

Probing histone deacetylases with a Capture Compound™ bearing suberoylanilide hydroxamic acid (SAHA) as the selectivity group

Capture Compound Mass Spectrometry (CCMS) is an innovative technology to reduce the complexity of biological samples through selective isolation of targeted protein or enzyme families. Small synthetic molecules (Capture Compounds™) interrogate native proteins, even lipophilic membrane proteins. CCMS technology enables an efficient complexity reduction of the proteome and allows discovering, isolating and profiling members of functional protein families within a variety of biological samples.

Introduction

The diverse and dynamic posttranslational modifications of histones of nucleosomes represent a key biochemical mechanism in the epigenetic control of gene expression and are the core of the histone code hypothesis (1). The posttranslational modifications, among them acetylation, methylation, ubiquitination, or phosphorylation alter the interactions of the core histones with DNA, to enable or prevent the transcription of target genes. While acetylation is thought to be associated with weakened histone-DNA interactions and increased transcriptional activity, the deacetylation of histones is associated with strengthened histone-DNA interactions and repression of transcription, as already hypothesized by Allfrey et al (2). Histone acetylation is dynamic and controlled by dedicated enzymes, the histone acetyl transferases, and the histone deacetylases (3). Histone deacetylases have drawn interest as drug targets because of their dysregulation in some types of cancer (4). Suberoylanilide hydroxamic acid (SAHA) is a broadband histone deacetylase inhibitor that binds

to the histone deacetylases HDAC 1, 2, 3, and 6 with nanomolar affinity. A new Capture Compound™ (CC) with SAHA coupled as the selectivity function to the Capture Compound™ scaffold was developed as a research tool to target SAHA-binding histone deacetylases in complex protein mixtures. The SAHA-CC was tested with a cell lysate from human hepatoma-derived cell line HepG2.

Materials

The SAHA-CC was used at a stock concentration of 100 μ M in water, free SAHA was used as the competitor at a stock concentration of 0.56 mM. Further standard components of caproKit™ was used: streptavidin magnetic beads, 5 x concentrated capture buffer 4, and 5 x concentrated wash buffer. Assays were prepared in PCR tube strips for volumes up to 200 μ l (Thermo Fisher, cat. No. AB-1114) as reaction vessels, conduct the capturing experiments, wash and isolate the magnetic beads. Irradiation of the samples for photo cross-linking was performed using the caproBox™, and the beads were isolated using the caproMag™. As the protein sample, HepG2

lysate at a stock concentration of 7 mg protein/ml was used. HepG2 cells were lysed by French Press in lysis buffer (6.7 mM MES, 6.7 mM NaOAc, 6.7 mM HEPES, pH 7.5, supplemented to 200 mM NaCl, 10 mM β -mercaptoethanol, 1 mM EDTA, and protease inhibitor cocktail). Debris was removed by centrifugation at 30,000 x g for 60 min at 4 °C. The supernatant filtrated through a 0.2 μ m filter, and was then dialysed against lysis buffer for the removal of small molecules (optional). After dialysis, centrifugation at 4000 x g was carried out to remove protein precipitated during dialysis.

Capture experiment

The capture experiments were carried out in the OffBead configuration as described in the Guideline (<http://www.caprotec.com/support/downloads>). In brief, the assays were prepared in the order of pipetting water and 5 x capture buffer into PCR tubes and the assays were vortexed thoroughly. Cell lysate was then added to a final concentration of 5 mg/ml, and vortexed thoroughly. To the competition sample, the SAHA Competitor was added and allowed to incubate with the proteins for 30 min at 4 °C under rotation in the dark. (Optional: Depletion of biotinylated proteins was achieved by adding streptavidin beads from 50 μ l solution and incubating together with the lysate and competitor. Before adding the SAHA Capture Compound the beads were removed using the caproMag™). The SAHA Capture Compound™ was added and allowed to interact with the target proteins for two hours at 4 °C under rotation in the dark. The tubes were then placed in the pre-cooled caproBox™. The lid of the caproBox™ was then closed. The samples were irradiated for ten minutes at 2-4 °C. Wash buffer 1 was added, and the samples were vortexed thoroughly again. Then, streptavidin magnetic beads were added, and the samples left rotating for 30 min at 4 °C. Capture Compound-protein conjugates were then isolated and washed using the caproMag™ procedure as described in the caproMag™ Guideline (<http://www.caprotec.com/support/downloads>). The capture samples were then subjected to OnBead tryptic digestion. For this purpose, the beads after the final washing step with wash buffer 1 were

washed twice with HPLC grade water and incubated with 10 μ l per assay of 50 mM NH_4HCO_3 , and supplemented with 0.5 μ g trypsin (promega sequencing grade). This bead suspension was transferred into a new PCR tube. Tryptic digest was allowed to proceed over night at 32 °C under vigorous shaking. Beads were then fixed at the tube walls using the caproMag™, and the supernatants with the peptides recovered, and then directly subjected to LC-MS/MS analysis.

