



The Marimastat caproKit™ enables profiling of metalloproteinases by Capture Compound Mass Spectrometry

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. Thus, CCMS allows for discovering, isolating and profiling of members of functional protein families within a variety of biological samples. Capture Compounds™, synthetic tri-functional small molecules, interrogate native proteins, even lipophilic membrane proteins. The key step is the formation of a photo-induced covalent crosslink between the Capture Compound™ and the target proteins.

Introduction

Zinc-dependent metalloproteinases are a diverse group of proteolytic enzymes defined by the presence of a metal ion, Zn^{2+} , in their catalytic site (1). The roles of these proteinases are manifold and extend from processing of pro-hormones to shedding of membrane proteins or degradation of extracellular matrix proteins. The latter function is exerted by a subclass of metallo-endopeptidases, the matrix metalloproteinases (MMPs) (2). There is evidence that members of the metalloproteinase family, and especially MMPs, are involved in pathological processes such as metastatic progression of cancer or inflammatory processes in the joints leading to arthritis (3, 4). Therefore, these proteins are considered to be potential drug targets and important biomarkers (5).

Marimastat is originally a pharmaceutical compound designed to inhibit MMPs (6). Its mechanism of action is to chelate the Zn^{2+} ion in the active site of metalloproteinases, thus rendering them inactive towards their substrate. caprotec now introduces a

Capture Compound™ that bears marimastat as the selectivity function (Marimastat-CC) to profile directly metalloproteinases from biological samples. This application note provides examples for the use of the Marimastat caproKit™.

Materials

MMP cocktails consisted of recombinant or purified human matrix metalloproteinases. Cocktail 1 (MMPs in the proenzyme form): MMP-1 (Uniprot P03956), MMP-2 (P08253), MMP-8 (P22894), MMP-9 (P14780), MMP-13 (P45452) and MMP-14 (P50281), all from Invitek GmbH, Berlin. 0.4 μ g per MMP were used in the cocktail for one assay. Cocktail 2 (MMP recombinant catalytic domains): MMP-3 (P08254, residues 100-273), MMP-13 (P45452, residues 104-274), MMP-14 (P50281, residues 89-265), MMP-15 (P51511, residues 91-267), MMP-16 (P51512, residues 91-279), MMP-17 (Q9ULZ9, residues 128-314), and MMP-24 (Q9Y5R, residues 156-351), all from Invitek GmbH, Berlin. Again, 0.4 μ g per MMP

were used in the cocktail for one assay. MMP-2 in the proenzyme form is supplied with the caproKit™ as positive control protein. As an additional chemical (not included in the Marimimastat caproKit™) for the *in vitro* activation of MMP proenzymes, 4-aminophenylmercuric acetate (APMA, Sigma Aldrich, product number A9563) was used. The Marimastat-CC (Figure 1) was used at a stock concentration of 20 μM in water (1 μM in the assay), free marimastat was used as competitor at a stock concentration of 10 mM (500 μM in the assay) and is also supplied with the caproKit™. The following components were used as supplied with the caproKit™: Streptavidin magnetic beads (SA-MB), 5x concentrated MMP capture buffer 3 (5x CB3; Note: CB3 differs from the composition of other CBs), and 5x concentrated wash buffer (5x WB1).

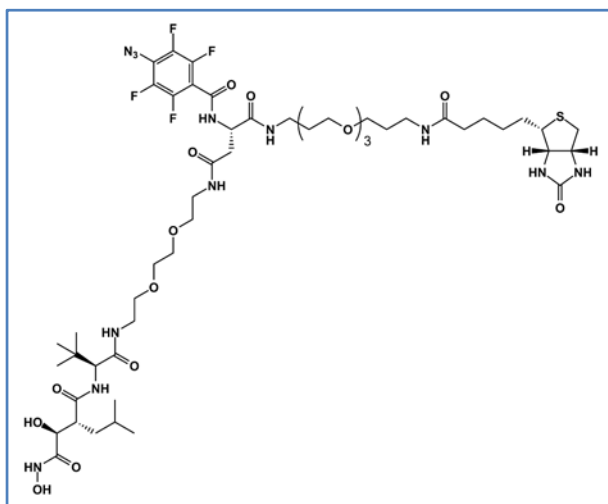


Figure 1. Structure of Marimastat Capture Compound™ (Marimastat-CC)

