loading and washing can be discarded. Elution should be done in a low binding tube.

▲ **CRITICAL STEP** Centrifugation speed and duration are merely estimates. Solutions should have entirely run through without drying of the column.

Step	Aim	Solution	Centrifugation
1	Conditioning	Methanol (50 μ L)	2 min 300 g
2	Conditioning	Stage tip solution B (50 μ L)	2 min 300 g
3	Conditioning	Stage tip solution A (50 μ L)	2 min 300 g
4	Loading	Load samples on stage tips	2 min 600 g
5	Washing	Stage tip solution A (100 μ L)	2 min 600 g
6	Switch to low binding tube		
7	Elution	Stage tip solution B (100 μ L)	2 min 600 g

41| Evaporate the solvent in a SpeedVac.

■ **PAUSE POINT** Store the samples at -20 °C until required.

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- 42| Reconstitute sample in 50 μL of LC-MS sample solution.
- 43| Prepare a QC sample by pooling 2 μL from each sample.

LC-IMS-MS analysis ● TIMING ~1 h per sample per replicate

- 44| Inject 1 μL of a sample onto the UPLC-IMS-MS system (see Equipment Setup) and perform at least duplicate LC-MS analysis of each sample.

▲ **CRITICAL STEP** To prevent drift in instrument performance influencing the results, make sure to randomize the measurement of biological replicates and perform technical replicates of QC samples to check the variation in LC-MS performance. It is recommend to run a QC sample after every 24 MS runs.²⁰

? TROUBLESHOOTING

Data processing and analysis ● TIMING ~5 h per 16 runs

- 45| Open Progenesis QI for proteomics. Create new label-free experiment. Choose Data type “Profile data” and Machine type “High resolution mass spectrometer”. Choose an experiment folder (use the same name as the experiment name).
- 46| Import data: select the .raw folder of each LC-MS run that must be compared. If samples are fractionated (step 5), analyze the fractions separately using the processing parameters specified below. Perform lock mass calibration with lock mass m/z 785.8426. Perform MS^E identification workflow (the default energy thresholds and elution start 10 min and elution end 65 min were used in this example. The optimal settings depend on the instrument and the LC gradient). Non-default settings are indicated with an asterisk:
- 47| Perform automatic processing while the raw data is importing. Assess all runs in the experiment for suitability as alignment reference. Automatically align the runs and perform peak picking with the default parameters. Set the parameters to identify peptides. Use the DataBank Editor to select the FASTA file (**Box 1**) and add with parsing rules: UNIPROT. Select trypsin as digest reagent, 2 missed cleavages, max protein mass 250 kDa, modifications carbamidomethyl C (fixed) and oxidation M (variable). Search tolerance parameters: set FDR to less than 1% and ion matching requirements: at least 2 fragments/peptide, 5 fragments/protein and 1 peptide/protein. Protein quantitation: select relative quantitation using Hi-N with N = 3 and use protein grouping. Depending on the sample complexity and number of runs being compared, processing may take up to one hour per sample.

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Parameter	Value
Lock mass m/z	785.8426
Low energy threshold	150 counts
Elevated energy threshold	30 counts
Elution start	Run start
Elution end	Run end
Alignment reference	Assess all runs in the experiment for suitability
Automatic alignment	Yes
Peak picking	Yes
FASTA file	See Box 1 *
Digest reagent	trypsin
Missed cleavages	Max 2*
Modifications	Fixed carbamidomethyl C, variable oxidation M
FDR less than	1%*
Fragments/peptide	2*
Fragments/protein	5*
Peptides/protein	1
Quantitation method	Relative quantitation using Hi-N
Number of peptides to measure per protein (N)	3
Protein grouping	Yes

Box 1 | Database generation

For an excellent tutorial on the bioinformatics behind protein identification, see Vaudel *et al.*⁴¹ Here are the steps to generate the database used in this study to search for protein identifications in Progenesis:

1. Go to www.uniprot.org.⁴² Under “Proteomes” search for “Mus musculus” and select the mouse proteome (ID UP000000589).
2. View all proteins, select the reviewed proteins and download as an uncompressed FASTA (canonical) file. **▲ CRITICAL STEP** For this study only the reviewed part of the mouse proteome was selected, because the unreviewed (TrEMBL) database contains many duplicate proteins. This makes identification of unique peptides more difficult. If your organism of interest is not as extensively reviewed as the mouse proteome however, or if you are searching for novel, unknown proteins, it might be better to use the unreviewed proteome.
3. Add expected contaminants to the database: trypsin, yeast enolase (peptide standard added to all samples) and avidin (from the on-bead digestion). Search the Uniprot database (accessions P00761, P00924 and P02701), go to the “sequence” tab and click the “FASTA” button. Paste this sequence into the mouse proteome fasta file.

