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

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Activity-based protein profiling (ABPP) has emerged as a valuable chemical proteomics method to guide the therapeutic development of covalent drugs by assessing their on-target engagement and off-target activity. We recently used ABPP to determine the serine hydrolase interaction landscape of the experimental drug BIA 10-2474, thereby providing a potential explanation for the adverse side effects observed with this compound. ABPP allows mapping of protein interaction landscapes of inhibitors in cells, tissues and animal models. Whereas our previous protocol described quantification of proteasome activity using stable-isotope labeling, this protocol describes the procedures for identifying the *in vivo* selectivity profile of covalent inhibitors with label-free quantitative proteomics. The optimization of our protocol for label-free quantification methods results in high proteome coverage and allows the comparison of multiple biological samples. We demonstrate our protocol by assessing the protein interaction landscape of the diacylglycerol lipase inhibitor DH376 in mouse brain, liver, kidney and testes. The stages of the protocol include tissue lysis, probe incubation, target enrichment, sample preparation, liquid chromatography–mass spectrometry (LC–MS) measurement, data processing and analysis. This approach can be used to study target engagement in a native proteome and to identify potential off targets for the inhibitor under investigation. The entire protocol takes at least 4 d, depending on the number of samples.

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 in selecting the best compound as a drug candidate (in terms of activity and selectivity) and may guide 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 (CETSAs) and positron emission tomography[1–4]. Recently, ABPP has emerged as a powerful chemical proteomics technology for mapping the interactions between small molecules and proteins on a global scale in living systems, including cells, animals and humans[5–7].

ABPP

ABPP is a technique pioneered by the Cravatt laboratory[5] that relies on active site-directed chemical probes that react, in a mechanism-based manner, 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. As this process requires a catalytically active protein, these chemical probes report on the abundance of active enzymes. ABPP enables the possibility of studying 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 most cases) a recognition element that 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[5].

A variety of ABPs have been described for different enzyme classes (**Table 1**)[8,9]. An ABP can be specific for one enzyme (tailored probe)[10] or can target a group of enzymes sharing recognition or reactivity (broad-spectrum probe)[11]. 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 is resolved and visualized by SDS-PAGE and in-gel fluorescence scanning. Pre-incubation of the proteome with an inhibitor will reduce the ABP labeling of proteins targeted by the inhibitor[12]. 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 LC–MS-based methods[13]. Compared with 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 must be validated with genetic knockout