Assays were prepared in PCR tube strips for volumes up to 200 μl (Thermo Scientific, cat. No. AB-1114) as reaction vessels, also used to conduct the capture experiments, to wash and to isolate the magnetic beads. Irradiation of the samples for photo cross-linking was performed using the caproBox™, and the magnetic SA-MB were isolated using the caproMag™ containing a high-performance neodymium magnet. HEK293 lysate and HepG2 lysate at a stock concentration of 11 and 9 mg protein/ml, respectively, were used as the cellular protein samples. HEK293 and 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 cCOMPLETE™ inhibitor cocktail from Roche Applied Science, 1 tablet/50 ml buffer. Debris was removed by centrifugation at 30,000 xg for 60 min at 4 °C. The supernatant was filtrated through a 0.2 μm filter, and was then dialysed against lysis buffer for the removal of small molecules (optional). After dialysis, centrifugation at 4000 xg was carried out to remove protein precipitated during dialysis.

Capture experiment

The capture experiments were carried out using the in-solution (“OffBead”) protocol as described in the respective guideline that can be downloaded at <http://www.caprotec.com/support/downloads>. In brief, the assays were prepared in the order of pipetting water and 5x CB3 into PCR tubes and the assays were vortexed thoroughly. In the control reaction, marimastat was added in 500-fold excess as competitor to the Marimastat-CC (final concentration 500 μM). Cell lysate was then added to a final concentration of 5 mg/ml total protein and gently mixed by inversion of sealed tubes. ZnCl₂ was added in 20% molar excess with respect to the EDTA present in the sample due to the lysate buffer. (alternatively, do not to use EDTA in the lysate buffer, in which case the Zn²⁺ present in CB3 is sufficient). Purified MMPs were used in a final concentration of 4 μg/ml (0.4 μg per assay, pro-MMPs were activated by 1 mM APMA). For capture experiments analyzed by SDS-PAGE/silver stain, 0.8 μg pro-MMP-2, activated by APMA (1 mM), was used per assay. Finally, the Marimastat-CC was added (final concentration 1 μM) and the samples were gently mixed by inversion of sealed tubes. To allow complete binding of the marimastat moiety of the Marimastat-CC to proteins (non-covalent, reversible), the samples were incubated for 10 min at 4 °C. The tubes with open lids were then placed in the pre-cooled (2 - 4 °C) caproBox™. To form the covalent crosslink of the tetrafluoroarylazide reactivity function of the Marimastat-CC to the bound proteins, the lid of the caproBox™ was closed and the samples were irradiated for 4 min at 2 - 4 °C. 5x WB1 was added, and the samples were mixed by inversion in closed tubes. Then, SA-MB were added

to bind the Marimastat-CC–protein conjugates *via* the biotin moiety, and the beads were kept in suspension by rotation for 60 min at 4 °C. Marimastat-CC–protein conjugates on the SA-MB were then isolated and washed with WB1 and water using the caproMag™ as described in the cartoon for using the caproMag™ (18 MB) (please refer to protocol sections at: <http://www.caprotec.com/support/downloads>).

SDS-PAGE samples were prepared by heating the suspended SA-MB in SDS-sample buffer (10 µl, 10 min, 95°C) and the whole suspension was transferred into the gel pocket. Samples to be analyzed by MS were additionally washed with 80 % acetonitrile. The capture samples were then subjected to on-bead tryptic digestion. For this purpose, the SA-MB were washed twice with HPLC grade water after the final washing step and incubated with 10 µl per assay of 50 mM NH₄HCO₃, supplemented with 0.5 µg trypsin (promega sequencing grade). Tryptic digest was allowed to proceed over night at 37 °C under vigorous shaking. Beads were then fixed at the tube walls with the help of the magnet of the caproMag™, and the supernatants with the peptides recovered, lyophilized and subjected to LC-MS/MS analysis.