Mass spectrometry

LC-MS/MS experiments were performed using a nanoflow HPLC system (Proxeon) coupled on-line to an ESI-LTQ-Orbitrap system. From the LC column, peptides were eluted during an 80-min linear gradient from 5 % ACN/0.1 % FA to 40 % ACN/0.1 % FA followed by additional 2 min to 100 % ACN/0.1 % FA and remaining at 100 % for another eight minutes with a controlled flow rate of 300 nl/min. MS/MS fragmentation was performed in a data-dependent mode. Proteins were identified by automated database searching against the human UniProtKB/Swiss-Prot database using the algorithms SEQUEST and X!-Tandem as integrated in the software Scaffold™ 2. Mass accuracy was required to be better than 5 ppm for precursor ions, 1 amu for fragment ions. Two missed cleavage sites were allowed. Phosphorylation at serine, threonine, and tyrosine, oxidation of methionines, deamidation at asparagines and glutamine, acetylation at lysine and serine, formylation at lysine, and methylation at arginine, lysine, serine, threonine and asparagine were allowed as variable modifications. No fixed modifications were used in database search. The minimum Scaffold protein probability was set to 99 %, the minimum peptide probability to 95 %.

Results

From 500 μ g of HepG2 lysate as complex protein mixture input, 14 proteins were specifically identified. This means that the proteins were identified robustly in the capture assay, but were absent or massively reduced in terms of unique

peptides identified in the competition sample that contained free SAHA to compete for binding of the Capture Compound™ to the target proteins. Two of the identified proteins were HDACs, namely HDAC1 and HDAC6 (see Fig. 1, table 1). All the other proteins that were specifically captured were reported interaction partners of HDACs, proteins annotated as containing N-acetyl lysine (and thus likely to represent HDAC substrates), as well as proteins that have not yet been functionally characterized.

As an example for a known HDAC 6 interaction partner (5), we report here the identification of the Parkinson’s Disease protein Park7/DJ-1 as a protein specifically identified in the capture sample (Fig. 1, table 1). We conclude from this that the SAHA-CC is not only highly selective for the isolation of HDACs from complex biological samples, but can also reveal HDAC interaction partners.

Table 1 Selection of specifically captured proteins from two independent capture experiments

protein	Uniprot acc. no	Unique peptides assay	Unique peptides control	binds
HDAC1	Q13547	3/3	0/2	SAHA
HDAC6	Q9UBN7	6/9	0/0	SAHA
DJ-1	Q99497	9/9	4/1	Likely HDAC6

