Fluorescence Probes

DOI: 10.1002/anie.200702811

Selective Activity-Based Probes for Cysteine Cathepsins**

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Proteases are critically involved in a multitude of vital processes, essential for cellular signaling and tissue homeostasis.[1–3] Since proteolytic activity has to remain under stringent control, most of the proteases are synthesized as inactive precursors (zymogens), which are activated by limited proteolysis. Consequently, most of the classical biochemical and proteomics methods fail to discriminate between the expression level and proteolytic activity of a given protease in vivo. Therefore, the development of chemical tools for biomolecular imaging of protease activity rather than protease expression has gained considerable interest in recent years, and a series of internally quenched activity-based probes (ABPs) for proteases has been developed.[4,5] These probes become fluorescent upon proteolytic cleavage.

Analysis of the function of a distinct protease in the complex milieu of the cell requires the development of selective and cell-permeable ABPs, which has been challenging. To present, internally quenched imaging probes for proteolytic enzymes have been derived from peptidic substrates, coupled to appropriate reporter groups which are spatially separated upon proteolytic cleavage of the cognate sequence.[4] The preferred substrates are commonly identified from positional scanning combinatorial libraries.[6] The synthesis and scanning of such libraries can be time consuming, and the peptidic nature of the resulting substrates may result in unspecific cleavage by other proteases when the probes are applied in cellular or whole animal imaging experiments. For proteases from the cysteine cathepsin family, cell-permeable activity-based probes have been designed as suicide substrates which label the enzyme covalently and inactivate it upon binding.[5,7] While such probes are powerful tools for the localization of proteases, the sensitive real-time monitoring of protease activity and inhibition in vivo would likely benefit from probes that are substrates to the enzyme and amplify the fluorescent signal upon proteolysis. Such probes are of high interest for drug discovery and diagnostics since they could enable direct observation of inhibitor efficacy by competition studies in pharmacologically relevant models.[4d–f]

We report here a new concept for the design of specific, stable, and cell-permeable ABPs. The probes that are described herein are substrates to the target enzyme and selectively differentiate between related cysteine cathepsins. Cysteine cathepsins have been established as pharmaceutical targets for the treatment of bone disorders and degenerative joint diseases (cathepsin K)[8,9] as well as immune disorders (cathepsin S).[10,11] Notably, the design of our probes is not based on peptide substrate information gained through positional scanning combinatorial libraries. We base the design on the structures of highly potent and selective cathepsin inhibitors which have undergone extensive medicinal chemistry optimization and thus display favorable pharmacokinetic properties in addition to potency and selectivity against related proteases.[1,11–14] These inhibitors commonly carry a reactive group (often a nitrile group) as an electrophilic mimic of the substrate's scissile bond. Once bound to a cathepsin, the electrophilic group is attacked by the catalytic cysteine residue of the enzyme, resulting in a covalent protein–inhibitor complex.[11–14] Since the catalytic mechanism is conserved within the cysteine cathepsin family, the selectivity of such inhibitors is most likely derived from their chemical scaffold. Consequently, we hypothesized that the replacement of the electrophilic group of such inhibitors with a cleavable peptide bond should result in a nonsuicide substrate with a selectivity pattern related to the original inhibitor. Subsequent derivatization with appropriate reporter groups would result in a selective activity-based imaging probe (Scheme 1). Starting from optimized inhibitors rather than peptide substrates, this concept allows one to access a significant knowledge base of existing structure–activity relationships and utilize it for the design of new selective ABPs.

Several selective inhibitors for cathepsin K have entered clinical trials.[13] To demonstrate our concept we chose nitrile inhibitor **1** to provide us with the basic scaffold for an ABP for cathepsin K (Scheme 1a). Compound **1** has a high affinity to cathepsin K ($IC_{50} = 0.5$ nM) while exhibiting good selectivity against the related cathepsins B ($IC_{50} = 6200$ nM) and S ($IC_{50} = 2200$ nM).[13] Inhibitor **3** has been reported as specific for cathepsin S and was therefore selected as a promising

