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Short Communication

Epoxysuccinyl Peptide-Derived Cathepsin B Inhibitors: Modulating Membrane Permeability by Conjugation with the C-Terminal Heptapeptide Segment of Penetratin

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Besides its physiological role in lysosomal protein breakdown, extralysosomal cathepsin B has recently been implicated in apoptotic cell death. Highly specific irreversible cathepsin B inhibitors that are readily cell-permeant should be useful tools to elucidate the effects of cathepsin B in the cytosol. We have covalently functionalised the poorly cell-permeant epoxysuccinyl-based cathepsin B inhibitor [R-Gly-Gly-Leu-(2*S*, 3*S*)-*t*Eps-Leu-Pro-OH; R=OMe] with the C-terminal heptapeptide segment of penetratin (R=εAhx-Arg-Arg-Nle-Lys-Trp-Lys-Lys-NH_2). The high inhibitory potency and selectivity for cathepsin B *versus* cathepsin L of the parent compound was not affected by the conjugation with the penetratin heptapeptide. The conjugate was shown to efficiently penetrate into MCF-7 cells as an active inhibitor, thereby circumventing an intracellular activation step that is required by other inhibitors, such as the prodrug-like epoxysuccinyl peptides E64d and CA074Me.

Key words: Antennapedia / CA074 / Cathepsin L / MCF-7 cells.

Papain-like cysteine proteases are involved in diverse physiological processes and, if disregulated, they contribute to a variety of disorders (Kirschke *et al.*, 1995; Otto *et al.*, 1997; Barrett *et al.*, 1998; Brömme, 1999). In particular, cathepsin B is proteolytically active in different compartments, depending on its role under physiological or pathological conditions (Mort and Buttle, 1997). Physiologically contributing to lysosomal protein degradation, this protease has been shown to be membrane-associated and/or secreted into the pericellular space in several tumor cell lines and is believed to promote metastasis and tumor progression (Elliott and Sloane, 1996; Frosch *et al.*, 1999). Moreover, according to very recent findings cytosolic cathepsin B seems to play a role in apoptotic cell death (Guicciardi *et al.*, 2000; Foghsgaard *et al.*, 2001; Mathiasen *et al.*, 2001).

In various attempts to elucidate the contribution of cathepsin B to these processes, specific inhibitors and affinity labels have been used. Depending on the localisation of cathepsin B in the pericellular/extracellular space or within the cell, it is of great importance to modulate the membrane permeability of these tools. In order to target extracellular cathepsin B, we have recently introduced the *endo*-epoxysuccinyl peptide **1** as well as affinity labels (compounds **2** and **3**) derived from this irreversible inhibitor (Figure 1) (Schaschke *et al.*, 1998, 2000a). These highly cathepsin B-selective and potent inhibitors are based on the CA030-like fragment HO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH as lead structure, which was elongated by the peptide stretch Leu-Gly-Gly derived from the cathepsin B propeptide (amino acids 46–48) to simultaneously address interactions with both the S and the S‘ subsites. A special feature of this parent inhibitor is that the terminal glycine residue is located solvent-exposed at the surface of the enzyme as proposed by modeling experiments (data not shown). Consequently, this position offers the possibility to further derivatise the parent inhibitor **1** with various functional groups without affecting its inhibitory potency. This concept has been confirmed by synthesis of the affinity labels **2** and **3** as well as of a cathepsin B-selective drug carrier system consisting of β-cyclodextrin as carrier- and the inhibitor as address-unit (Schaschke *et al.*, 2000b).

To render inhibitor **1** membrane-permeable two general strategies seem promising. On one hand it seems possible to convert **1** into a permeable prodrug-like form by synthesising the proline methyl ester. This approach has been successfully applied to transform the well-known cathepsin B inhibitor CA074 into the cell permeable pro-inhibitor CA074Me (Buttle *et al.*, 1992). On the other hand, an alternative strategy is to conjugate the inhibitor with a cell-penetrating peptide (Lindgren *et al.*, 2000). Here, we report the synthesis and functional characterisation of a penetratin-epoxysuccinyl peptide conjugate