Mass spectrometry

LC-MS/MS experiments were performed using a nanoflow HPLC system (Proxeon) coupled to an ESI-LTQ-Orbitrap system. From the LC column, peptides were eluted during an 80-min linear gradient from 5 % acetonitrile (ACN)/0.1 % formic acid (FA) to 40 % ACN/0.1 % FA followed by additional 2 min to 100 % ACN/0.1 % FA and remaining at 100 % for another 8 min 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 & Conclusion

As an initial experiment, the performance of the Marimastat caproKit™ was tested towards isolated MMP-2. Marimastat can bind only to activated matrix metalloproteinases. To mimic natural activation of pro-MMP-2, 4-aminophenylmercuric acetate (APMA) can be used to enable marimastat access to the active site of MMP-2 (7). We tested the conditions for the activation of MMP-2 by APMA and found that incubation of 0.8 µg pro-MMP-2 with 1 mM APMA (from a stock solution of 10-20 mM APMA in DMSO) at 37 °C for 1.5 hours leads to a complete activation of MMP-2, as deducible from the molecular weight shift from the 72 kDa protein band for the pro-enzyme to the 62 kDa protein band for the activated enzyme (Figure 2). Activated MMP-2, but not the proenzyme used in a control sample, is robustly and specifically captured by the Marimastat-CC under these conditions.

In order to investigate the capability of the Marimastat-CC to capture different MMPs, two different cocktails of recombinant or purified human MMPs were tested. Cocktail 1 contained MMPs in the proenzyme form, cocktail 2 contained catalytic domains of human MMPs. Having shown that successful capturing of MMP proenzymes by the Marimastat-CC requires activation of the proenzymes by APMA, capturing in cocktail 1 was performed after APMA treatment (1 mM, over night). In contrast, successful capturing of MMP catalytic domains in cocktail 2 should not require APMA treatment. This was indeed observed for cocktail 1 (Figure 3). With this sample, APMA-activated MMPs were captured. MMPs from cocktail 2 were readily captured without APMA activation (Figure 4). In both cases, the capturing of the MMPs by the Marimastat-CC was robustly inhibited in the samples with marimastat competitor.

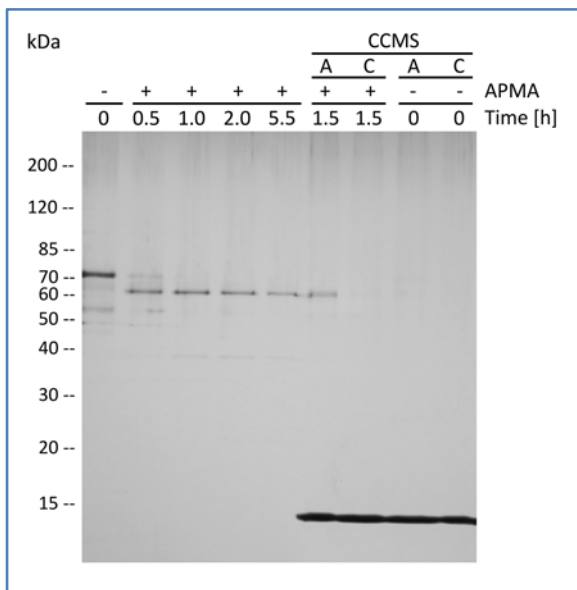


Figure 2. SDS-PAGE/silver stain analysis of pro-MMP-2 activation by APMA and successive capture experiments. Lanes 1-5: Time course of activation of pro-MMP-2 (72 kDa) by APMA treatment resulting in the activated MMP-2 form with lower MW (62 kDa). Analyzed samples contained 0.08 µg protein and were drawn from a 0.2 mg/ml pro-MMP-2 solution treated with 1 mM APMA at 37°C. 1.5 h treatment was considered sufficient for further experiments.

Lanes 6-9: Capturing activated MMP-2 (lane 6 and 7; A, assay; C, competition control) and attempt to capture pro-MMP-2 (lane 8 and 9) using the Marimastat-CC. APMA-activation is necessary (compare lanes 6 and 8); capturing is fully competed by an excess of marimastat used as competitor to the Marimastat-CC in the control (compare lane 6 and 7). The band below 15 kDa in lanes 6-9 originates from streptavidin monomer cleaved from the streptavidin coated magnetic beads used to isolate the MMP-2–Marimastat-CC crosslink.

