



# 3rd International Electronic Conference on Medicinal Chemistry

1-30 November 2017

chaired by Dr. Jean Jacques Vanden Eynde

sponsored by



pharmaceuticals

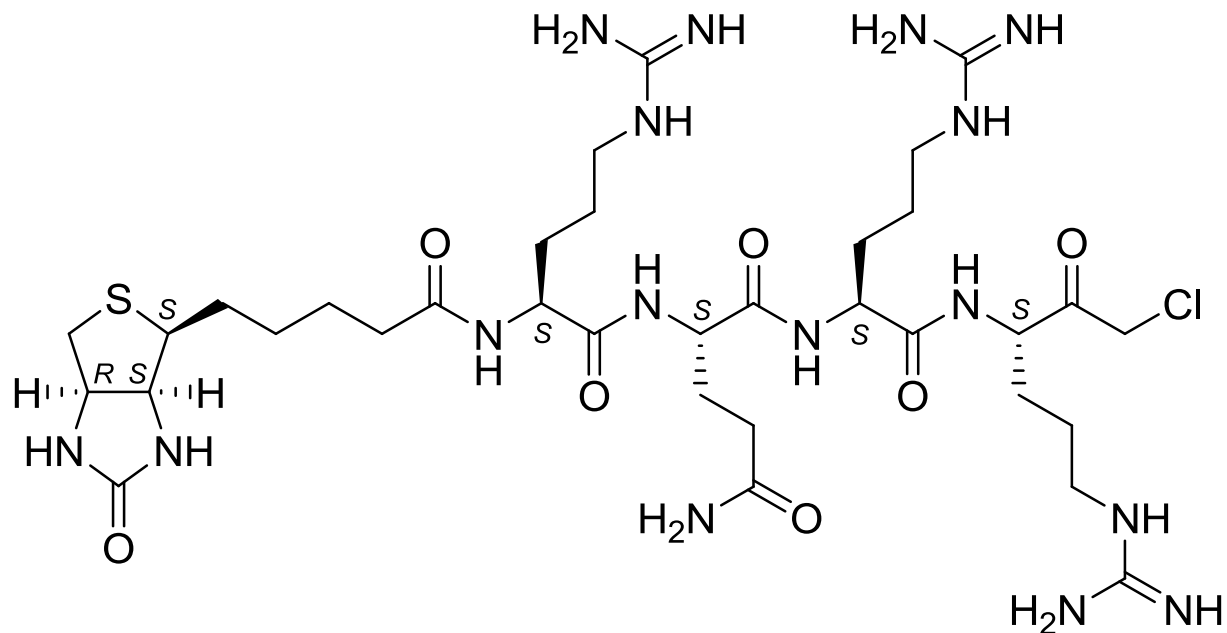
## A Short Peptide Inhibitor as an Activity-Based Probe for Matriptase-2

Martin Mangold <sup>1,\*</sup>, Michael Gütschow <sup>1</sup>, and Marit Stirnberg <sup>1</sup>

<sup>1</sup> Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany.