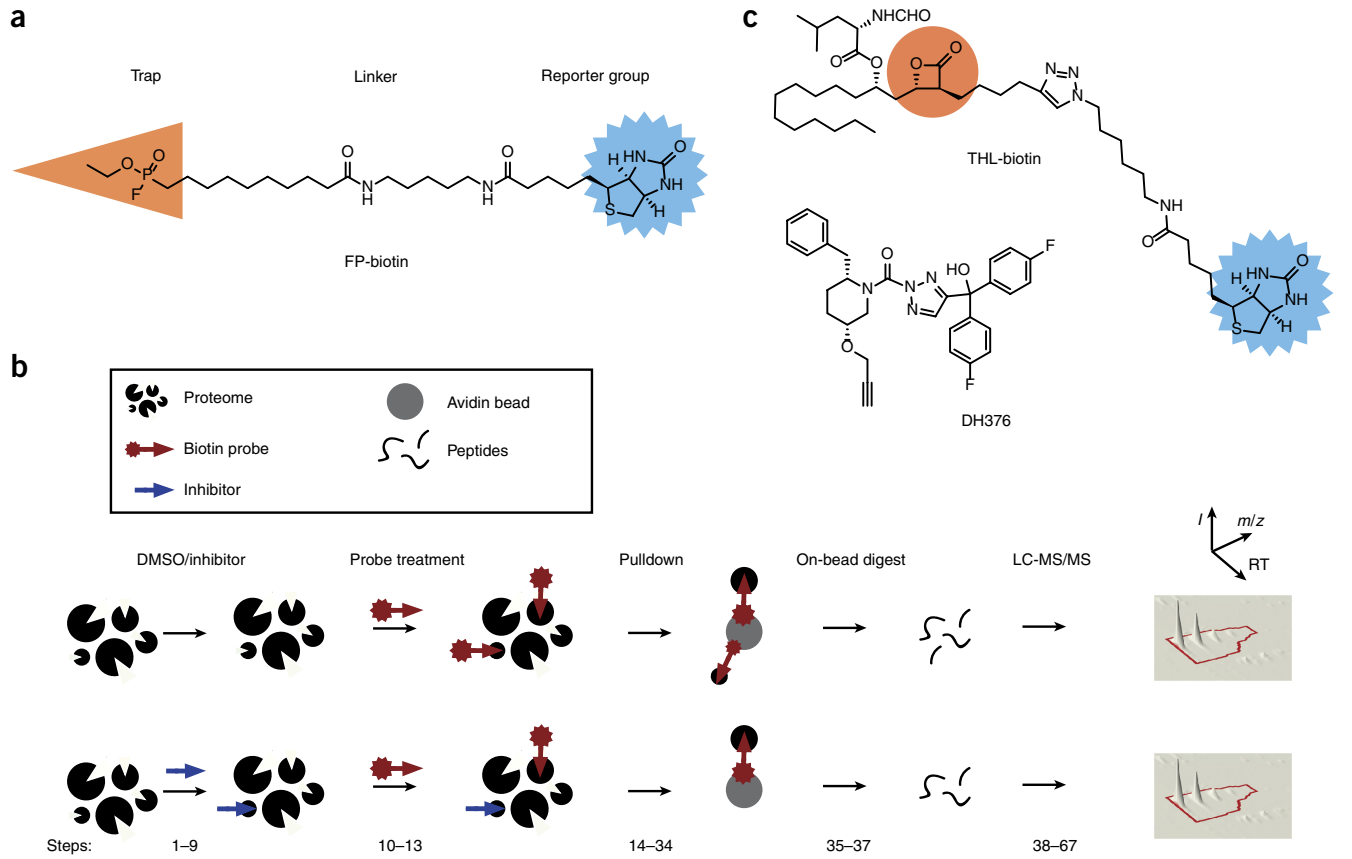

Figure 1 | Chemical proteomics workflow with inhibitor and probes used in this study. (**a**) General activity-based probe (ABP) design, illustrated with FP-biotin Orange: trap (fluorophosphonate). Blue: reporter group (biotin). (**b**) Workflow of competitive ABPP followed by chemical proteomics: after treatment with inhibitor (or DMSO control), a proteome is labeled with a biotin-containing ABP and enriched using avidin (pulldown), which is 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. RT, retention time.

or well-characterized inhibitors). The disadvantages of mass spectrometry-based ABPP are the necessity of more elaborate sample preparation (typically, a gel-based ABPP experiment can be performed in 3 h, whereas a pulldown assay takes 2 d) and the advanced instrumentation required[14].

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 Scientific	88311
Desthiobiotin-GTP	GTPases	Thermo Fisher Scientific	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[15,16]. Recently, label-free quantification approaches have gained interest as a suitable alternative, because they allow for a more simplistic experimental setup, avoiding expensive and time-consuming labeling steps, and do not require analysis of complex mass spectra[15,17–19]. 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 LC separation and spray conditions, and the need for technical replicates. Furthermore, data-processing time is increased by the requirement to align 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[11,20,21].

Whereas quantification of protein activity in our previous protocol was performed by dimethyl labeling[12], we now describe a label-free quantification protocol[17] with the use of data-independent acquisition (DIA) and ion mobility separation (IMS), based on the report of Distler *et al.*[19]. In data-dependent acquisition, 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[22]. 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 IMS, ions are separated in the mass spectrometer according to their mobility in a buffer gas, thereby providing an additional dimension of separation after LC[23]. In addition, precursor ions can be coupled to their fragments on the basis of their drift time in IMS, increasing the number of identified peptides markedly[24].

Overview of the procedure

Here, we demonstrate our ABPP protocol 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**). After tissue lysis (Steps 1–9), enzymes are labeled by incubation with a cocktail of the two biotinylated ABPs (Steps 10–13), 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 (Steps 38–44). Label-free quantification is used to compare the different conditions (vehicle versus heat inactivated, vehicle versus 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, because of the lack of multiplicity.

Applications of competitive ABPP

Here, 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 (ref. 25). We performed competitive ABPP studies with two different ABPs: the broad-spectrum serine hydrolase-directed probe fluorophosphonate–biotin (FP-biotin, **Fig. 1a**) and the tailored probe MB108 (THL-biotin, **Fig. 1c**)[26,27]. The latter probe preferentially reacts with endocannabinoid hydrolases diacylglycerol lipase-α (DAGLα), ABHD6 and ABHD12, as well as with several other enzymes. Together, the two probes enabled target engagement assays for fatty acid amide hydrolase (FAAH) and a broad array (>50) of other brain serine hydrolases[25–31]. Using this competitive ABPP assay, we confirmed that BIA 10-2474 interacted with FAAH and FAAH2 in human cells[25]. Furthermore, we discovered that BIA 10-2474 is 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[25].

Using an adapted version of the protocol, we had previously discovered and optimized LEI105 (an α-ketoheterocycle)[27] as a selective and reversible inhibitor of DAGL, an enzyme that catalyzes the conversion of diacylglycerol to the endocannabinoid 2-arachidonoylglycerol (2-AG)[28]. Comparative ABPP was used to map the activity of different endocannabinoid hydrolases in various brain regions, both in cannabinoid type 1 receptor knockout and wild-type brain tissue[29]. In addition, competitive ABPP was critical to the identification of the first brain-active DAGL inhibitor DH376 (refs. 30,31) (**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[30]. 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[30,31].

Limitations and comparison with 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 allows the determination of inhibitor selectivity for only the proteins targeted by the ABP. In this protocol, we demonstrate the use of a cocktail of two probes 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 false-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 of studying the target interaction profile of covalent inhibitors in living organisms[1,3]. One possibility is to turn the inhibitor of interest into an ABP by attaching a reporter group[2]. 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 we recommend confirming the activity of the modified inhibitor in an in vitro assay. Several complementary approaches have been developed that rely on the observation that inhibitor binding stabilizes the target protein. The CETSA relies on thermal stabilization of the target proteins by inhibitor binding[4]. Drug affinity–responsive target stability is an approach that relies on the assumption that inhibitor targets are more resistant to proteolysis[32]. As 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 our previous protocol[12]. The main advantage of gel-based ABPP, as compared with LC–MS methods, is the higher

throughput of samples. However, LC–MS-based ABPP is unrivaled in its depth of analysis, resolution and sensitivity.

Ion mobility separation is a powerful method of increasing the analytical depth of the proteomic analysis. Unfortunately, open-source software is not yet available to process raw ion mobility data. In this protocol, we describe the use of the vendor software Progenesis for data processing. Open-source software has been published for the label-free data analysis, but this workflow still depends on the vendor software PLGS for raw data processing[33]. 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.

