A BODIPY-Tagged Phosphono Peptide as Activity-Based Probe for Human Leukocyte Elastase

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S Supporting Information

ABSTRACT: Human leukocyte elastase plays a crucial role in a variety of inflammatory disorders and represents an important subject of biomedical studies. The chemotype of peptidic phosphonates was employed for the design of a new activity-based probe for human leukocyte elastase. Its structure combines the phosphonate warhead with an adequate peptide portion and a BODIPY fluorophore with a clickable ethynylphenyl moiety at *meso* position. The probe **6** was assembled by copper-catalyzed alkyne–azide 1,3-dipolar cycloaddition. It was characterized as an active site-directed elastase inhibitor exhibiting a second-order rate constant of inactivation of 88400 M^{−1}s^{−1}. The suitability of **6** as a fluorescent probe for human leukocyte elastase was demonstrated by in-gel fluorescence analysis. Labeling experiments and inhibition data toward a panel of related proteases underlined the selectivity of the probe for the targeted leukocyte elastase.

KEYWORDS: BODIPY derivatives, copper-catalyzed azide–alkyne cycloaddition, human leukocyte elastase, phosphonates, serine proteases

Human leukocyte elastase (HLE) is a serine protease stored as an active enzyme within the azurophilic granules of polymorphonuclear neutrophils. After the fusion of azurophilic granules with vacuoles carrying phagocytosed bacteria, HLE functions in intracellular host defense by degrading bacterial membrane proteins. Beside this primary role, HLE also acts extracellularly. Upon neutrophil activation by chemokines, chemoattractants, or bacterial lipopolysaccharides, HLE is released extracellularly. As a membrane bound or free protease, it can then break down matrix proteins, such as elastin, fibronectin, laminin, and collagen. Moreover, HLE activates other proteases or deactivates their endogenous inhibitors, releases cytokines from their precursors, or liberates growth factors.^{1–5}

The catalytic activity of HLE is controlled by endogenous serine protease inhibitors, such as α_1 -proteinase inhibitor, α_2 -macroglobulin, or secretory leukocyte peptidase inhibitor. However, an out-of-balance activity might produce deleterious effects and contributes to the onset and progression of inflammatory lung diseases. Thus, the upregulated HLE activity is involved in the pathophysiology of chronic obstructive pulmonary disease, acute lung injury, respiratory distress syndrome, and inflammatory bowel disease.^{1,2,6} HLE represents an enzyme of diagnostic potential,⁷ e.g., as a fecal marker of intestinal inflammation.⁸

HLE has a primary substrate specificity to cleave peptides with small aliphatic residues such as alanine or valine in the P1 position.^{1,2} The cleavage is initiated by the nucleophilic attack

of the active-site serine at the carbonyl carbon of the scissile bond and the subsequent hydrolysis of the resulting acyl enzyme.^{1,2,6} The only synthetic HLE inhibitor that has reached the market, sivelestat, interacts similarly in the course of an acyl transfer. Its ester bond is enzymatically cleaved by HLE, and a pivaloylated enzyme is generated.⁹

The crucial role of HLE in multiple forms of inflammatory disorders has provided the impetus for the development of fluorescent reporters,^{10,11} as well as synthetic inhibitors. The different chemotypes of HLE inhibitors include peptidic chloromethyl and trifluoromethyl ketones,¹² 4-oxo- β -lactams,¹³ kojic acid derivatives,¹⁴ saccharines,¹⁵ and isothiazolones.¹⁶

The α -aminoalkylphosphonates represent phosphonic analogues of naturally occurring amino acids. Combined with a tailored peptide portion, sufficiently stable and potentially selective serine protease inhibitors can be achieved.¹⁷ Due to the tetrahedral configuration of the phosphorus atom, α -aminophosphonates mimic the transition state of the enzymatic reaction. Two aryloxy groups on the phosphorus atom were introduced to maintain reactivity toward the active-site serine. The nucleophilic attack of the catalytic serine residue and the release of a phenoxy group forms a tetravalent derivative. This serine phosphono diester undergoes a slow “aging” process upon the loss of the second phenoxy group to form a stable

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Scheme 1. Synthesis of the Activity-Based Probe 6^a

^aReagents and conditions: (a) 50% TFA/CH₂Cl₂ (v/v), rt; (b) HBTU, DIPEA, MeCN, rt; (c) CuSO₄·5 H₂O, sodium ascorbate, H₂O, DMSO, rt.