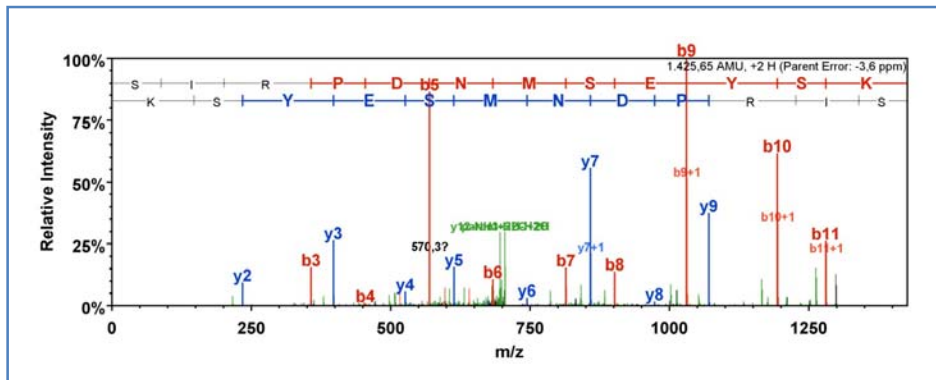
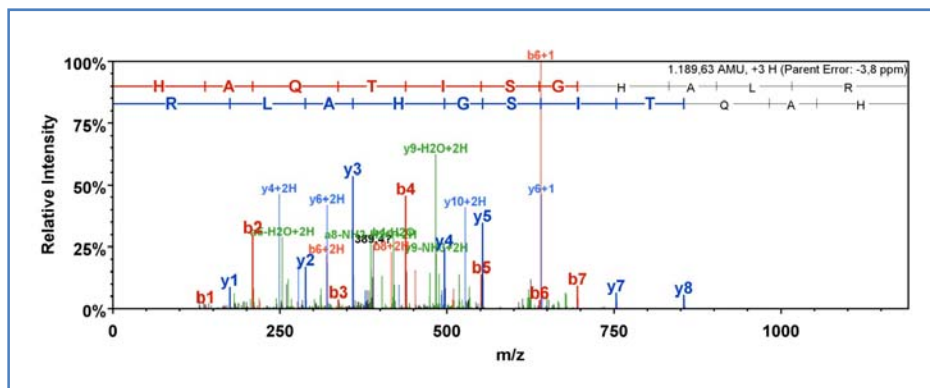
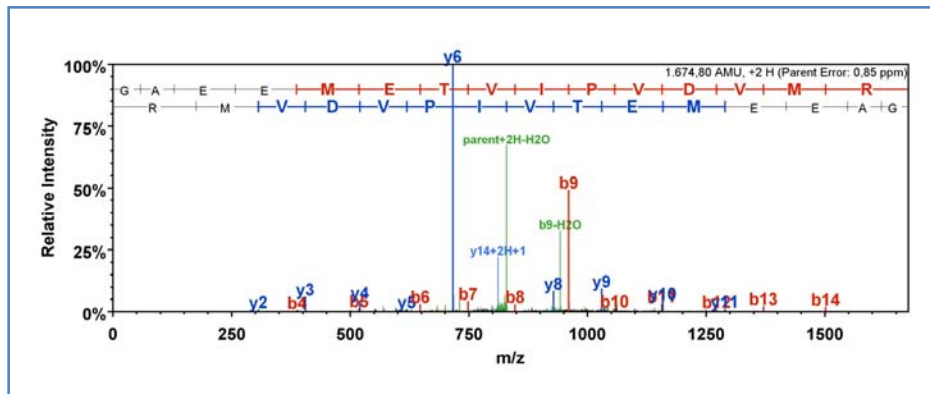


Figure 1 Selected MS/MS spectra of HDAC-derived tryptic peptides: Peptide SIRPDNMSEYSK from HDAC 1 (uniprot accession Q13547)



Peptide HAQTISGHALR from HDAC 6 (uniprot accession Q9UBN7)



Peptide GAEMETVIPDVMR from Park7/DJ-1 (uniprot accession Q99497)

References:

- 1) Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. *Nature* **403**, 41-45.
- 2) Allfrey, V.G., Faulkner, R., and Mirsky, A.E. (1964). Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Nat. Acad. Sci USA* **51**, 786-794.
- 3) Kouzarides, T. (2007). Chromatin modifications and their function. *Cell* **128**, 693-705.
- 4) Gallinari, P., Di Marco, S., Jones, P., Pallaoro, M., and Steinkühler, C. (2007). *Cell Res.* **17**, 195-211.
- 5) Olzmann, J.A., Li, L., Chudaev, M.V., Chen, J., Perez, F.A., Palmiter, R.D., and Chin, L.S. (2007). Parkin-mediated K63-linked polyubiquitination targets misfolded DJ-1 to aggresomes via binding to HDAC6. *J. Cell. Biol.* **178**, 1025-1038.

Downloads:

www.caprotec.com/support/downloads

For additional information please Email:

support@caprotec.com

Contact and order information:

Headquarters caprotec bioanalytics GmbH

Volmerstrasse 5
D-12489 Berlin

Phone: +49 30 63 92 39 90

Fax: +49 30 63 92 39 89

Web: www.caprotec.com

Email: sales@caprotec.com

Ordering information:

Item Nr.	Description
1-1070-050	SAHA caproKit™ 50 reactions
1-1070-010	SAHA caproKit™ 10 reactions

The caproKit includes the SAHA specific Capture Compound, all buffers, protein controls, SAHA competitor, and Streptavidin magnetic beads.

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Or contact us. Email: info@caprotec.com

Phone: +49 30 6392 3990

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