As chemical proteomics is a multidisciplinary field in which chemistry, biology and mass spectrometry expertise is needed, we hope that this protocol can 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 the 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 was only identified by using sucrose gradient fractionation of mouse brain membrane[34]. Comparison of samples to controls is necessary to distinguish specific binders from contaminants and background. In this protocol, we use heat-inactivated vehicle-treated controls to determine whether a protein is identified in an activity-based manner. To determine whether an enzyme target is enriched as compared with the heat-inactivated control, we use the cutoff values of ANOVA (P) < 0.05 and ratio (active/inactive) > 2. Furthermore, we used a cocktail of FP-biotin and THL-biotin 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.

In our opinion, the most cumbersome part of this protocol (which can lead to the highest sample variation) is the methanol–chloroform precipitation (Steps 14–21)[35]. This step is necessary to remove excess probe before avidin enrichment. We recommend practicing this step (on any 1.0 mg/ml protein solution) before performing it on valuable samples. The first day of this protocol (up until overnight trypsinization in Step 37) is time-consuming; however, there is one optional pause point (Step 23).

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[36]. In our experience, Top3 quantification provides reliable quantification data only when low-scoring identified peptides are filtered out. We use the Benjamini–Hochberg procedure to correct for multiple comparisons, using a false-discovery rate (FDR) of 10% (ref. 37).

MATERIALS

REAGENTS

- Acetonitrile, ULC/MS grade (Biosolve, cat. no. 012041)
  ! CAUTION Acetonitrile is flammable and harmful if inhaled or swallowed, or upon contact with skin or eyes. Handle in a fume hood and wear a lab coat and safety glasses.
- Activity-based probe of interest: in this example, we describe the use of FP-biotin (Santa Cruz Biotechnology, cat. no. sc-215056A) and THL-biotin (MB108; synthesized as described[26] and available from the authors 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-Aldrich, cat. no. A9207)
- Benzonase nuclease (Santa Cruz Biotechnology, cat. no. sc-202391)
- BSA (Sigma-Aldrich, cat. no. A9647)
- Bradford reagent (Bio-Rad, cat. no. 500-0006)
- Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$; Merck Millipore, cat. no. 102382)
- Chloroform (Sigma-Aldrich, cat. no. 32211-M) **! CAUTION** Chloroform is a suspected carcinogen and is toxic if inhaled; handle in a fume hood and wear a lab coat and safety glasses.
- DMSO (Sigma-Aldrich, cat. no. 34943-M)
- DTT (BioChemica, cat. no. A1101) **! CAUTION** DTT is an eye and skin irritant.
- Empore C18 47-mm extraction disk (3M Purification, model 2215)
- Formic acid, LC–MS grade (Actu-All Chemicals, art. no. 8060128A1)
  ! CAUTION This compound can cause severe burns; handle in a fume hood, and wear a lab coat and safety glasses.
- [Glu^1]-fibrinopeptide B (GluFib; Waters, product no. 700004729)
- Glycerol, 85% (vol/vol) (Merck Millipore, cat. no. 104092)
- Hydrochloric acid (HCl; Sigma-Aldrich, cat. no. 30721-M)
  ! CAUTION Can cause severe burns; handle in a fume hood and wear a lab coat and safety glasses.
- HEPES, free acid (Millipore, cat. no. 391340)
- Inhibitor of interest: in this example, we describe the use of the diacylglycerol lipase inhibitor DH376 (synthesized as described[30] and available from the authors upon request).
- Iodoacetamide (IAA; Sigma-Aldrich, 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 ($MgCl_2{\cdot}6H_2O$; Acros Organics, cat. no. 413415000)
- Methanol, reagent grade (Sigma-Aldrich, cat. no. 32213-M) or ULC/MS grade (Biosolve, cat. no. 136841) **! CAUTION** Methanol is flammable and toxic. Handle it 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, cat. no. C57BL/6NCrl) treated with vehicle or DH376 were used (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 no. 14137).
- 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 lab coat and safety glasses.
- Tris(hydroxymethyl)aminomethane (Tris; Acros Organics, cat. no. 16762)

- Trypsin, sequencing grade (Promega, cat. no. V5111)
- Urea (Sigma-Aldrich, cat. no. 33247)
- Water, ULC/MS grade (Biosolve, cat. no. 232141) and Milli-Q water (Equipment) ▲ CRITICAL Avoid using autoclaved water, because it may contain high chemical background.
- Yeast enolase (Waters, product no. 186002325; UniProt P00924)

EQUIPMENT

- Analytical column (HSS-T3 C18, 1.8 µM, 75 µM × 250 mm; Waters, part no. 186007474)
- Bio-Spin columns (Bio-Rad, cat. no. 7326204)
- Centrifuge for 15-ml tubes, 2,500*g* required (Heraeus Megafuge, model no. 1.0R)
- Centrifuge for 1.5- to 2-ml tubes, 18,400*g* required (Eppendorf, model no. 5415D)
- Dounce homogenizer (Wheaton, model no. 357422)
- Eppendorf ThermoMixer C (Eppendorf, cat. no. 5382000015)
- Example data sets: the mass spectrometry proteomics data used in this protocol have been deposited to the ProteomeXchange Consortium via the PRIDE[38] partner repository with the dataset identifier PXD007965. The data used for analysis are available in **Supplementary Data 1–4**.
- Insulin syringe (Terumo, model no. 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, model no. 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, gray (Sarstedt, order no. 70.1130.600)
- Probe sonicator (Branson, Digital Sonifier)
- SpeedVac (Eppendorf, model no. concentrator 5301)
- Suction pump (Meyvis BV)
- SYNAPT G2-Si high-definition mass spectrometer (Waters)
- Tecan GENios microplate reader (Tecan Trading)
- Trap column (C18, 100 Å, 5 µM, 180 µM × 20 mm; Waters, part no. 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, model no. Optima L-90K)
- Vials, LC–MS (Waters, part no. 600000671CV)
- Vortex mixer (VWR, cat. no. 10153-838)

Software

- Progenesis (v3.0: http://www.nonlinear.com/progenesis/qi-for-proteomics/)
- Excel (Microsoft, v2010)
- KNIME (3.2.1: http://www.knime.com)

REAGENT SETUP

▲ CRITICAL All reagents are made with 18.2 MΩ Milli-Q water unless indicated otherwise.