\* martinmangold@uni-bonn.de

# A Short Peptide Inhibitor as an Activity-Based Probe for Matriptase-2



## **Abstract:**

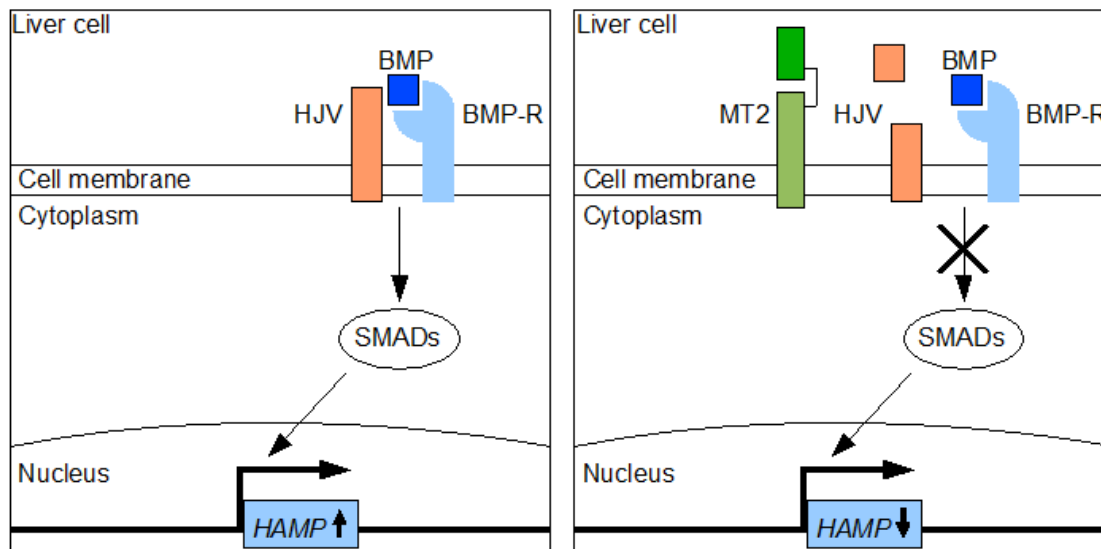
Matriptase-2 is a type II transmembrane serine protease and the key regulator of systemic iron homeostasis. After its synthesis, matriptase-2 is present on the cell surface as an inactive zymogen which is activated by processing. Two processing steps lead to the release of a 55 kDa enzymatically active fragment of the protein into the cell culture supernatant. Since the underlying mechanism of these processing steps is not yet fully understood there is strong need for analytical tools that help to distinguish active and inactive matriptase-2. For this purpose, we present a short biotinylated peptide probe with a chloromethyl ketone group as an inhibitor and activity-based probe for matriptase-2. This probe was characterized in kinetic experiments and applied for in-gel detection of matriptase-2.

**Keywords: matriptase-2; activity-based probe; chloromethyl ketone**



# Introduction

Owing to its influence on the peptide hormone hepcidin, matriptase-2 (MT2) is the key regulator of systemic iron homeostasis [1]. MT2 reduces hepcidin transcription (corresponding gene name: *HAMP*) by the proteolysis of the membrane protein hemojuvelin (HJV), the co-receptor of the BMP receptor. Low MT2 levels lead to upregulated hepcidin expression and reduced plasma iron levels. High amounts of active MT2 repress the hepcidin expression resulting in an increase in plasma iron levels.

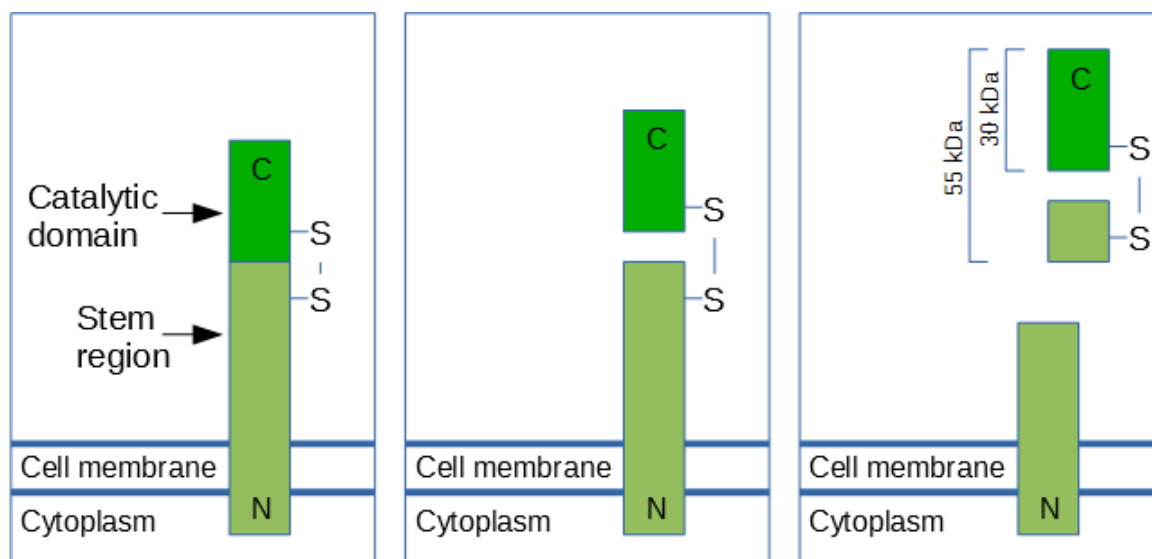


[1] Wang, C.; Meynard, D.; Lin, H. *Front. Pharmacol.* **2014**, *5*, 114.