Table 1. Inhibition of Proteases by Phosphono Peptides 1A, 1B, and 6

compd ^a	k_{inac}/K_i (M ⁻¹ s ⁻¹) ^b							
	HLE	PPE	bovine chymotrypsin	bovine factor Xa	human thrombin	bovine trypsin	human cathepsin B	human cathepsin L
1A (S,S,R)	399000 ± 55000	7990 ± 1180	NI ^c	NI	NI	NI	NI	NI
1B (S,S,S)	1000 ± 150	NI	NI	NI	NI	NI	NI	NI
6 (S,S,R)	88400 ± 10400	1440 ± 70	565 ± 52	NI	NI	NI	NI	NI

^aThe stereochemistry is given in parentheses. The first entry refers to the configuration at Val, the second at Pro, and the third at the aminophosphonate. ^bProgress curves were analyzed using the equation $[P] = v_i (1 - \exp(-k_{\text{obs}}t))/k_{\text{obs}} + d$, where $[P]$ is the product concentration, v_i the initial rate, k_{obs} the observed first-order rate constant, and d the offset. The k_{obs} values (means from duplicate measurements) were plotted against the inhibitor concentrations, $[I]$. Second-order rate constants of inactivation, k_{inac}/K_i , were determined according to $k_{\text{inac}}/K_i = k_{\text{obs}}/[I](1 + [S]/K_m) + d$, where $[S]$ is the substrate concentration, K_m the Michaelis–Menten constant, and d the offset. Standard errors (±SEM) refer to this linear regression. ^cNI: No inhibition relates to more than 90% product formation after 60 min at an inhibitor concentration of 1 μM in duplicate measurements.

serine phosphono monoester, leading to irreversible inhibition.¹⁸

Because of their irreversible mode of action, peptidic phosphonates provide an excellent structural basis for the design of activity-based probes for different serine proteases.^{19–25} Such probes contain a phosphonic analogue of an amino acid (AA^P) in P1 position. Phosphonate-based probes for elastase and the related neutrophil enzyme proteinase-3 have been reported with *rac*-Val^P carrying either biotin or cyanine 5 as label.^{26,27} An extended P3–P1 portion, Val–Pro–*rac*-Leu^P, was employed for a biotinylated probe targeting the neutrophil serine proteases cathepsin G and elastase.²⁸ Another biotin-labeled probe for elastase contains a tetrapeptide unit composed of unnatural amino acids with the phosphonate analogue of 4-aminobutyric acid in P1 position.²⁹

In this study, we have designed a new activity-based probe for neutrophil elastase with a defined configuration at the α-carbon of the aminophosphonate substructure. The tripeptidic P3–P1 sequence, Val–Pro–Val^P, was chosen on the basis of highly active elastase inactivators bearing two 4-methylthiophenoxy groups at the phosphonate warhead.³⁰ The structure of our probe was further assembled to contain a P4 glycine moiety whose nitrogen is incorporated into a triazole ring accessible via copper-catalyzed alkyne–azide 1,3-dipolar cyclo-

addition (click reaction). Click chemistry has already been successfully utilized for the development of activity-based probes for serine proteases.^{13,22} It was intended to equip our probe with a boron-dipyrromethene (BODIPY) label, which should be introduced through the aforementioned click reaction. BODIPYs have excellent features for biological applications, such as chemical robustness, photochemical stability, high quantum yields, and appropriate excitation and emission properties.^{31,32} The linear synthesis of our activity-based probe started with an Oleksyszyn reaction employing the three components tris(4-(methylthio)phenyl)phosphite, available from phosphorus trichloride and 4-(methylthio)phenol, isobutyraldehyde, and benzyl carbamate. The resulting *rac* aminoalkylphosphonate was deprotected with HBr in acetic acid and coupled in turn with Boc-D-Pro-OH and, after deprotection, with Boc-D-Val-OH. This led to a diastereomeric mixture, which was separated by column chromatography to obtain the epimers 1A (Scheme 1) and 1B, respectively.³⁰ The code A or B refers to the differences in ³¹P NMR spectra with A exhibiting the stronger upfield shifted signal (1A, 17.87 ppm; 1B, 18.25 ppm).

In order to elucidate the effect of the configuration on the biological activity, the tripeptidic phosphonates 1A and 1B were evaluated for their inhibitory potency against HLE and for

Figure 1. Detection of HLE after labeling with the fluorescent probe **6** and SDS-PAGE. (A, C, E) In-gel fluorescence detection. (B, D, F) Coomassie staining. (A, B) Increasing amounts of HLE were incubated with $2.5 \mu\text{M}$ **6**. (C, D) HEK cell lysate ($9 \mu\text{g}$, first lane) or HLE (600 ng , second lane) was incubated with $2.5 \mu\text{M}$ **6**. HEK cell lysate ($9 \mu\text{g}$) was added after (third lane) or before (fourth lane) incubation of HLE (600 ng) with $2.5 \mu\text{M}$ **6**. The horizontal line indicates the incubation period; the HEK cell lysate was added either before or after the incubation time. (E, F) HLE (600 ng) was incubated with or without $5 \mu\text{M}$ sivelestat (Siv) followed by incubation with $2.5 \mu\text{M}$ **6**. M, molecular mass marker.

