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Protease Probes

A Fluorescent-Labeled Phosphono Bisbenzguanidine As an Activity-Based Probe for Matriptase

Daniela Häußler,[a] Anna-Christina Schulz-Fincke,[a] Anna-Madeleine Beckmann,[a] Aline Keils,[b] Erik Gilberg,[a, c] Martin Mangold,[a] Jürgen Bajorath,[c] Marit Stirnberg,[a] Torsten Steinmetzer,[b] and Michael Gütschow*[a]

Abstract: Activity-based probes are compounds that exclusively form covalent bonds with active enzymes. They can be utilized to profile enzyme activities in vivo, to identify target enzymes and to characterize their function. The design of a new activity-based probe for matriptase, a member of the type II transmembrane serine proteases, is based on linker-connected bis-benzguanidines. An amino acid, introduced as linker, bears the coumarin fluorophore. Moreover, an incorporated phosphonate allows for a covalent interaction with the active-site serine. The resulting irreversible mode of action was demonstrated, leading to enzyme inactivation and, simultaneously, to a fluorescence labeling of matriptase. The ten-step synthetic approach to a coumarin-labeled bis-benzguanidine and its evaluation as activity-based probe for matriptase based on in-gel fluorescence and fluorescence HPLC is reported. HPLC fluorescence detection as a new application for activity-based probes for proteases is demonstrated herein for the first time.

Matriptase, the eponymous enzyme of the matriptase subfamily, is a member of the type II transmembrane serine proteases (TTSPs), a family of mammalian cell surface-associated serine proteases with a unique modular structure. These membrane-anchored proteases are structurally defined by a cytoplasmic N-terminal tail, a transmembrane domain, a stem region that contains various functional domains, and a C-terminal extracellular serine protease domain, characterized by the catalytic triad, serine, histidine and aspartate, essential for proteolytic activity.[1] Matriptase, one of the best characterized TTSPs, is mainly expressed in epithelia, such as epidermis or thymic stoma.[2–4] The proteolytic activity of matriptase is regulated by the Kunitz type hepatocyte growth factor activator inhibitors HAI-1 and HAI-2.[5,6] Matriptase is expressed as inactive zymogen precursor and has to be converted in an autocatalytic manner into its active form.[7]

Deregulation of matriptase is related to a variety of epithelial cancers and enhanced metastasis; increased matriptase activities often correlate with a poor disease outcome. Matriptase processes and activates several substrates, which themselves play critical roles in tumorigenesis, such as hepatocyte growth factor/scatter factor, urokinase-type plasminogen activator and protease activated receptor 2.[8–10]

Furthermore, matriptase, as an inducer and activator of procollagenases, was found to be a key initiator of cartilage destruction in osteoarthritis. It is able to activate selective promatrix metalloproteinases and to induce collagenase expression.[11] Several intestinal diseases are linked to a reduced matriptase activity and to an impaired intestinal barrier function.[12,13] Recent findings propose that matriptase activates hemagglutinin of certain H9N2 and H1N1 influenza A viruses and promotes viral replication. Hence, inhibition of matriptase significantly blocked influenza virus replication.[14–16]

Activity-based probes (ABPs) have emerged as a powerful tool in protein identification and profiling. The probes' ability to selectively visualize only the active forms of proteases is advantageous because expression levels often do not correlate with their enzymatic activity, for example, due to post-translational regulation.[17,18] Typically, the probes consist of three essential components, that is, a reactive group for covalent interaction, a recognition element that controls the probe's selectivity and a detectable agent. Several radioactive, fluorescent and biotin labels are established reporter tags for ABPs.[17,18] While fluorescent ABPs for matriptase have not been reported so far, a biotinylated chloromethyl ketone peptide has been used for the detection of active matriptase.[19] Moreover, biotin-labeled ABPs for trypsin-like enzymes with a phosphonate warhead and one benzamidine moiety have been developed.[20]

In continuation of a previous study,[24] our design of an ABP for matriptase is based on two linker-connected benzguanidines, a known substructure of matriptase inhibitors.[21–23] One benzguanidine moiety is very likely to be oriented towards the