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- 48| Review alignment: check if the automatic alignment algorithm has allocated vectors and make sure the part of the chromatogram where peptides elute has good alignment quality.

? TROUBLESHOOTING

- 49| Filtering: select ions with charges 2, 3, 4, 5, 6 and 7+ and delete non-matching peptide ions (roughly 20-25%).
- 50| Review normalization: check if certain samples deviate from the normalization reference.
- 51| Experiment design setup: choose between-subject design. Create separate designs for vehicle vs heat and vehicle vs inhibitor.
- 52| QC metrics: use the QC metrics to quickly identify possible errors in sample preparation or acquisition due to the visualization of complex data across the separate runs.
- 53| Refine identifications: use PLGS score less than 6.0 as batch deletion criterion and delete matching search results (only peptides with a score of 6.0 or more should be used for protein quantitation).

▲ CRITICAL STEP This step is crucial to obtain reliable quantitative data. The search algorithm tries to identify as many peptides as possible, and the low-scoring peptides are very unreliable, resulting in unreliable quantified proteins.

- 54| Review proteins: export protein measurements for each experimental design setup. The protein data is exported as .csv files and the analysis can be continued with Excel, for example (step 57). For proteins of interest, view peptide measurements. Each peptide has a unique identifier which can be used to find the spectrum and chromatogram in “Review peak picking”. The fragmentation spectrum can be found in “Identify peptides”.
- 55| Check if the data clusters according to the experimental conditions using PCA under Protein statistics.
- 56| Repeat steps 45-55 for each fraction. Open Progenesis and go to “Combine analysed fractions”. Select “Recombine analysed fractions”. Import data and recombine samples. Go to “Experiment design setup” and select the sample grouping. Go to “review proteins” and export protein

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measurements. Check if the data clusters according to the experimental conditions using PCA under Protein statistics.

57| Use Excel to open the “vehicle-vs-heat” .csv file created in step 56. Save as .xlsx file (for data management, extend the file name with “-analysis”, and keep the .csv file as “raw” data from Progenesis).

58| Insert a column and extract the gene name from description using (for example) the following Excel formula (K4 is the cell containing the description in this example):

=MID(K4,SEARCH("GN=",K4)+3,SEARCH("PE=",K4)-SEARCH("GN=",K4)-4)

Applying this formula to the string “Fatty-acid amide hydrolase 1 OS=Mus musculus GN=Faah PE=1 SV=1” should return “Faah”.

59| Delete proteins with 0 unique peptides or a peptide count < 2. Calculate the average normalized abundance for the vehicle and heat treated control samples. Use these values to calculate the ratio of vehicle/heat.

60| Select the proteins with Anova(p) < 0.05 and ratio > 2 (enriched in vehicle).

61| Use Excel to open the “vehicle-vs-inhibitor” .csv file created in step 56. Save as .xlsx file (for data management, extend the file name with “-analysis”, and keep the .csv file as “raw” data from Progenesis).

62| Extract the gene names as in step 58. Copy the gene names from the proteins selected in step 60 and paste into a second sheet called “heat-filtered”.

63| Use the option “Advanced filter” (under Data > Sort & Filter > Advanced). Select the first sheet as “List range” and the heat-filtered genes as “Criteria range” to only show the proteins that are enriched compared to the heat-inactivated control.

64| Filter this selection again using the putative targets list. A small database of putative probe targets from previous experiments using these probes was generated,^{28,30} a phylogenetic tree of α , β -

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hydrolase fold proteins combined with annotated catalytic nucleophiles in Uniprot.⁴²

- 65| Apply the Benjamini-Hochberg correction with a FDR of 10% ($q = 0.1$): (1) List all anova(p) values from lowest to highest. (2) Calculate the B-H statistic as $q \cdot \text{position in the list} / \text{number of tests}$. The choice of q is arbitrary (the lower the value the stricter the correction), but decide on this value before performing the analysis! (3) Select all proteins with a p value smaller than the B-H statistic (with $q = 0.1$, these are now corrected for a FDR of 10%).
- 66| For each protein, calculate the average normalized abundance of vehicle and inhibitor treated samples, percentage of inhibition ($\text{inhibitor} / \text{vehicle} \cdot 100\%$) and the error of ratio using the following formula (with x = average inhibitor, y = average vehicle and σ = standard deviation).
- $$\text{error of ratio} = \frac{x}{y} \cdot \sqrt{\left(\frac{\sigma_x}{x}\right)^2 + \left(\frac{\sigma_y}{y}\right)^2}$$
- 67| Repeat steps **57-66** for each tissue.
- 68| To compare the relative activity of each probe target across the different tissues, select the proteins from each tissue from step **65**. Calculate the average normalized abundance for brain, kidney, liver and testis (as described in step **66**). Calculate the relative intensity of each protein by dividing the average intensity of each tissue by the maximum intensity of that protein. Use hierarchical clustering (node in KNIME 3.2.1, agglomerative algorithm, Euclidian distance function, single linkage type) (**Fig. 2**).