Finally, the Marimastat caproKit™ was applied to the analysis of complex cell lysates. While activated MMP-2 spiked into HEK293 cell lysate can be captured, the proenzyme is not captured. However, when the proenzyme is spiked into the lysate and APMA added then, activation fails, presumably due to the nonspecific interaction of APMA with any sulfhydryl group in the lysate (data not shown). This makes the Marimastat caproKit™ most suitable for the investigation of endogenously activated MMPs. From the lysates of HEK293 cells as well as from HepG2 cells, the most obvious specifically captured protein was the metalloprotease dipeptidylpeptidase 3 (DPP3). DPP3 was robustly and specifically captured by the Marimastat-CC (Table 1), regardless of the presence of APMA. This

is in agreement with reports by others using an activity-based compound (8).

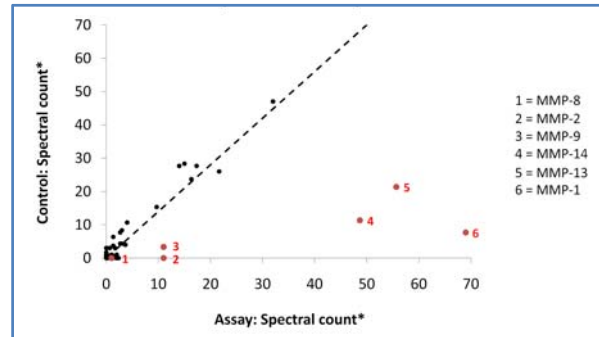


Figure 3. CCMS analysis of MMP cocktail 1. A wide range of MMPs are captured after APMA-activation of proenzymes in cocktail 1. Red dots represent MMPs, black dots represent additional non-MMP proteins present in the preparation. Three technical replicates were performed. Deviation of the proteins from the approx. diagonal (dotted line) towards the x-axis indicates successful competition of the capture reaction by an excess of marimastat used as competitor to the Marimastat-CC.

* Total number of peptide spectra acquired for a given protein.

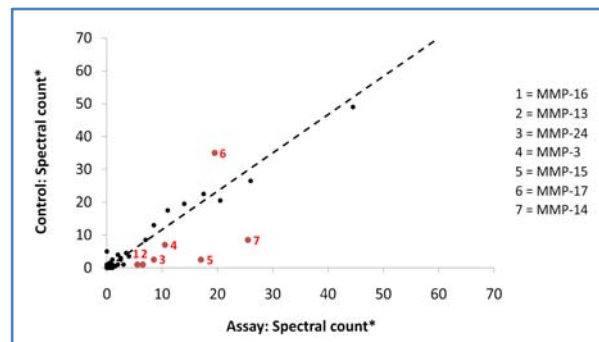


Figure 4. CCMS analysis of MMP cocktail 2. MMP catalytic domains are captured from cocktail 2. Red dots represent MMPs, black dots represent additional non-MMP proteins present in the preparation. Two technical replicates were performed. Deviation of the proteins from the approx. diagonal (dotted line) towards the x-axis indicates successful competition of the capture reaction by an excess of marimastat used as competitor to the Marimastat-CC.

* Total number of peptide spectra acquired for a given protein.

Summarized, these results demonstrate that the Marimastat caproKit™ is a powerful tool to investigate metalloproteinases.

Table 1. The metalloproteinase dipeptidyl peptidase 3 (DPP3, Uniprot Q9NY33) is specifically captured from HEK293 cell lysate or from HepG2 cell lysate. Numbers of unique peptides identified are given. A, assay; C, competition control.

	-APMA		+APMA	
	A	C	A	C
HEK293	12	0	14	0
HepG2	9	0	7	0

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Downloads:

www.caprotec.com/support/downloads

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Ordering information:

Item Nr.	Description
1-1060-050	Marimastat caproKit™ 50 rxn
1-1060-010	Marimastat caproKit™ 10 rxn

The caproKit includes the Marimastat Capture Compound, all buffers, positive control protein (pro-MMP-2), marimastat competitor, and streptavidin magnetic beads. Mind that APMA required for activation of pro-MMP-2 has to be purchased from a different supplier.

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