[a] Dr. D. Häußler, A.-C. Schulz-Fincke, Dr. A.-M. Beckmann, E. Gilberg, M. Mangold, Dr. M. Stirnberg, Prof. Dr. M. Gütschow
Pharmaceutical Institute, Pharmaceutical Chemistry I
University of Bonn, An der Immenburg 4
53121 Bonn (Germany)
E-mail: guetschow@uni-bonn.de

[b] A. Keils, Prof. Dr. T. Steinmetzer
Institute of Pharmaceutical Chemistry
Philipps University of Marburg, Marbacher Weg 6
35032 Marburg (Germany)

[c] E. Gilberg, Prof. Dr. J. Bajorath
Department of Life Science Informatics
B-IT, LIMES Program Unit Chemical Biology and Medicinal Chemistry
University of Bonn, Dahlmannstr. 2, 53113 Bonn (Germany)

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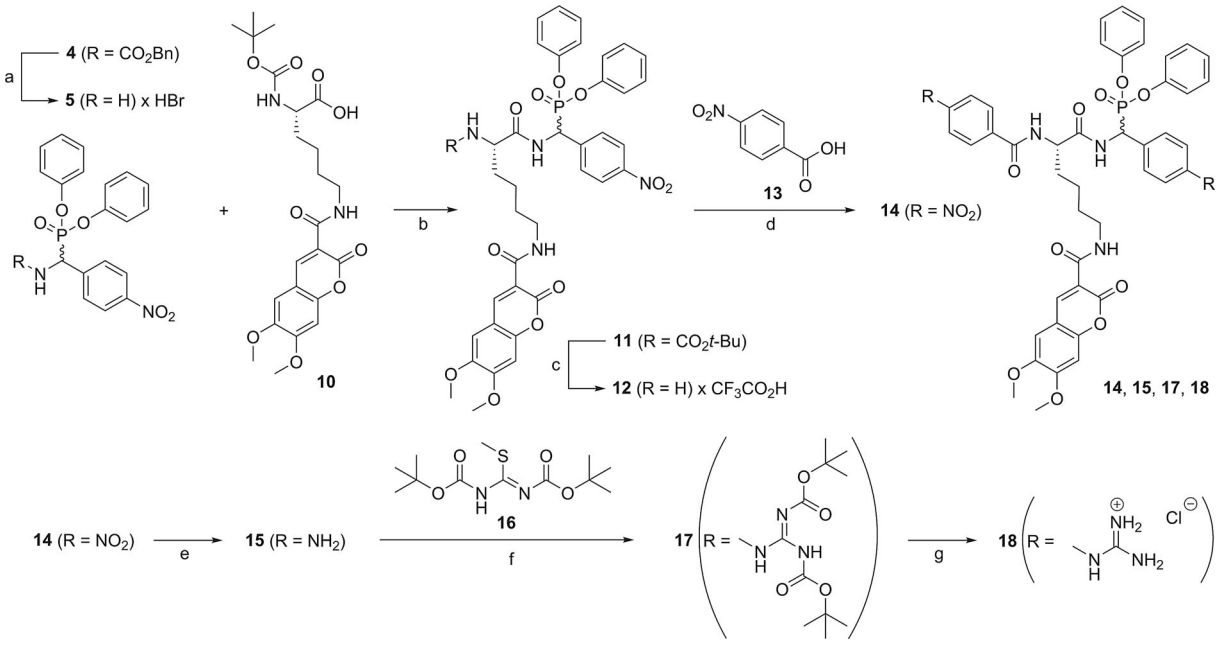
deep, negatively charged S1 pocket.[25,26] However, the remaining benzguanidine is either able to address the S3/S4 or the S2 pocket of matriptase.[4,27–29] The assembly of our probe was inspired by several potent dibasic matriptase inhibitors, such as peptidic ketobenzothiazoles derived from the natural Arg-Gln-Ala-Arg autoactivation sequence of matriptase,[27,28] bis-benzamidines,[4,29] or sulfonylated 3-amidinophenylalanine derivatives.[30] To achieve covalent interaction, a phosphonate group was attached. The α-aminoalkylphosphonates represent phosphonic analogues of naturally occurring amino acids which can be further extended by a suitable peptide moiety to improve target selectivity. Such peptidic phosphonates are well established irreversible inhibitors of serine proteases.[31–39] The attachment of a coumarin fluorophore was accomplished by the introduction of the trifunctional amino acid lysine. Coumarins represent a widely used class of fluorescent dyes, distinguished by their small molecular size, low bleaching and large Stokes shifts.[40–44]