● TIMING

Steps **1-9**, tissue lysis: ~4 h for 12 tissues.

Steps **10-37**; pulldown: ~11 h for 24 samples + overnight digestion.

Steps **38-43**, sample preparation: ~3 h

Step **44**, LC-IMS-MS analysis: ~1 h per sample per replicate

Steps **45-68**, data processing and analysis: depends highly on number of runs and sample complexity (~5 h per 16 runs)

Box 1, database generation: ~5 min.

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? TROUBLESHOOTING

Table 2 | Troubleshooting advice.

Step	Problem	Possible reason	Solution
18	The protein pellet breaks	The protein concentration was too low	Use more protein per sample (250 – 2000 µg)
23	The protein does not redissolve	The pellet has dried out The protein amount was too high	Add urea directly after removing methanol Use more urea buffer
44	Low signal Loss in sensitivity	Unsuccessful pulldown The column or instrument was contaminated	Optimize the protein amount, probe concentration and amount of beads Replace the trap and/or analytical column, clean the instrument
48	Poor alignment	The algorithm did not place vectors correctly Samples are too different	Choose a mix sample as alignment reference Minimize differences arising from sample preparation If the alignment quality is poor, you can consider manually placing vectors. However, this is very time-consuming and makes the acquired results irreproducible. It can help automatic alignment to select a QC sample (mix of samples being compared) as an alignment reference in step 47.

Anticipated results

Probe cocktail. In previous studies using FP- and THL-based biotinylated probes,^{28,30} a separate sample for each probe was prepared, duplicating the amount of samples. The Venn diagram in **Figure 3** summarizes the result of comparing the identified probe targets in samples of mouse brain membrane proteome treated with THL-biotin or FP-biotin separately, or mixed (probe cocktail; twice the amount of avidin beads used). All proteins identified in the samples treated with each probe separately are also identified in the probe cocktail sample. Furthermore, several putative probe targets are identified only in the probe cocktail sample. A possible explanation for this observation could be that these enzymes are shared probe targets for which both probes have a low potency. The additive effect of peptides being picked up by two probes might push these hits over the detection threshold. Depending on the experimental design and enzymes of interest, it can be worthwhile to combine biotinylated probes for one pulldown.

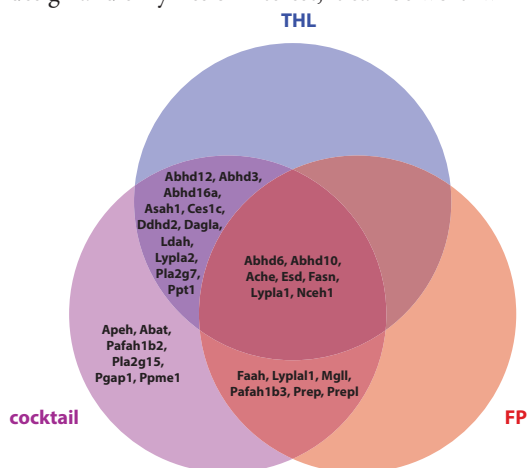


Figure 3 | Venn diagram of putative probe targets identified in mouse brain membrane proteome with THL-biotin (blue), FP-biotin (red) or a mix of both (cocktail; purple).

Competitive ABPP. Using this label-free quantitative proteomics protocol, it is confirmed that DH376 inhibits Dagla and Abhd6 *in vivo* and identify several novel off-targets (**Fig. 4**). Using heat-inactivated controls is helpful in separating probe targets from background binders. Combining this heat-filter with a putative probe target filter (Step 64), 81 proteins are identified that are picked up by the probe cocktail in an activity-based manner across four murine tissues. The results of the competitive ABPP experiment are summarized in **Figure 4**.