Ammonium bicarbonate buffer Dissolve 198 mg of 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 $MgCl_2$ and 20 mM NaCl). Aliquots of this solution can be stored at −20 °C for at least 6 months.

$CaCl_2$ stock Dissolve 147 mg of calcium chloride dehydrate in 1 ml of water to prepare a 1 M $CaCl_2$ solution. This solution can be stored at room temperature (18–24 °C) for up to 1 month.

DTT stock Dissolve 1.54 g of 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 1 year.

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

HCl stock Prepare a 1 mM HCl solution (pH 3) by diluting 37% (wt/vol) HCl (~12 M) in water. Dilute 1 ml of 12 M HCl in 119 ml of water for a 0.1 M solution; dilute 0.5 ml of this solution in 49.5 ml of water for a 1 mM solution. These solutions can be stored at room temperature for up to 1 month.

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

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

IAA stock Dissolve 92 mg of iodoacetamide in 1 ml of 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 1,900 µl of ULC/MS-grade water, 60 µl of acetonitrile, 2 µl of formic acid and 40 µl of 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 of water. Aliquots of this solution can be stored at −20 °C for up to 1 year.

Lock mass 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 of GFP stock solution and 6 µl of 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 1 month.

Lysis buffer For lysing 12 tissues, prepare 30 ml of lysis buffer by combining 29.2 ml of water, 0.6 ml of HEPES stock, 60 µl of DTT stock, 30 µl of $MgCl_2$ stock and 75 µl of benzonase (final concentrations: 20 mM HEPES, 2 mM DTT, 1 mM $MgCl_2$ and 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.

$MgCl_2$ stock Dissolve 2.0 g of $MgCl_2 \cdot 6\,H_2O$ 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 1 month.

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 1 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 1 month.

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

On-bead digestion buffer (OB-Dig) For 24 samples, combine 4,668 µl of water, 600 µl of Tris stock, 600 µl of NaCl stock, 6 µl of $CaCl_2$ stock and 120 µl of 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 10× stock Dissolve 68.05 g of KH_2PO_4 in 500 ml of water (heat at 40 °C until dissolved), dissolve 261.23 g of K_2HPO_4 in 1,500 ml of water, mix and add 877 g of NaCl, add water to a final volume of 10 liters and filter over a 0.22-µM 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 3 months.

PBS Dilute PBS 10× stock ten times in Milli-Q water (pH should be 7.5). This solution can be stored at room temperature for up to 1 month.

PBS/SDS Add 50 ml of SDS stock to 950 ml of PBS (final concentration: 0.5% (wt/vol) SDS). This solution can be stored at room temperature for up to 1 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 1 year.

SDS stock Prepare a 10% (wt/vol) SDS solution in water. This solution can be stored at room temperature for up to 1 year.
Seal wash Prepare a 10% (vol/vol) acetonitrile solution in water (both ULC/MS grade).
StageTip solution A Prepare a 0.5% (vol/vol) formic acid solution in water. This solution can be stored at room temperature for up to 1 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 1 month.
StageTips See 'Equipment Setup'.
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 1 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 1 month.
Trypsin solution Dissolve 20 µg of trypsin in 40 µl of 1 mM HCl stock (final concentration: 0.5 µg/µl). Store this solution at −20 °C for up to 1 month and avoid freeze–thaw cycles.
Urea buffer Add 1 ml of ammonium bicarbonate buffer (250 mM) to 3.6 g of 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 of yeast enolase in 1 ml of 3% (vol/vol) acetonitrile in water. This solution can be stored at room temperature for at least 1 year.

EQUIPMENT SETUP

StageTips As a final step in sample preparation, we use StageTips. Their preparation and use are described in *Nature Protocols* by Rappsilber *et al.*[39]. We use Empore C18 47-mm extraction disks (Reagents) to fabricate our StageTips. Typically, we stack two disks on top of each other to make StageTips with two layers of column material, inserted into 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 Our LC–MS method is based on the approach described by Distler *et al.*[19]. A summary of changes in our approach is given here. We do not add DMSO to the LC solvents. Therefore, we use a lower source temperature (80 °C instead of 100 °C). As the affinity chromatography step (pulldown) makes the samples less complex, our gradient is shorter. We use a trap–elute protocol, in which 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.*[19]. The column is re-equilibrated to initial conditions after washing with 90% solvent B. The detailed protocol is specified below:

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. [Glu[1]]-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 µl/min).
MS acquisition method A UDMS[e] method is set up as described in Distler *et al.*[19]. Briefly, the mass range is set from 50 to 2,000 Da, with a scan time of 0.6 s 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[33]. The lock mass is sampled every 30 s.

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 numbers of freeze–thaw cycles for example).

2| Manually lyse the tissue in 2 ml of precooled lysis buffer using a dounce homogenizer. The number of strokes required depends on both the type of tissue and the size. Typically, for the brain (soft tissue) and heart (tough tissue), we use 5 and 25 strokes, respectively, for complete homogenization. Transfer the lysed tissue to a 2-ml tube.

3| Incubate the homogenized tissue on ice for 15 min.