# Introduction

MT2 is synthesized and transported to the cell surface as an inactive zymogen which is activated by processing. Processing takes place in two steps by autocatalytic cleavages in the catalytic domain and in the stem region of the enzyme [2]. An 55 kDa enzymatically active fragment of MT2 is released into the supernatant.



[2] Stirnberg, M.; Maurer, E.; Horstmeyer, A.; et al. *Biochem. J.* **2010**, *430*, 87-95.



# Introduction

As a biochemical tool compound and to distinguish the active and inactive protease, an activity-based probe could be of vital importance for the MT2-related research. This is even more the case as specific antibodies for MT2 are lacking. Since MT2 prefers substrates with arginine at P1, P2 or P3 and P4 positions [3-5]. this knowledge can be used to design small peptide inhibitors and peptidomimetic activity-based probes. Here we present a short biotinylated peptide probe with a chloromethyl ketone (CMK) warhead as an irreversible inhibitor and activity-based probe for MT2. This probe was originally developed for the related enzyme matriptase, [6] which exhibits a similar substrate specificity [7].

[3] Wysocka, M.; Gruba, N.; Miecznikowska, A.; et al. *Biochimie* **2014**, *19*, 1052-1061.

[4] Béliveau, F.; Désilets, A.; Leduc, R. *FEBS J.* **2009**, *276*, 2213-2226.

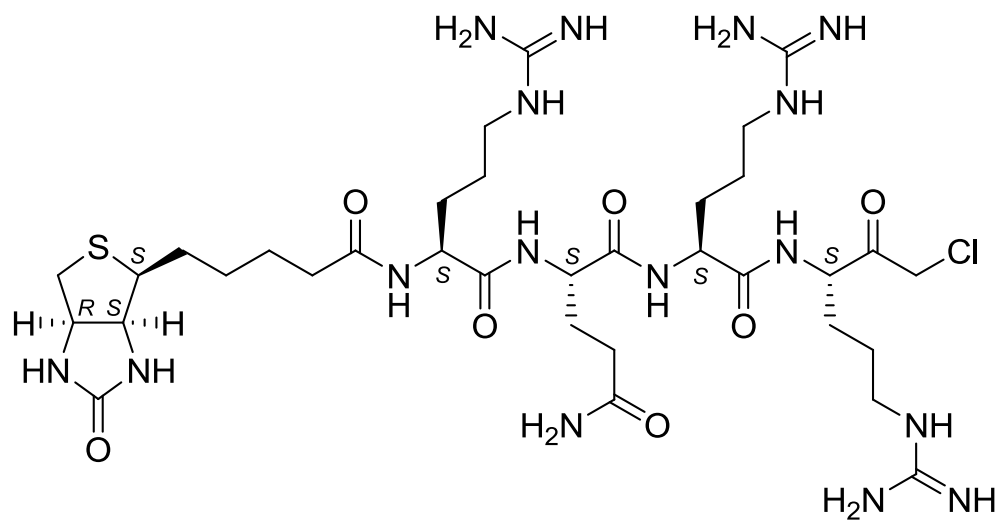
[5] Stirnberg, M.; Gütschow, M. *Curr. Pharm. Des.* **2013**, *19*, 1052-1061.

[6] Godiksen, S.; Soendergaard, C.; Friis, S.; et al. *PLoS ONE* **2013**, *8*, e77146.

[7] Häußler, D.; Schulz-Fincke, AC.; Beckmann, AM.; et al. *Chem. Eur. J.* **2017**, *23*, 5205-5209.