Scheme 1 outlines the convergent synthetic approach to the ABP **18**, similar to the one that has previously been established for other benzguanidino phosphonates.[24] Details are given in the Supporting Information. The racemic Cbz-protected α-aminophosphonate **4** was prepared by a three-component reaction, comprising triphenyl phosphite (**1**), benzyl carbamate (**2**) and 4-nitrobenzaldehyde (**3**).[32–34] Deprotection of the aminophosphonate **4** was accomplished with a solution of HBr in acetic acid.[36–38] The bromide salt **5** was used in the next step without further purification. To produce the coumarin-labeled amino acid **10**, a Knoevenagel reaction was performed with 2-hydroxy-4,5-dimethoxybenzaldehyde (**6**) and Meldrum's acid (**7**),[41] and the resulting 6,7-dimethoxy-coumarin-3-carboxylic acid (**8**) was reacted with N_α-Boc-protected lysine (**9**).[44] Compound **10** was then coupled with the before produced aminophosphonate **5**.[36,38] This led to **11**, a dipeptide intermediate containing a coumarin tag and the phosphonate group. The ammonium salt **12**, obtained by deprotection of **11**, was converted to the free amine and reacted with 4-nitrobenzoic acid (**13**) in order to incorporate a second nitro moiety. A HATU-promoted coupling yielded the bis-nitro compound **14**. The reduction of the nitro groups was carried out with $SnCl_2$.[33,39] Following a literature protocol,[33,37] the amino groups of **15** were converted to protected guanidine moieties in intermediate **17**. For this purpose, a $HgCl_2$-mediated reaction with *N*,*N*′-di-Boc-*S*-methylisothiourea (**16**) was applied. After the removal of the Boc-protecting groups with trifluoroacetic acid, the desired bis-benzguanidine **18** was received and purified by preparative HPLC. The hydrochloride salt was obtained by adding HCl to an aqueous solution of the guanidinium trifluoroacetate, followed by lyophilisation.

The ABP **18** was characterized for its spectroscopic properties in buffer and exhibited an absorption maximum of 369 nm, an emission maximum of 448 nm, and thus a Stokes shift of 79 nm (Figure S1 in the Supporting Information).

The inhibitory potency of the ABP **18** against matriptase was determined with the fluorogenic substrate Mes-D-Arg-Pro-Arg-AMC. The probe showed time-dependent inhibition (Figure 1), and non-linear regression of the progress curves gave pseudo-first order rate constants of irreversible inhibition, k_{obs}. These values were plotted versus the inhibitor concentrations (Figure 1, right inset) and non-linear regression gave k_{inac}/K_i. This value, which characterizes the ability of a covalent irreversible inhibitor to interact with a target was 576 $M^{-1}s^{-1}$. ABP **18** was also evaluated at related serine proteases. At a concentration of 30 μM, it did not inhibit human thrombin and bovine



Scheme 1. Synthesis of compound **18**. Reaction conditions: a) AcOH/HBr, RT; b) HATU, DIPEA, DMF, RT, 38% yield from **4** to **11**; c) TFA, CH_2Cl_2, RT; d) HATU, DIPEA, DMF, RT, 74% yield from **11** to **14**; e) $SnCl_2{\cdot}2H_2O$, H_2O, EtOAc, reflux, 15% yield; f) $HgCl_2$, NEt_3, CH_2Cl_2, RT; g) TFA, CH_2Cl_2, RT, prep. HPLC, 21% yield from **15** to **18**. DIPEA = *N*,*N*-Diisopropylethylamine, TFA = trifluoroacetic acid.