4| Pellet the cell debris by centrifugation (3 min, 2,500*g*, 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, 100,000*g*, 4 °C).
! CAUTION The tubes should be undamaged, properly balanced and sealed. The rotor should be undamaged, clean and dry.
▲ CRITICAL STEP Depending on the abundance and distribution of proteins of interest, this fractionation step can be omitted or elaborated (Experimental design).

6| Collect the supernatant into a tube as the cytosolic fraction.

7| Resuspend the pellet (membrane fraction) in 1–2 ml of HEPES/DTT buffer (amount depends on the size of the pellet; in our experience, 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 suction the membrane fraction and push it through the needle once for homogenization.

8| According to the manufacturer's protocol (bulletin no. 4110065, Bio-Rad), perform the Bradford assay to determine the protein concentration, or alternatively, one can use a microBCA assay (Thermo Scientific, cat. no. 23225). We usually obtain approximately the following protein amounts:

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, divide 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 be snap-frozen and that freeze–thaw cycles be avoided.
■ PAUSE POINT The lysates can be stored at –80 °C for at least 6 months. For some enzymes, freshly prepared lysate may provide 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) to each clear 1.5-ml tube. Prepare one tube for each inhibitor-treated sample, and two tubes for each vehicle sample.

11| To prepare heat-inactivated control samples, incubate one of the vehicle-treated samples for 5 min at 100 °C. We advise adding 25 µl of 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 r.p.m.), followed by a short spin down (600*g*, 20 °C, 10 s).

Methanol/chloroform precipitation ● TIMING ~1.5 h for 24 samples
! CAUTION Perform Steps 14–21 in a fume hood and discard the supernatants obtained in Steps 18 and 21 as halogenated organic waste.

14| Add 250 µl of water to each sample for a final volume of 500 µl.
▲ **CRITICAL STEP** If the protein sample from Step 13 has a different volume than 250 µl, adjust the amount of water added to obtain a final volume of 500 µl.

15| Add 666 µl of MeOH and briefly vortex.

16| Add 166 µl of $CHCl_3$ and briefly vortex.

17| Add 150 µl of water and briefly vortex; this should result in a cloudy suspension (**Supplementary Fig. 1a**).

18| Pellet the precipitated protein by centrifugation (10 min, 1,500*g* at room temperature), this should result in a floating pellet (**Supplementary Fig. 1b**). 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; see **Supplementary Fig. 1c**).
▲ **CRITICAL STEP** Handle the samples carefully to prevent disrupting the protein pellet.
? TROUBLESHOOTING

19| Add 600 µl of MeOH to the pellet.

20| Resuspend the pellet by sonication with a probe sonicator (10 s, 30% amplitude), this should result in a suspension without any visible protein pellet (**Supplementary Fig. 1e**).
▲ **CRITICAL STEP** The tip of the probe sonicator should be positioned just above the bottom of the tube (**Supplementary Fig. 1d**).

21| Pellet the protein by centrifugation (5 min, 18,400*g* at room temperature) (**Supplementary Fig. 1f**) 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 of 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 µl)
? TROUBLESHOOTING
■ **PAUSE POINT** The solubilized protein samples can be stored at –80 °C for at least 1 month.

24| Add 2.5 µl of DTT stock, vortex briefly, spin down briefly (600*g*, 20 °C, 10 s) and incubate for 15 min at 65 °C while shaking (600 r.p.m.).

25| Let the samples cool to room temperature (at least 5 min).

26| Add 20 µl of IAA stock, vortex briefly and incubate for 30 min at room temperature in the dark (e.g., in a drawer or wrapped in aluminum foil).

27| Add 70 µl of SDS stock, vortex briefly and incubate for 5 min at 65 °C.

28| For 24 samples, take 2.4 ml of avidin beads from a 50% slurry (100 µl of slurry per sample) and divide them over four 15-ml tubes (600 µl per tube). Be sure to properly homogenize the slurry before pipetting. When testing the individual probes (Step 12), 50 µl of slurry can be used per sample.

29| Wash the beads three times with 10 ml of PBS. Pellet the beads by centrifugation (2 min, 20 °C, 2,500*g*) and remove the supernatant with a suction pump.
▲ **CRITICAL STEP** Be careful not to suction up the beads.

30| Resuspend the beads in 6 ml of PBS per tube.

31| For 24 samples, prepare 24 tubes (15 ml) with 2 ml of PBS and 1 ml of 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.

Washing of beads ● TIMING ~1.5 h for 24 samples

▲ **CRITICAL** Steps 33 and 34 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, 2,500*g* at room temperature) and remove the supernatant.

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

34| Wash the beads once with 6 ml of PBS/SDS, followed by three times with 6 ml of PBS. Pellet the beads by centrifugation (2 min, 2,500*g* at room temperature) after each washing step and remove the supernatant.

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

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

35| Add 250 µl of 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 µl (500 ng) of trypsin solution per sample. The trypsin stock can also be diluted (24 µl in 6 ml of OB-Dig buffer) to allow pipetting of larger volumes (250 µl)—this might improve consistency.

37| Digest overnight at 37 °C with vigorous shaking (950 r.p.m.).

Sample preparation ● TIMING ~3 h

38| Spin down briefly (600*g*, 20 °C, 10 s) and add 12.5 µl of formic acid, briefly vortex and spin down again (600*g*, 20 °C, 10 s).

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

40| Condition the StageTips (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.