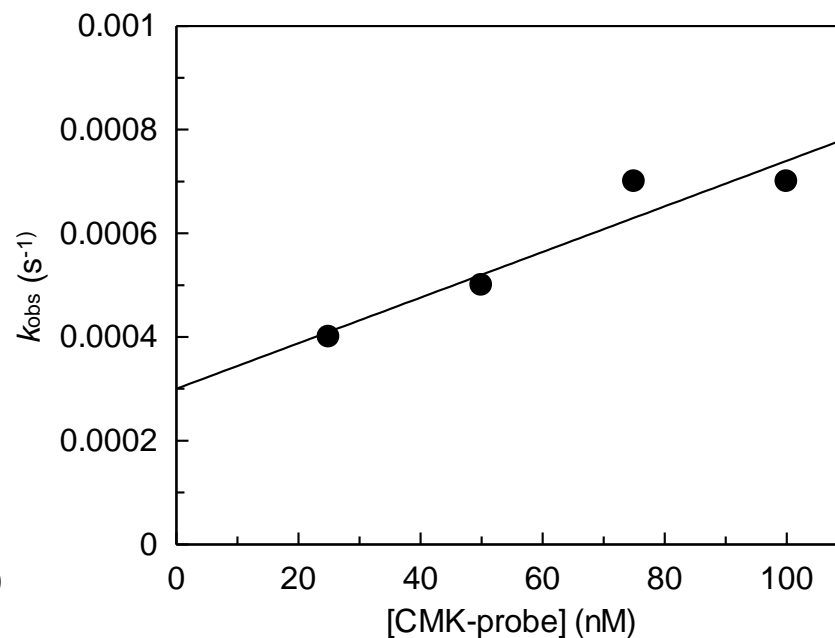
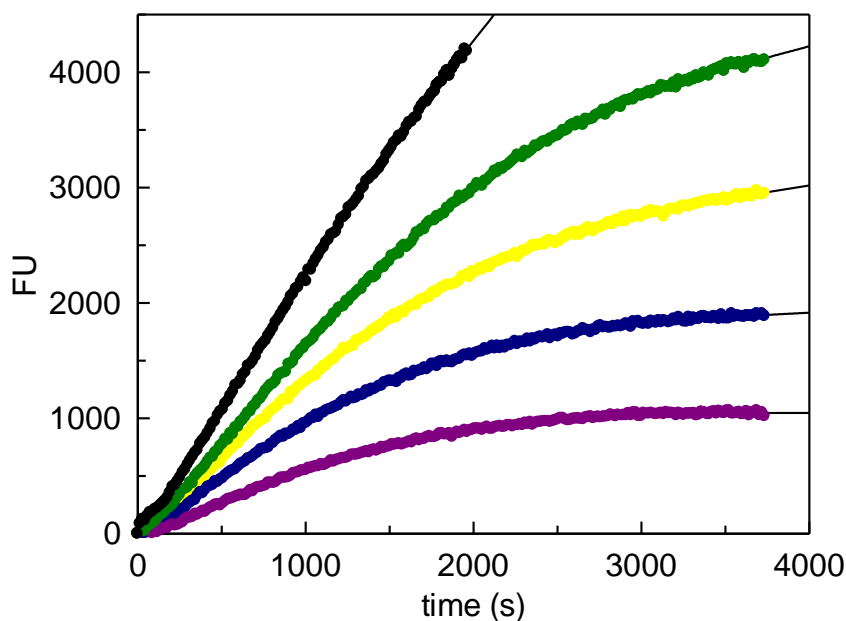
# Introduction



Chemical structure of the CMK-probe.