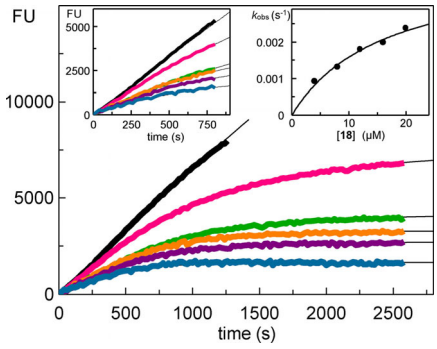


Figure 1. Inhibition of human matriptase by activity-based probe **18**. Black: uninhibited reaction; magenta: 4 μM; green: 8 μM; orange: 12 μM; purple: 16 μM; blue: 20 μM. Values k_{obs} were obtained from non-linear regression of the progress curves using the equation $[P] = v_i\,(1-e^{-kobs\cdot t})/k_{obs} + d$, where [P] is the product concentration, v_i is the initial rate and d is the offset. The left inset shows the first 800 s of the progress curves. The right inset shows a plot of k_{obs} values (mean of duplicate measurements) versus the inhibitor concentrations, [I]. For non-linear regression, the following equation was used $k_{obs} = k_{inac}\cdot[I]/(K_i\,(1+[S]/K_m)+[I])$, where k_{inac} is the first-order inactivation rate constant, K_i is the inhibition constant and [S] is the substrate concentration. A k_{inac}/K_i value of $576 \pm 151\ M^{-1}s^{-1}$ was obtained. The standard error refers to the non-linear regression of the k_{obs} versus [I] plot.

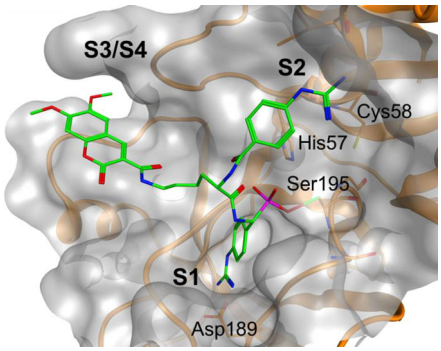


Figure 2. Modeled covalent complex of **18** and matriptase.

factor Xa, where more than 30% product formation was observed after 30 min. However, the closest relative of matriptase, that is, matriptase-2, was also inhibited, although to a lesser extent ($k_{inac}/K_i \pm SEM = 79.3 \pm 3.6\ M^{-1}s^{-1}$). An applicability of the probe to label matriptase should nevertheless be possible, because, in comparison to matriptase, the liver enzyme matriptase-2 has a limited tissue distribution.

The initial parts of the progress curves of the matriptase-catalyzed reaction in the presence of **18** were nearly linear (Figure 1, left inset), indicating a slow irreversible inhibition at the beginning of the measurement. We assume a rapid equilibrium between the free enzyme and two reversible enzyme-inhibitor complexes, from which one is non-productive with respect to the inactivation step. In such a complex, the "left" benzguanidine moiety might occupy the S1 pocket. However, when the "right" benzguanidine moiety binds to the S1 pocket, an inactivation would be facilitated because the phosphonate warhead would be directed to the active site serine residue.

In order to propose possible binding modes of probe **18**, molecular docking calculations were performed, using the crystal structure of a complex of matriptase with a bis-benzamidine inhibitor (PDB-ID: 4JZI).[4] The interaction of serine proteases with diphenyl phosphonate inhibitors includes the formation of a serine phosphono diester through the nucleophilic attack of the active site residue Ser195 at the phosphorus and the release of one phenoxy group, followed by a hydrolytic "aging" into a phosphono monoester.[45–47] Such a monoester complex with phosphorus in a tetrahedral configuration was generated by covalent docking as follows. Both phenoxy groups were removed from the inhibitor and the single bond between the oxygen of Ser195 and the phosphorus was manually built. In the presence of this covalent constraint, the inhibitor was flexibly docked and the Ser195 residue was also free to move. The calculations yielded a plausible binding mode as shown in Figure 2. In accordance with previous reports,[45–47] one of the (partially) negatively charged oxygen atoms of the phosphonate is proposed to form ionic interactions with the protonated ε^2-nitrogen of His57, whereas the other is orientated towards the oxyanion hole. The "right" benzguanidinium moiety, as an arginine mimetic, expectedly interacts with Asp189 in the deep S1 pocket by charge-assisted hydrogen bonding. Possible interactions in the S2 and S3/S4 pockets were modeled with lower confidence. In the putative binding mode depicted in Figure 2, the "left" benzguanidinium group occupies the upper S2 pocket forming putative hydrogen bonds with the carbonyl oxygen atoms of His57 and Cys58, and the coumarin moiety is oriented towards the S3/S4