	Stage	Buffer	Centrifugation
1	Conditioning 1	50 µl of MeOH	2 min, 300*g* at room temperature (RT)
2	Conditioning 2	50 µl of StageTip solution B	2 min, 300*g* at RT
3	Conditioning 3	50 µl of StageTip solution A	2 min, 300*g* at RT
4	Loading	Sample from Step 39	2 min, 600*g* at RT
5	Washing	100 µl of StageTip solution A	2 min, 600*g* at RT
6	Switch to low-binding tube		
7	Elution	100 µl of StageTip solution B	2 min, 600*g* at RT

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

41| Evaporate the solvent in a SpeedVac.

■ **PAUSE POINT** Store the samples at –20 °C until required. Samples can be stored up to 1 year.

42| Reconstitute the 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 (Equipment Setup) and perform at least duplicate LC–MS analysis of each sample.

▲ CRITICAL STEP To prevent drift in instrument performance from 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. We recommend running a QC sample after every 24 MS runs[19].

? TROUBLESHOOTING

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

45| Open Progenesis QI for proteomics. Create a new label-free experiment. Choose Data type 'Profile data' and Machine type 'High resolution mass spectrometer'. Choose an experiment folder (for data management, we recommend giving this folder 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 the alternating low- and high-energy collision scan mass spectrometry (MS^E) identification workflow (we use the default energy thresholds, and elution start = 10 min and elution end = 65 min. The optimal settings depend on the instrument and the LC gradient). Nondefault settings are indicated with an asterisk:

Parameter	Value
Lock mass *m/z* value	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 **Supplementary Data 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

47| Perform automatic processing while the raw data are 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 it with 'UNIPROT' as parsing rules. Select trypsin as digest reagent, two missed cleavages, max protein mass = 250 kDa, modifications carbamidomethyl C (fixed) and oxidation M (variable). Search tolerance parameters: set 'FDR' to <1% and ion-matching requirements to at least two fragments/peptide, five fragments/protein and one peptide/protein. For 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 1 h per sample.

48| Review alignment: check whether the automatic alignment algorithm has allocated vectors and make sure that the part of the chromatogram in which peptides elute has good alignment quality.
? TROUBLESHOOTING

49| Under 'Filtering': select ions with charges 2, 3, 4, 5, 6 and 7+ and delete nonmatching peptide ions (in our experience, ~20–25%).

50| Review normalization: check whether certain samples deviate from the normalization reference.

51| Experiment design setup: choose between-subject design. Create separate designs for vehicle versus heat and vehicle versus 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 <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** In our experience, this step is critical to obtaining 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 are 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 that can be used to find the spectrum and chromatogram under 'Review peak picking'. The fragmentation spectrum can be found under 'Identify peptides'.

55| Check whether the data cluster according to the experimental conditions using 'PCA' under 'Protein statistics'.

Box 1 | Database generation ● TIMING ~5 min

For an excellent tutorial on the bioinformatics behind protein identification, see Vaudel *et al.*[40]. Here, we briefly explain how we generate our database, which was used in this study to search for protein identifications in Progenesis (**Supplementary Data 1**).

1. Go to http://www.uniprot.org[41]. 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, we chose to select only the reviewed part of the mouse proteome, 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 (accession nos. P00761, P00924 and P02701), go to the 'sequence' tab and click the 'FASTA' button. Paste this sequence into the mouse proteome .fasta file. See **Supplementary Data 1** for the database used in this protocol.

56| Repeat Steps 45–55 for each fraction. Open Progenesis and go to 'Combine analysed fractions'. Select 'Recombine analysed fractions'. Import data and recombine the samples. Go to 'Experiment design setup' and select the sample grouping. Go to 'review proteins' and export protein measurements. Check whether the data cluster according to the experimental conditions using 'PCA' under 'Protein statistics'. Protein measurements from the example dataset are available in **Supplementary Data 2**.

57| Use Excel to open the 'vehicle-versus-heat' .csv file created in Step 56. Save as a .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`' will return 'Faah'.

59| Delete proteins with zero 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-versus-inhibitor' .csv file created in Step 56. Save this as a .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 as compared with the heat-inactivated control.

64| Filter this selection again using the putative targets list. We generated a small database of putative probe targets from our previous experiments using these probes[27,29], and a phylogenetic tree of α,β-hydrolase fold proteins combined with annotated catalytic nucleophiles in UniProt[40]. See **Supplementary Data 4** for the list of probe targets used in this protocol.

65| Apply the Benjamini–Hochberg correction with an FDR of 10% (q = 0.1): (i) List all ANOVA (P) values from lowest to highest. (ii) Calculate the B–H statistic as q × position in the list/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! (iii) Select all proteins with a P value smaller than the B–H statistic (with q = 0.1; these are now corrected for an FDR of 10%).

66| For each protein, calculate the average normalized abundance of vehicle- and inhibitor-treated samples, percentage of inhibition (inhibitor/vehicle × 100%) and the error of ratio, using the following formula (with x = average inhibitor, y = average vehicle and σ = s.d.) (see **Supplementary Fig. 2**).

$$\text{error of ratio} = \frac{x}{y} \cdot \sqrt{\left(\frac{\sigma_x}{x}\right)^2 + \left(\frac{\sigma_y}{y}\right)^2}$$

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, and single-linkage type) (**Fig. 2**).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
18	The protein pellet breaks	The protein concentration was too low	Use more protein per sample; we recommend 250–2,000 µg
23	The protein does not redissolve	• The pellet has dried out	• Add urea directly after removing methanol
		• The protein amount was too high	• Use more urea buffer
44	Low signal	• Unsuccessful pulldown	• Optimize the protein amount, probe concentration and amount of beads
	•Loss in sensitivity	• The column or instrument was contaminated	• Replace the trap and/or analytical column, and clean the instrument
48	Poor alignment	• The algorithm did not place vectors correctly	• Choose a mixed sample as alignment reference
		• Samples are too different	• Minimize differences arising from sample preparation
			• If the alignment quality is poor, you can consider manually placing vectors. However, in our experience, 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

For further troubleshooting advice (especially regarding the LC–MS analysis), see Distler *et al*[19].