their selectivity versus five serine proteases, i.e., porcine pancreatic elastase (PPE), chymotrypsin, factor Xa, thrombin, and trypsin, as well as two cysteine proteases, i.e., cathepsin B and L (Table 1). The protease activities were assayed photometrically or fluorometrically and the reactions were followed over 60 min.^{16,23,33}

Epimer **1A** showed a strong inhibition of HLE with a second-order rate constant of $399000 \text{ M}^{-1}\text{s}^{-1}$, a moderate effect against PPE, and inactivity against the other proteases (Table 1). Compound **1B** exhibited no inhibitory activity against the investigated proteases except HLE, which was inhibited with a weak second-order rate constant of $1000 \text{ M}^{-1}\text{s}^{-1}$ (Table 1). The obtained progress curves with HLE showed time-dependent inhibition (Figures S1 and S2, Supporting Information), which is in accordance with the expected irreversible mode of action. Analysis of the progress curves gave the aforementioned second-order rate constants, k_{inac}/K_i , as the main characteristic of irreversible inhibitors.

The (S,S,R)- and (S,S,S)-configuration was assigned to **1A** and **1B**, respectively, for the following reasons: (i) the full agreement of our data (Table 1) with those of a previous report by Winiarski et al.³⁰ with respect to the much stronger anti-elastase activity of the (S,S,R)-configured **1A** compared to the (S,S,S)-configured epimer **1B**, (ii) the assignment of the active phosphonate epimer as matriptase-2 and trypsin inhibitors

achieved by molecular docking,²³ (iii) literature data on aminophosphonates as serine protease inhibitors in which, in four of the five cases, the active, (R)-configured epimer had ^{31}P NMR resonances at higher field than the (S)-epimer,³⁰ (iv) an asymmetric synthesis of aminophosphonates with the (R)-enantiomers as superior inactivators of human cathepsin G, chymotrypsin, and HLE.³⁴ The assignment of the respective configuration at the CHPO chiral center for **1A** and **1B** can moreover be expected because the (R)-configured aminophosphonate substructures have the same molecular geometry as natural (S)-configured amino acids.

The efficient inactivation of HLE by the phosphono peptide **1A** provided the impetus for the design and synthesis of an activity-based probe of this chemotype. For this purpose, an N-terminal elongation of valine and the incorporation of a fluorescence tag through click chemistry was performed. The 1,2,3-triazole-containing linker structure may participate actively in hydrogen bond formation and dipole–dipole interactions within the active site cleft.^{13,35} The synthesis of the probe is outlined in Scheme 1 and started with the deprotection of compound **1A**. The azido component **3** was prepared from bromoacetic acid, and a HBTU-mediated coupling reaction with the ammonium salt **2** yielded compound **4**. For the synthesis of ethynyl-BODIPY **5**, 4-ethynylbenzaldehyde was reacted with 4-dimethylpyrrole, and the resulting

intermediate was further treated with boron trifluoride diethyl etherate in the presence of *p*-chloranil in a base-catalyzed complexation.³⁶ Compound **5** has been investigated in several physicochemical and synthetic studies and utilized for the generation of one DNA probe.^{37,38} To combine the fluorescent tag with the inhibitor substructure, a click-chemistry approach was performed through the reaction of the azido peptide **4** and ethynyl-BODIPY **5**, using a Cu^{II} salt and sodium ascorbate as reducing agent.³⁹ This yielded the desired activity-based probe **6** with a phosphonate warhead for an irreversible modification of HLE, a peptidic linker structure capable of interacting with the enzyme's binding pockets, a BODIPY as fluorescence reporter group, and the preferred (*R*)-configuration at the crucial amino phosphonate moiety. The NMR data of **6** (e.g., ³¹P NMR, δ = 17.85 ppm) resembled that of the aforementioned precursor **1A**.

The results of the kinetic evaluation of probe **6** are given in Table 1. Again, no inhibition was observed for factor Xa, thrombin, trypsin, and the two cathepsins. Chymotrypsin was inhibited to some extent. The inactivation of HLE and PPE confirmed the desired preference of the probe for elastases.

The progress curves of the substrate consumption of both elastase enzymes in the presence of different concentrations of **6** revealed that **6** maintained the irreversible mode of action, a prerequisite for an activity-based probe (see Figure S3 for HLE). Probe **6** was approximately 5-fold less potent than its precursor **1A** (Table 1). However, with the k_{inac}/K_i value of 88400 M⁻¹s⁻¹, our probe showed a still extraordinary potency against the target HLE. The strong selectivity of **1A** for HLE versus PPE was retained in the case of **6** (88400 M⁻¹s⁻¹ versus 1440 M⁻¹s⁻¹).