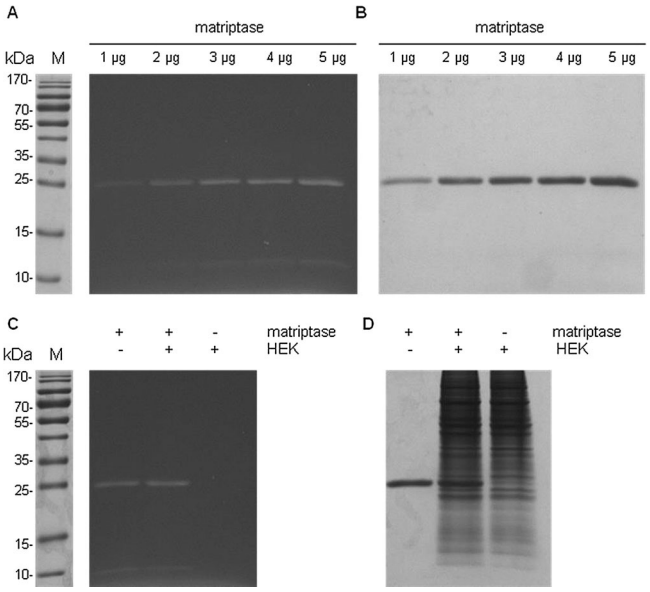


Figure 3. Imaging of human matriptase with the fluorescent probe **18**. Different amounts of matriptase (1–5 μg) were incubated with 50 μM of **18**. Labeling reactions were performed for 45 min at 37 °C. The proteins were separated by SDS–PAGE and visualized by (A) fluorescence detection and (B) Coomassie staining. Matriptase (3 μg), matriptase (3 μg) spiked with human embryonic kidney (HEK) lysate (25 μg), and HEK lysate (25 μg) alone were incubated for 45 min at 37 °C with 50 μM of **18**. The proteins were separated by SDS–PAGE and visualized by (C) fluorescence detection and (D) Coomassie staining. M, molecular mass marker.

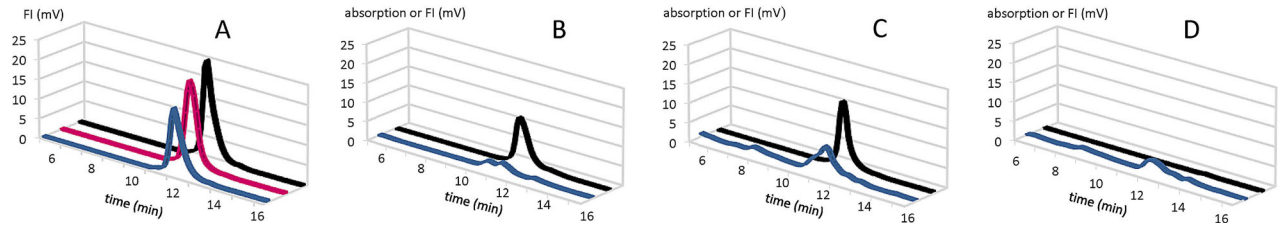


Figure 4. Size-exclusion chromatograms of human matriptase labeled with the fluorescent probe **18**. The fluorescence detector gain was adjusted to 10. (A) Matriptase (5 μg) was incubated for 15 min (blue), 30 min (magenta) or 45 min (black) at 37 °C with 50 μM of **18**. The protein was subjected to HPLC and visualized by fluorescence detection (λ_{ex} = 369 nm, λ_{em} = 448 nm). (B–D) Proteins were separated by size-exclusion chromatography and simultaneously visualized by UV (λ_{abs} = 280 nm) and fluorescence detection (λ_{ex} = 369 nm, λ_{em} = 448 nm). Blue: UV channel; black: fluorescence channel. (B) Matriptase (3 μg), (C) HEK lysate (25 μg) spiked with matriptase (3 μg), and (D) HEK lysate (25 μg) alone were incubated for 45 min at 37 °C with 50 μM of **18**.

region, but does not engage in well-defined ligand–protein interactions.