● 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

ANTICIPATED RESULTS

Probe cocktail

In our previous studies using FP- and THL-based biotinylated probes[27,29], we prepared a separate sample for each probe, duplicating the amount of samples. The Venn diagram in **Supplementary 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.

Competitive ABPP

Using our label-free quantitative proteomics protocol, we confirmed that DH376 inhibits Dagla and ABHD6 *in vivo* and identified several novel off targets (**Fig. 2**). We found that 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), we identified 81

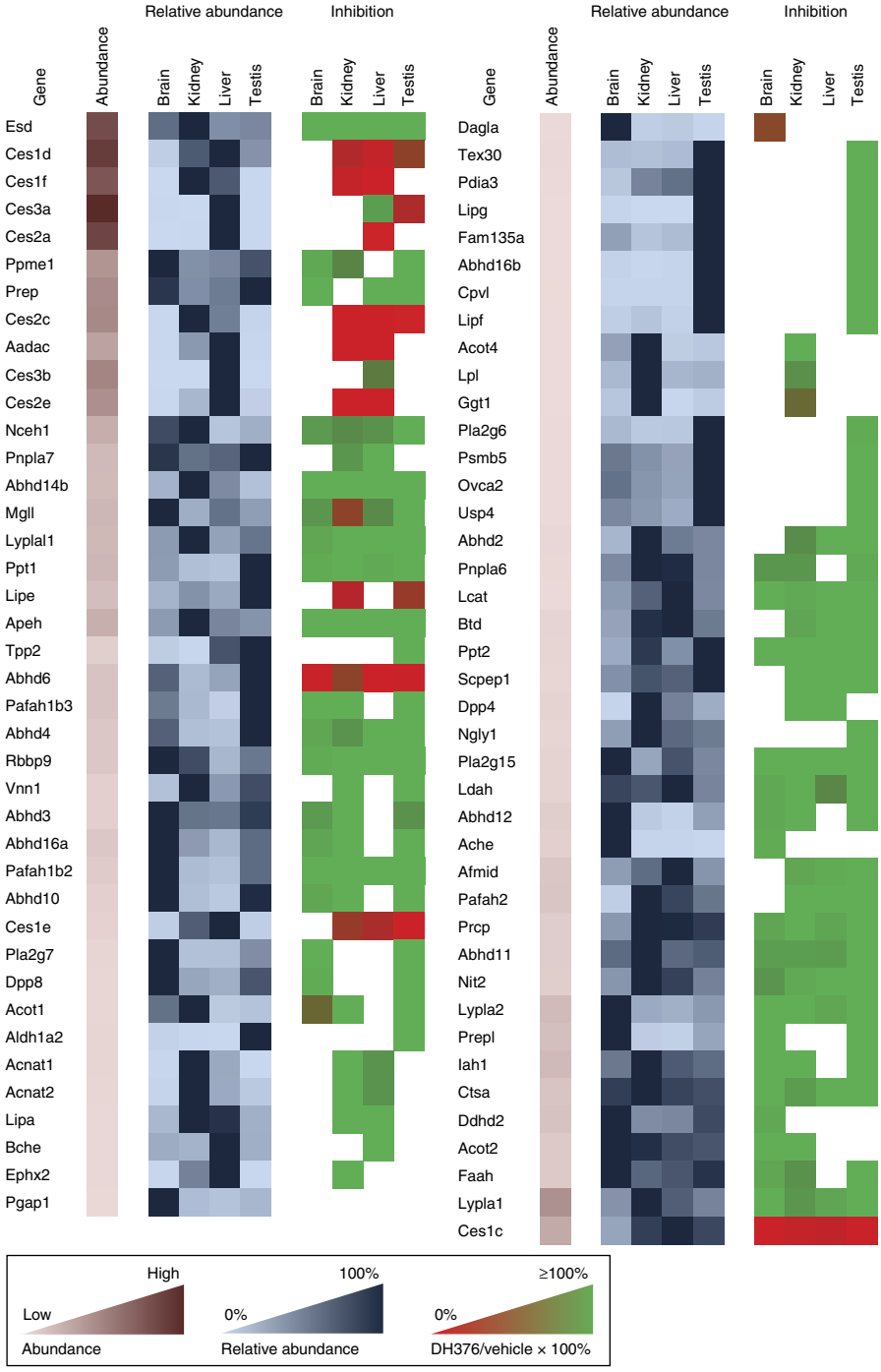

Figure 2 | Results of the competitive ABPP experiment in mice with DH376 and the probe cocktail, with hierarchical clustering of probe targets. Highest normalized abundance is shown in brown. The relative abundance of each protein in each tissue is shown in blue. The inhibition by DH376 is shown in red (inhibition) and green (no inhibition). The animal experiments were conducted in accordance with the ethical committee of Leiden University (DEC no. 14137).

proteins that are picked up by our probe cocktail in an activity-based manner across four murine tissues. The results of our competitive ABPP experiment are summarized in **Figure 2** (see **Supplementary Data 3** and **4** for protein and peptide quantification data, respectively, and **Supplementary Figs. 2** and **4** for inhibition data per tissue). 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. 3a**). Several other carboxylesterases with high sequence similarity, i.e., Ces1d, Ces1e, Ces1f, Ces2a and Ces2c, were also identified as novel targets of DH376 in the other tissues. Furthermore, off targets are Aadac and Lipe (both involved in triglyceride hydrolysis). An apparent discrepancy in the activity profile of DH376 between different tissues was observed for