In the brain, the known targets of DH376 are found to be inhibited: Dagla and Abhd6. Ces1c, a carboxylesterase, is inhibited in all four tissues (**Fig. 5a**). Several other carboxylesterases with high sequence

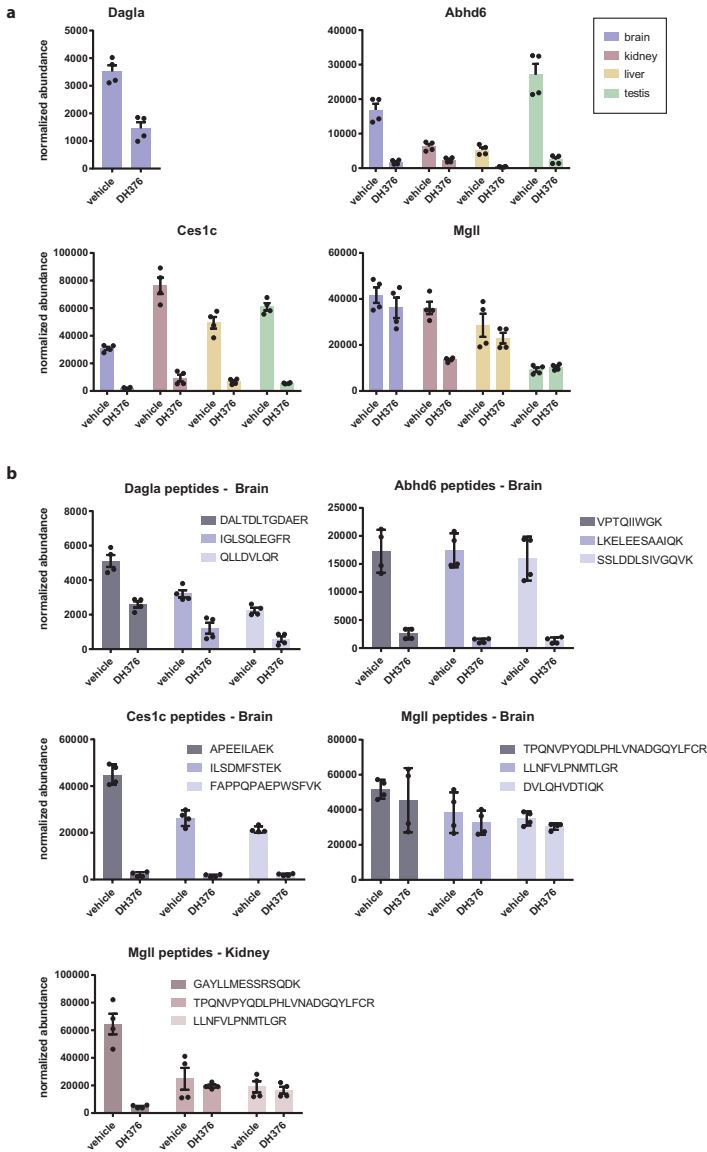
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Figure 4 | Results of the competitive ABPP experiment in mice with DH376 and the probe cocktail, with hierarchical clustering of probe targets. Highest normalized abundance is shown in brown. The relative abundance of each protein in each tissue is shown in blue. The inhibition by DH376 is shown in red (inhibition) and green (no inhibition). The animal experiments were conducted in accordance with the ethical committee of Leiden University (DEC no. 14137).

similarity, i.e. Ces1d, Ces1e, Ces1f, Ces2a and Ces2c, were also identified as novel targets of DH376 in the other tissues. Further off-targets are Aadam and Lipe (both involved in triglyceride hydrolysis). An apparent discrepancy in the activity profile of DH376 between different tissues was observed for monoglyceride lipase (Mgll). DH376 inhibited Mgll in the kidney, but not in the other three tissues (**Fig. 5a**). In **Figure 5b**, the peptides used for quantification of brain Dagla, Abhd6, Ces1c and Mgll show consistent inhibition profiles. However, only one Mgll peptide was significantly different between the vehicle and inhibitor treated kidney samples (**Fig. 5b**) Combined with results obtained via an orthogonal method of measuring Mgll inhibition³¹ this finding is denoted as a false positive. In a similar vein, Acot1 seems to be significantly inhibited in the brain, but not in the kidney and testis. Therefore, this might also be a false positive. These observations indicate that quality controls at the level of peptide quantification (in conjunction with orthogonal assays) will aid to establish the selectivity profile. Finally, the generation of the *in vivo* off-target profile of inhibitors using label-free quantitative activity-based proteomics will help to understand the *in vivo* mode of action of pharmacological tool compounds and guide the dose selection of drug candidates.

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