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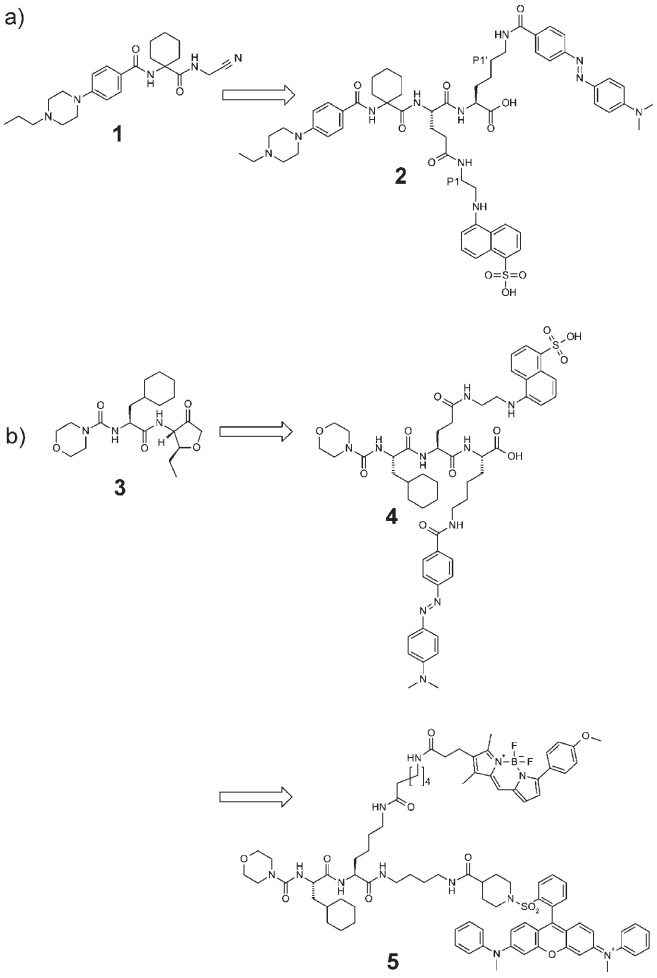
[**] This work was supported by the European Commission Framework VI Program (CAMP project, LSHG-2006-018830). We thank Prof. Markus Grütter and Dr. Marc Nazare for critical comments on this work.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 1. Converting of cathepsin inhibitors into activity-based probes (ABPs). a) Selective cathepsin K inhibitor **1**[13] and related ABP **2**. In **2** the electrophilic nitrile group is converted into a peptide bond. Fluorophore 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid and quencher 4-{[4-(dimethylamino)-phenyl] azo}benzoic acid are attached in the P1 and P1′ position, respectively. b) Selective cathepsin S inhibitor **3**[11] and corresponding ABP **4**. ABP **5** is a corresponding red fluorescent probe for in vivo studies containing Bodipy TMR-X and QSY-7 as fluorophore/quencher pair.

starting point to build an ABP directed towards this enzyme (Scheme 1 b).[11]

For the design of the desired ABPs for cathepsins K and S, we retained the core structures of inhibitors **1** and **3**, respectively. The electrophilic groups were converted into cleavable peptide bonds (Scheme 1). Cocrystal structures of cathepsins K and S inhibitors indicate that the P1 and P1′ moieties are openly accessible from the surrounding solvent.[15] We therefore decided to attach the spatially demanding fluorophore and quencher groups in these positions. The cleavable peptide bond between the P1′ lysine residue functionalized with the corresponding quencher (dabcyl) and the glutamic acid carrying the fluorophore (Edans) in P1 should be precisely oriented in the active site to be cleaved by cathepsin K (probe **2**) or cathepsin S (probe **4**), respectively. Substrates **2** and **4** were synthesized by standard solid-phase peptide synthesis.[16]

While cathepsin K can be secreted to the extracellular medium,[10] cathepsin S has been found to be predominantly localized in lysosomes where it is critical for antigen processing and presentation.[10,11] In vivo imaging of cathepsin S activity therefore requires cell-permeable probes with spectroscopic properties compatible with cellular imaging. To match these requirements, the fluorophore/quencher pair of **4** was exchanged to Bodipy TMR-X and QSY-7, resulting in an internally quenched red-shifted fluorescent probe **5**. Substrate **5** was synthesized by using a combination of solid- and solution-phase chemistry.[16]

Enzymatic turnover and selectivity of ABPs **2** and **4** were first tested in vitro with the related cysteine cathepsins S, K, B and L.[17] Probe **2** was selectively cleaved by cathepsin K as shown in Figure 1 a. Within the detection limit of the assay, absolute specificity against cathepsin S was observed. Likewise, ABP **4** was cleaved by cathepsins S and L, whereas no increase of fluorescence was obtained with the related