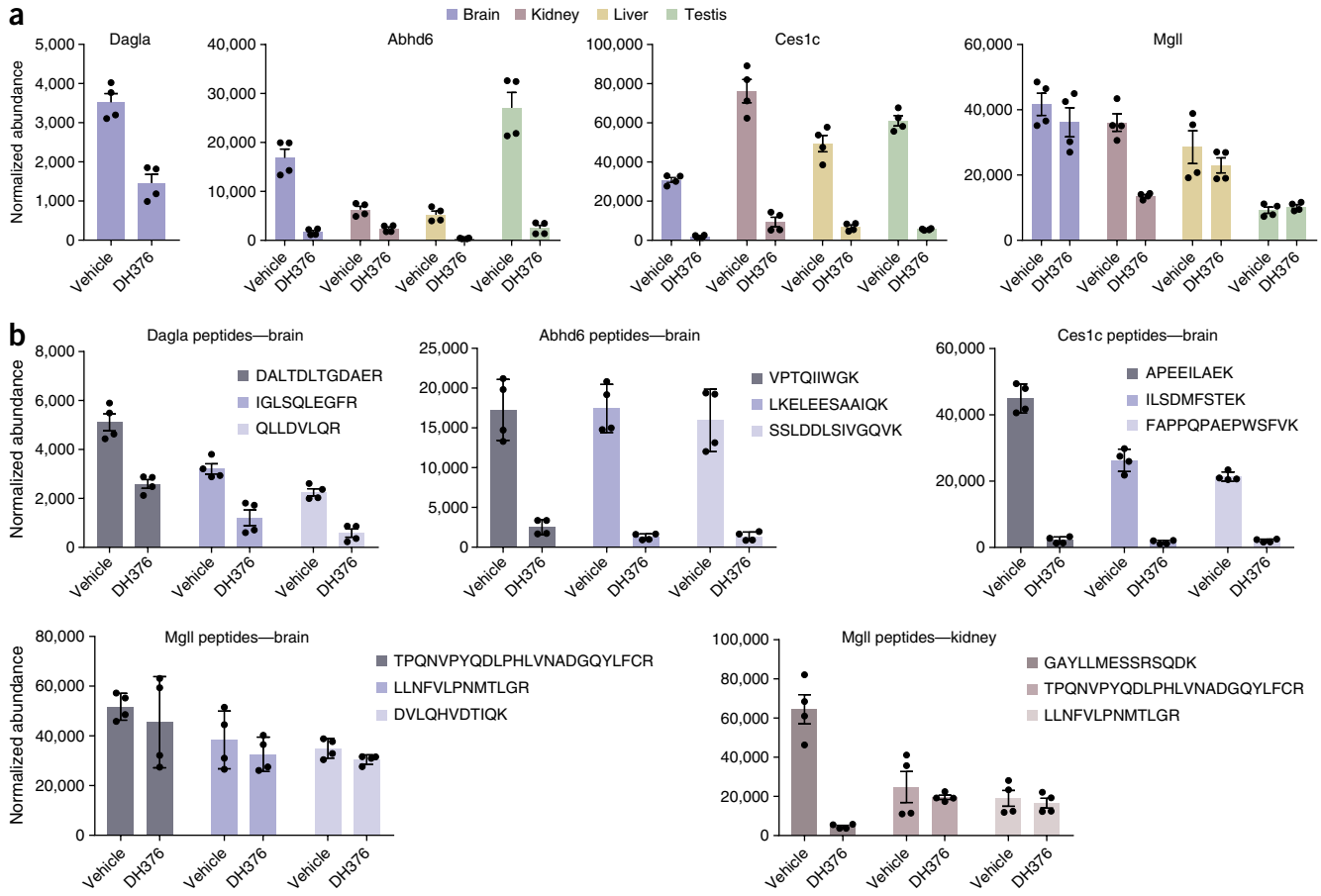

Figure 3 | Protein and peptide abundance data for selected probe targets. (**a**) Normalized protein abundance for Dagla, Abhd6, Ces1c and Mgll in different conditions. The normalized protein abundance is calculated from the averaged normalized abundance of the top three most abundant peptides of each protein. (**b**) Normalized abundance of peptides used in quantification of brain Dagla, Abhd6, Ces1c and Mgll, and kidney Mgll. Individual data points, mean and s.d. are shown. The peptide ion abundances are normalized between runs in Progenesis (Step 50).

monoglyceride lipase (Mgll). DH376 inhibited Mgll in the kidney, but not in the other three tissues (**Fig. 3a**). In **Figure 3b**, the peptides used for quantification of brain Dagla, Abhd6, Ces1c and Mgll show consistent inhibition profiles. However, only one Mgll peptide was substantially different between the vehicle- and inhibitor-treated kidney samples (**Fig. 3b**) Combined with the results obtained via an orthogonal method of measuring Mgll inhibition[30], we denote this finding 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 in establishing the selectivity profile. Finally, the generation of the *in vivo* off-target profile of inhibitors using label-free quantitative activity-based proteomics will help in understanding the *in vivo* mode of action of pharmacological tool compounds and guide the dose selection of drug candidates.

Further information on experimental design is available in the **Life Sciences Reporting Summary**.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS E.J.v.R. designed and performed experiments, analyzed data and wrote the paper; B.I.F., H.D. and J.Z. treated the mice; B.I.F. performed experiments and wrote the paper; M.P.B. and A.C.M.v.E. developed the initial protocol, and H.S.O and M.v.d.S. wrote the paper.

COMPETING INTERESTS
The authors declare no competing interests.

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