## Results and discussion

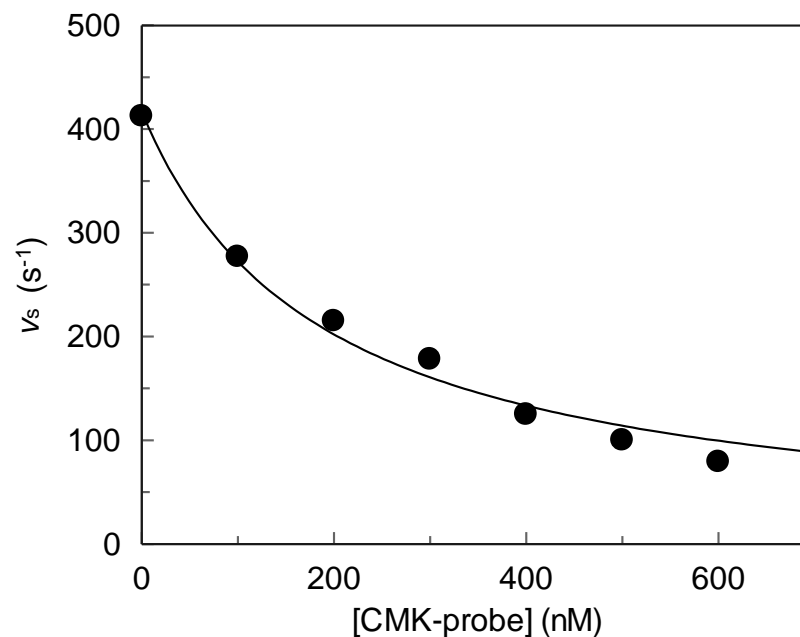
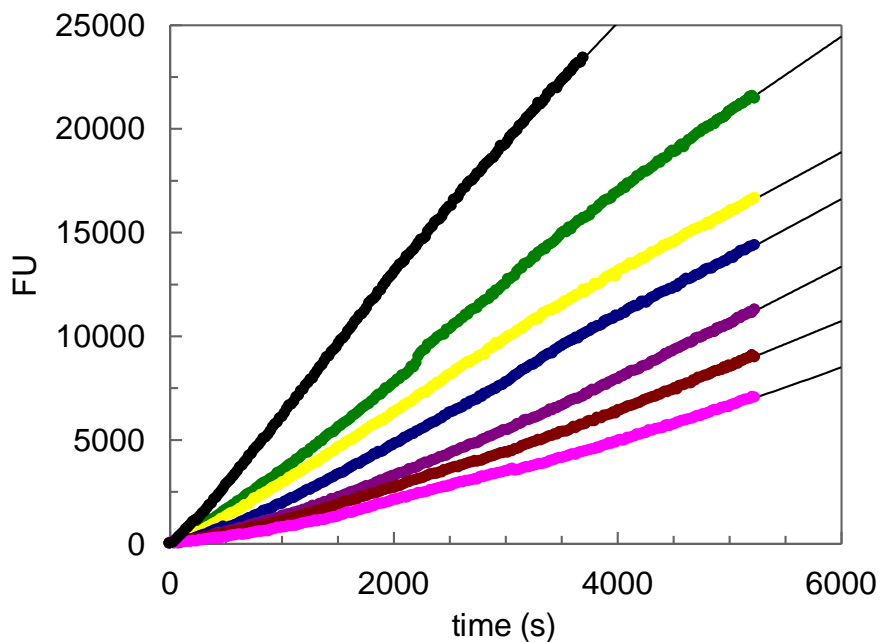


To assess the inhibitory activity, we determined MT2 activity in the supernatant of transfected HEK cells in the presence of increasing concentrations of the CMK probe (● uninhibited reaction; ● 25 nM; ● 50 nM; ● 75 nM; ● 100 nM). A concentration dependent decrease in MT2 activity was observed. The progress curves reflected an irreversible binding behavior with a  $k_{inac}/K_i$  value of  $10800 M^{-1} s^{-1}$ .





## Results and discussion



We also investigated the inhibitory activity of the CMK probe on the surface of transfected HEK cells expressing MT2. Overall, higher probe concentration were needed to inhibit MT2 (● uninhibited reaction; ● 100 nM; ● 200 nM; ● 300 nM; ● 400 nM; ● 500 nM; ● 600 nM) and no irreversible binding behavior was observed. This could be due to further unknown interaction partners at the cell surface and the ability of cells to produce activated MT2 over the time course of the experiment. A  $K_i$  value of 89 nM was obtained.