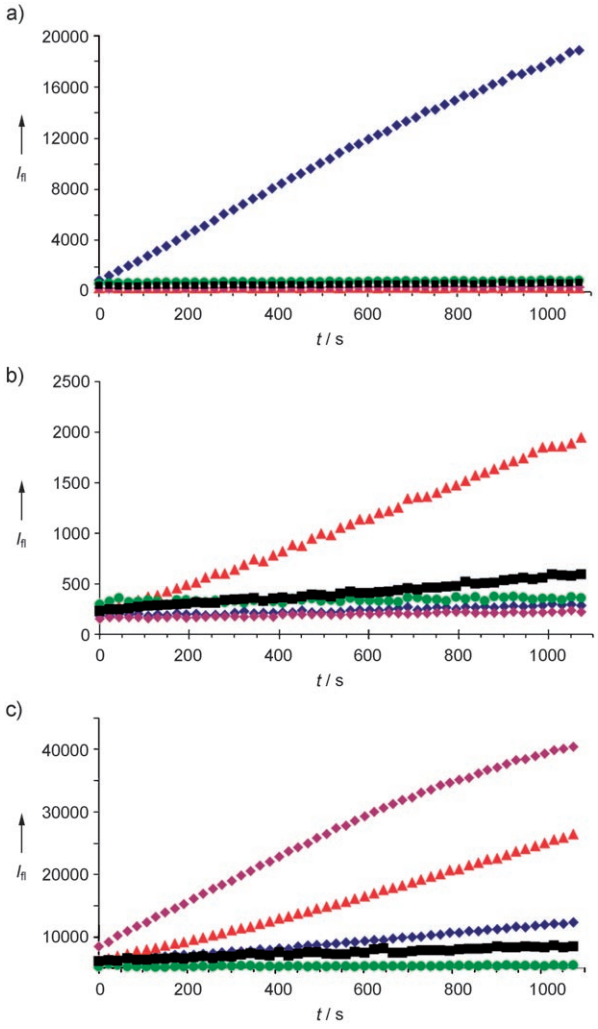


Figure 1. Enyzmatic turnover of probes **2**, **4**, and **5** with the cysteine cathepsins S (▲), K (◆), B(●), L (◆), and papain (■); $c = 10$ nM.[17] a) Progress curve for probe **2** (excitation wavelength $\lambda_{ex} = 336$ nm, emission wavelength $\lambda_{em} = 468$ nm); b) Progress curve for probe **4** ($\lambda_{ex} = 336$ nm, $\lambda_{em} = 468$ nm); c) Progress curve for probe **5** ($\lambda_{ex} = 544$ nm, $\lambda_{em} = 573$ nm).

cathepsins K and B under the conditions used (Figure 1b), thus reflecting the selectivity of the underlying inhibitors, on which these ABPs are based structurally.[11] Both probes are stable in human plasma, and the turnover numbers are comparable with commercially available substrates when incubated with their target enzyme (Table 1). Corresponding to the affinities of the underlying inhibitors **1** and **3**, ABPs **2** and **4** exhibit low K_M values of 35 μM and 1.3 μM for their targets cathepsin K and S, respectively.

Table 1: Kinetic parameters of probes **2**, **4**, and **5** with different cathepsins.[17][a]

		Cath S	Cath K	Cath B	Cath L	Papain
K_M [M]	**2**	n.d.	3.5×10^{-5}	8.8×10^{-4}	7.1×10^{-3}	6.3×10^{-6}
v_{max} [mmol s^{-1}]	**2**	n.d.	7.3	2.6	9.7	0.04
k_{cat}/K_M [M^{-1} s^{-1}]	**2**	n.d.	5420	283	424	296
K_M [M]	**4**	1.3×10^{-6}	8.9×10^{-5}	3.2×10^{-3}	8.4×10^{-4}	1.6×10^{-5}
v_{max} [mmol s^{-1}]	**4**	0.5	0.6	2.7	19.2	0.1
k_{cat}/K_M [M^{-1} s^{-1}]	**4**	15100	187	332	7070	581
K_M [M]	**5**	8.8×10^{-6}	5.6×10^{-4}	n.d.	3.5×10^{-6}	1.4×10^{-4}
v_{max} [mmol s^{-1}]	**5**	0.35	3.3	n.d.	0.3	0.9
k_{cat}/K_M [M^{-1} s^{-1}]	**5**	3930	569	n.d.	7930	680

[a] Kinetic efficiencies of probes **2**, **4**, and **5**. Probes **2** and **4** exhibit good selectivity amongst the related cysteine cathepsins S, K, B, L, and papain (n.d. = no turnover detectable). Probe **5** is selective for cathepsins S and L. All errors are within 10%.