In contrast to compounds **1A**, **1B**, and **6**, sivelestat showed a kinetic "slow binding" behavior, with concentration-dependent steady-state rates for the HLE-catalyzed reaction (Figure S4). This is in agreement with the covalent-reversible mode of action of sivelestat.⁹ We obtained a K_i value of 37 nM (k_{on} = 30300 ± 3600 M⁻¹s⁻¹, k_{off} = 0.00113 ± 0.00021 s⁻¹). Next, we investigated the reactivation of HLE inhibited by sivelestat, **1A**, and **6**, respectively. Unlike in the case of sivelestat, the enzymatic activity was not regained when a large excess of substrate was added after incubation of HLE with the phosphonates **1A** and **6** (Figure S5). This finding confirmed the irreversible character of the interaction of probe **6** with HLE.

The spectroscopic properties of **6** were determined in methanol, dichloromethane, and water as solvents (Figure S6). The fluorescence spectra showed excitation maxima at 495–509 nm and emission maxima at 503–513 nm, typical for BODIPY derivatives. Structure **6** features four methyl groups at pyrrolic positions and a biaryl moiety as *meso* substituent in an expected orthogonal configuration relative to the BODIPY core, giving rise to a bathochromic shift and a more efficient fluorescence.^{31,40}

The characteristics of our compound **6** encouraged us to evaluate its suitability as an activity-based probe for HLE. To assess the direct in-gel fluorescence visualization, varying amounts of HLE were incubated with 2.5 μM probe **6** and subjected to SDS-PAGE. Fluorescent bands at approximately 29 kDa were detected, and their intensities correlated with the amount of HLE (Figure 1A). Even the lowest protease amount employed in this experiment, i.e., 160 ng, could be effectively visualized. Moreover, in each lane, three fluorescent bands, representing three labeled forms of HLE, were observed. Such

bands have been demonstrated to represent distinct, catalytically active HLE isoforms, which differ in their carbohydrate content and have been resolved by SDS-PAGE.^{41–43} Moreover, the self-cleavage of elastase from murine and human neutrophils generates variants whose catalytic activity was shown by means of a biotinylated probe.⁴⁴ The occurrence of the pattern of three elastase bands (Figure 1) has been observed in several previous studies.^{27–29,41–44} Thus, it can be attributed to either differences in the carbohydrate contents or to an autoprocessing of elastase. Compared to the Coomassie Brilliant Blue staining, probe **6** produced a much stronger labeling of HLE (Figure 1A versus B).

The selectivity of activity-based probe **6** was investigated by adding HEK cell lysate after or prior to the incubation time of 20 min to two mixtures containing HLE and **6**. SDS-PAGE and subsequent fluorescence analysis revealed a selective elastase labeling as only HLE was imaged (Figure 1C). The following Coomassie staining (Figure 1D) indicated that the ratio between HLE and the HEK lysate proteins was appropriate to demonstrate the selective labeling by **6**. In addition, the binding mode of **6** was explored in a competition experiment (Figure 1E,F). HLE was preincubated with the active site-directed, covalent inhibitor sivelestat in order to protect the enzyme from a reaction with **6**. As a control, **6** was incubated with HLE in the absence of sivelestat. A clearly reduced fluorescence signal was observed in the presence of sivelestat due to the competition of our probe with sivelestat for the active site of the target HLE.

As a conclusion, the activity-based probe **6** was designed on the basis of a highly potent elastase inhibitor. The BODIPY fluorophore was introduced through click chemistry yielding a compound with a second-order rate constant of 88400 M⁻¹s⁻¹, making it one of the most potent fluorescently labeled inactivators of human leukocyte elastase. We have demonstrated that the phosphono peptide **6** reacts with the active site of HLE and allows for a selective in-gel fluorescence labeling of low amounts, down to 160 ng, of this protease. The new probe is expected to serve as a valuable tool to further investigate the pathophysiological role of leukocyte elastase in several inflammatory disorders.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.7b00533.

General methods and materials. Synthetic procedures and characterization of all compounds. General enzymatic methods. Enzyme inhibition assays. Inhibition of HLE with **1A**, **1B**, probe **6**, and sivelestat. Reactivation experiments. Normalized absorption and emission spectra of probe **6**. Detection of HLE with probe **6**. NMR spectra and LC/MS data of synthesized compounds (PDF)

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The manuscript was written through contributions of all authors, who have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AA, amino acid; BODIPY, boron-dipyrromethene; DIPEA, *N,N*-diisopropylethylamine; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEK, human embryonic kidney; HLE, human leukocyte elastase; PPE, porcine pancreatic elastase

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