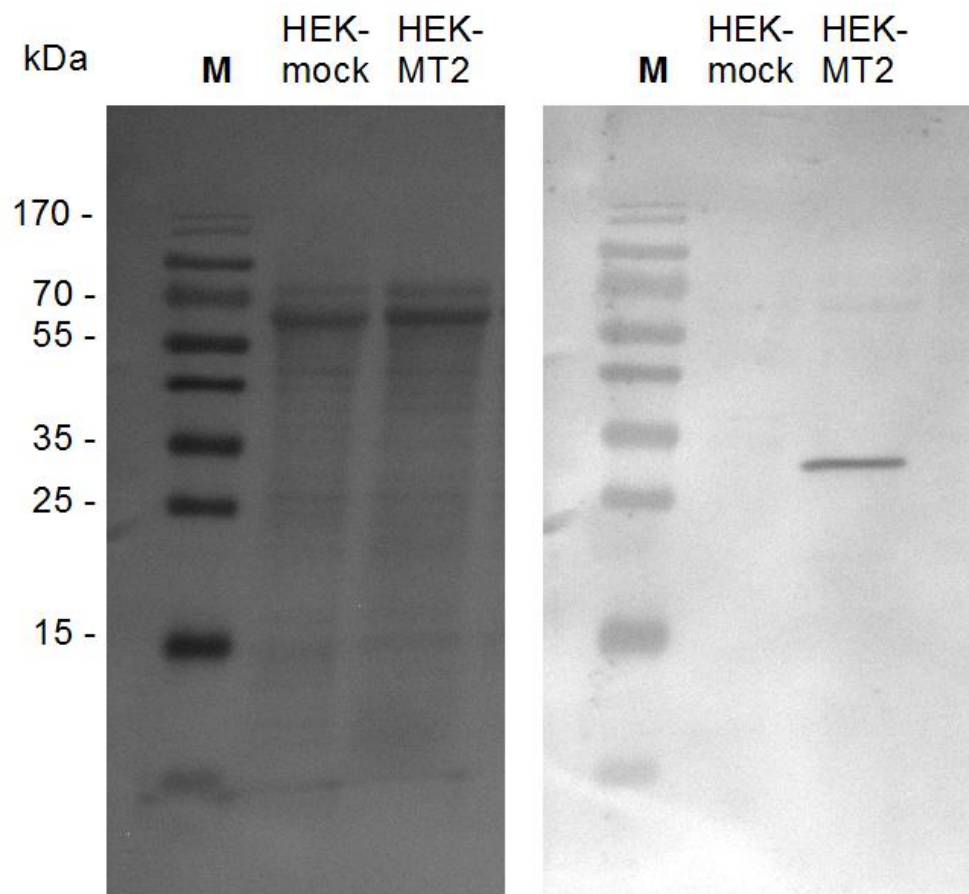
## Experimental procedures

HEK MT2 cells were cultivated in DMEM medium until a confluence of approximately 70% was reached. Afterwards cells were incubated in OptiMEM medium for two days at 37 °C. The medium, representing the supernatant, was removed and centrifuged at 2000 g for 10 min, while the cells were washed two times with PBS buffer. The experiments were started by adding assay buffer (NaCl 150 mM, TRIS 50 mM, pH 8.0), different amounts of the CMK probe in DMSO, or just DMSO (final DMSO concentration of 1%), and the substrate Boc-Gln-Ala-Arg-AMC ( $K_m = 32.2 \mu\text{M}$ ) in a concentration of 40  $\mu\text{M}$  to the supernatant or to the intact HEK cells. Values  $k_{\text{obs}}$  were obtained from non-linear regression of the progress curves of the supernatant experiment using the equation  $[P] = v_0 (1 - e^{-k_{\text{obs}} t})/k_{\text{obs}} + d$ , where  $[P]$  is the product concentration,  $v_0$  is the initial rate and  $d$  is the offset.  $k_{\text{inac}}/K_i$  values were obtained using the equation  $k_{\text{inac}}/K_i = (k_{\text{obs}}/[I]) (1 + ([S]/K_m))$ , where  $k_{\text{inac}}$  is the first-order inactivation rate constant,  $K_i$  is the inhibition constant,  $[S]$  is the substrate concentration and  $K_m$  is the Michaelis constant. The  $K_i$  value for the cell surface experiments was obtained from the linear regression of the progress curves and by using the equation  $K_i = [I]/(((v_0/v_i) - 1)(1 + ([S]/K_m)))$ , where  $v_i$  is the rate in the presence of the inhibitor.