ABP **5** contains a fluorophore/quencher pair with a spectral range compatible with cellular imaging. HaCaT cells (spontaneously immortalized human keratinocytes) were incubated with a 8 μM solution of **5**. Consistent with the reported lysosomal expression of cathepsins S and L in this cell line,[18] we observed enrichment of fluorescence in a lysosome-compatible staining pattern (Figure 2a). However, when HaCaT cells were preincubated with either 15 μM E-64d (a broad-spectrum cysteine cathepsin inhibitor) or 15 μM of an inhibitor selective for cathepsin S and L,[19] the fluorescence was almost completely supressed (Figure 2b,c), suggesting that the fluorescence signal results from the cathepsin S and/or cathepsin L. Similar results were obtained in the U937 human monocyte cell line (data not shown). These results demonstrate that ABP **5**, which was obtained by redesigning a selective cathepsin inhibitor into a cleavable substrate, allows one to selectively monitor the activity of two cysteine cathepsins in living cells.

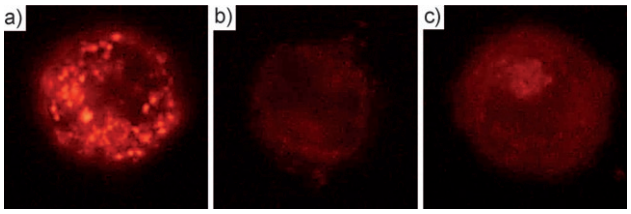


Figure 2. Monitoring of cathepsin activity in HaCaT cell line. a) After 3 h incubation with 8 μM of **5**, human keratinocytes exhibit a lysosome-compatible fluorescence pattern. b) When preincubated with 15 μM E64d, no fluorescence is observed. c) When preincubated with 15 μM selective cathepsin S/L inhibitor,[19] no fluorescence is observed.

In summary, compounds **2**, **4**, and **5** demonstrate that desirable properties of mechanism-based inhibitors for cysteine cathepsins can be transferred to cell-permeable and selective ABPs when the electrophilic group is exchanged for a cleavable peptide bond. The substrate nature and cell permeability of the newly designed probes allows one to monitor the activity of selected proteases in living cells. By transforming an optimized inhibitor to a substrate ABP, we have taken the reverse pathway compared to that which protease drug discovery commonly applies when transforming preferred substrates into inhibitors, which are subsequently optimized by the means of medicinal chemistry. This concept of "reverse design" retains optimized properties of the inhibitor in the ABP and may provide a general route towards selective probes for cysteine cathepsins and other cysteine proteases. "Reverse design" makes a significant reservoir of existing structure–activity relationships accessible for the design of new ABPs. Notably, with this concept not only the selectivity but also the K_M value of the ABP should be tunable on the basis of the properties of the parent inhibitor. The low K_M values of probes **2**, **4**, and **5** render them attractive for in vivo studies.

Received: June 25, 2007
Revised: September 19, 2007
Published online: November 19, 2007

Keywords: enzymes · fluorescent probes · inhibitors · medicinal chemistry

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[16] ABPs **2** and **4** were synthesized by standard solid-phase peptide synthesis using the 2-chlorotrityl chloride resin. ABP **5** was generated using a combination of solid- and solution-phase chemistry. For details, see the Supporting Information.
[17] For the in vitro enzyme activity assay, the active cysteine proteases were dissolved in AHNP buffer (150 mM acetate/2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) pH 6.5, 300 mM NaCl; 0.001 % pluronic, 5–100 mM cysteine depending on the enzyme) at a final concentration of 10 nM. The substrates **2**, **4**, and **5**, dissolved in DMSO, were added at concentrations of 98 μM (**2**) and 24 μM (**4** and **5**), and fluorescence was measured with a Tecan SAFIRE II spectrometer. The final DMSO concentration in the assay did not exceed 1 % (v/v). Steady-state kinetics were fitted by nonlinear least-squares regression using $v = [A]V/(K_M+(1+([A]/K_{si}))[A])$, where v is the initial velocity, V is the maximal rate, K_M is the Michaelis–Menten constant, and K_{si} is the constant for substrate inhibition.
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