The feasibility of ABP **18** for direct in-gel fluorescence detection of matriptase was demonstrated. In addition, we have examined whether matriptase could be visualized after labeling with **18** and passage through an HPLC system equipped with a fluorescence detector. Different amounts of matriptase (1–5 μg) were treated with 50 μM of **18** for 45 min. After performing the SDS–PAGE (Figure 3A), a band at 26 kDa could be detected and its intensity correlated well with the increasing amount of matriptase. At the lowest protease amount of 1 μg, detection was still possible. The presence of matriptase was confirmed by Coomassie Brilliant Blue staining (Figure 3B).

The mixture of matriptase (5 μg) and **18** (50 μM) was subjected to size-exclusion chromatography after different incubation times of 15, 30, and 45 min and analyzed by means of fluorescence measurement. We obtained a clear single peak for the enzyme–probe complex. The signal increased depending on the incubation time, reflecting the successive increase of the complex concentration (Figure 4A). When lower amounts of matriptase (1 or 3 μg) were treated with **18** (50 μM), the observed intensities of the signals were less distinct after incubation times of 15, 30, and 45 min (Figure S2 in the Supporting Information). Even an amount of only 1 μg of matriptase led to a chromatogram with an excellent signal-to-noise ratio.

For illustration of the selectivity of ABP **18**, it was applied in the presence of other proteins, spiked with matriptase. Herein, HEK lysate (25 μg) was used as protein source. As controls, matriptase and HEK lysate were separately incubated with **18** for 45 min. The incubation mixtures were subjected to SDS–PAGE and visualized by fluorescence imaging (Figure 3C) and protein staining (Figure 3D). This analysis revealed selective labeling of the target matriptase within a mixture of excess proteins without detectable nonspecific interactions of **18**. No labeling was observed in the case of the HEK lysate in the absence of matriptase.

For the corresponding HPLC analysis, we applied the UV detection to show the mixture of proteins. The lysate, in the presence or absence of matriptase, did not produce separate protein peaks (Figure 4C or 4D). However, an intensive signal for the labeled matriptase was observed in the fluorescence channel (Figure 4B and 4C). The fluorescence chromatogram obtained with matriptase in the presence of a large excess of lysate proteins (Figure 4C) clearly demonstrated the suitability and selectivity of ABP **18**.

By applying the chemotype of phosphono bis-benzguanidines, the first fluorescent ABP of matriptase was successfully employed for direct in-gel fluorescence readout and HPLC size-exclusion chromatography coupled to fluorescence detection. The probe described herein is expected to serve as a valuable tool compound for future investigations of matriptase, an enzyme of strong therapeutic importance.

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COMMUNICATION

Protease Probes

*D. Häußler, A.-C. Schulz-Fincke, A.-M. Beckmann, A. Keils, E. Gilberg, M. Mangold, J. Bajorath, M. Stirnberg, T. Steinmetzer, M. Gütschow**

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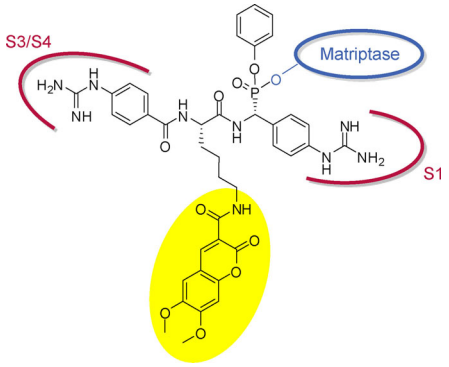
A Fluorescent-Labeled Phosphono Bisbenzguanidine As an Activity-Based Probe for Matriptase



An activity-based probe for matriptase, a therapeutically important transmembrane serine protease, was designed, synthesized and simultaneously evaluated by in-gel fluorescence analysis and HPLC fluorescence detection.