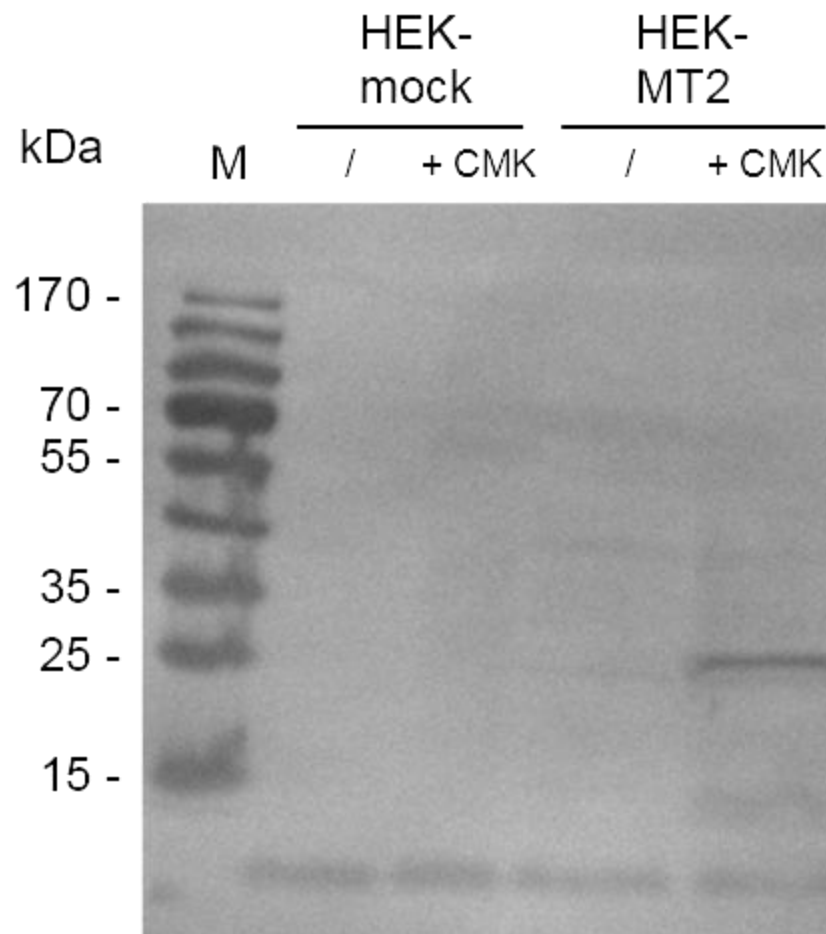
## Results and discussion

MT2 was visualized in Western blot experiments in the culture supernatant of HEK cells by using a antibody against the myc tag of the transfected protein. A Ponceau S staining of the blot is included as reference of total protein amounts (left panel). MT2 was detected as a 30 kDa protein after running the SDS gel under reducing conditions and applying the anti-myc antibody (right panel). This band represents the catalytic domain of the enzyme. The corresponding band was lacking in the culture supernatant of HEK mock cells expressing an empty vector.



## Results and discussion

Similar to antibody staining we employed the biotin tag of the CMK probe for the detection of MT2 after Western blotting. For this purpose, 30  $\mu\text{g}$  of HEK mock or HEK MT2 supernatants were incubated with the CMK probe in a concentration of 50  $\mu\text{M}$ . After the transfer, the blot was incubated with Strep-Tactin AP conjugate. MT2 was visualized as a 30 kDa protein band. This band was only observed in the supernatant of HEK cells expressing active MT2.



## Experimental procedures

For Western blot analysis, 30  $\mu\text{g}$  of HEK MT2 or HEK mock supernatant adjusted to a volume of 28.5  $\mu\text{l}$  were incubated with the CMK probe in a concentration of 50  $\mu\text{M}$  (final DMSO concentration of 5%) for 1 hour at 37  $^{\circ}\text{C}$ . After the addition of reducing sample buffer, the protein mixture was separated by SDS PAGE and blotted to a nitrocellulose membrane. The membrane was incubated in a blocking solution of 3% BSA in PBS-Tween for 1 hour and washed two times with PBS-Tween, followed by an incubation step in 3% BSA PBS-Tween with Strep-Tactin AP conjugate (dilution of 1:5000) overnight at 4  $^{\circ}\text{C}$ . The blot was washed again, one time with PBS-Tween and two times with PBS. To visualize the Strep-AP-CMK complex, the membrane was incubated in 20 ml reaction buffer ( $\text{MgCl}_2$  5 mM, NaCl 100 mM, TRIS 100 mM, pH 8.8) containing 7.5% 4-nitro blue tetrazolium chloride and 20% 5-bromo-4-chloro-3-indolyl phosphate.



## Conclusions

In conclusion we were able to identify and characterize a CMK peptide as an activity-based probe for MT2. Inhibition of MT2 activity in the supernatant as well as on the surface of transfected HEK cells with this CMK probe was demonstrated. A second-order rate constant of inhibition of  $10800 \text{ M}^{-1} \text{ s}^{-1}$  and an inhibition constant in the nanomolar range, respectively, were determined. The protein itself could be detected in Western blot experiments, using the biotin tag of the CMK probe. Further experiments to apply the probe for the visualization of MT2 by confocal microscopy are intended.



# Acknowledgment

Financial support by the Maria von Linden program of the University of Bonn, the IPID4all program of the Bonn International Graduate Schools (BIGS) and the German Research Foundation (DFG) is acknowledged.