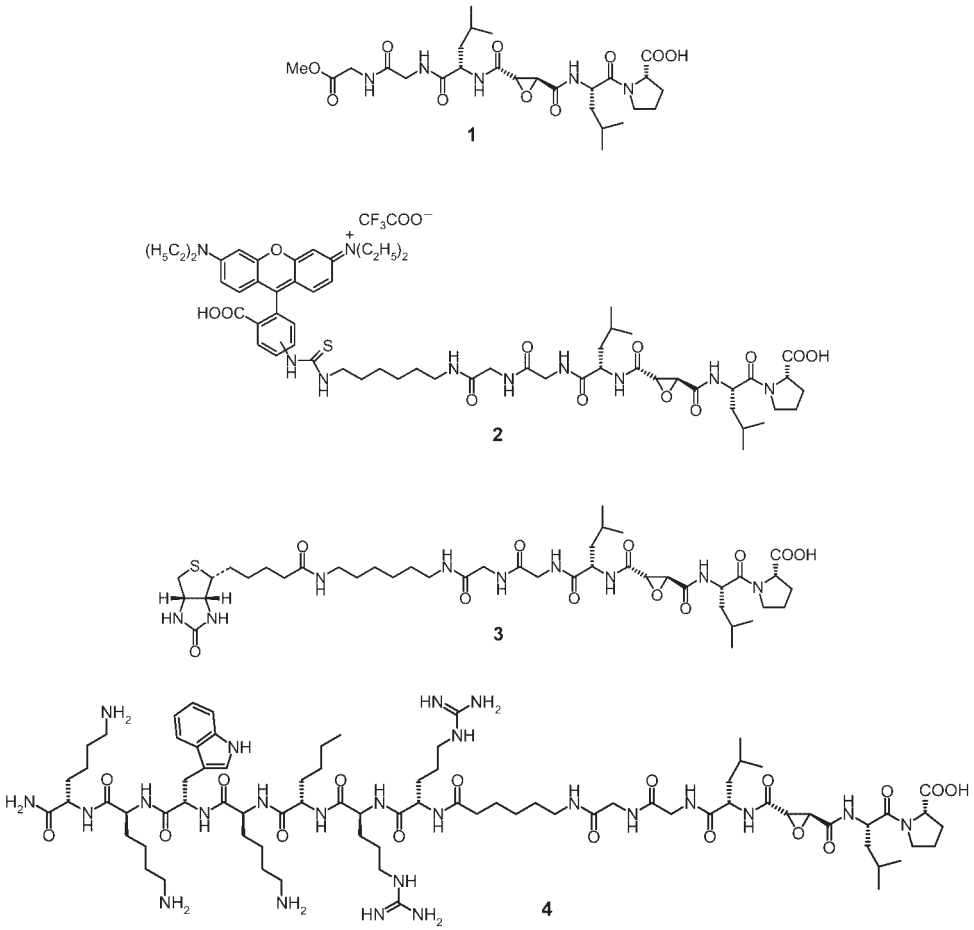

Fig. 1 Structure of Epoxysuccinyl Peptide **1** and the Effector-Functionalised Inhibitors **2**, **3** and **4**.

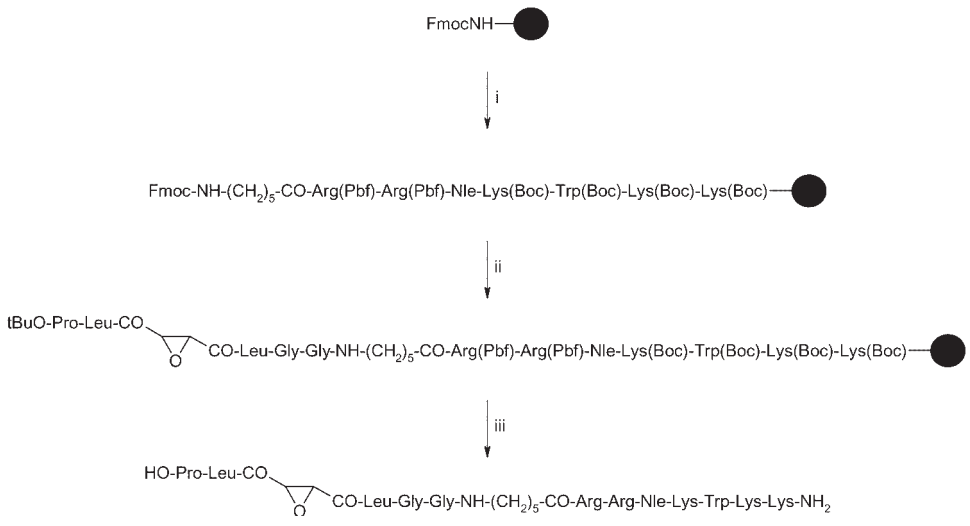

Fig. 2 Solid-Phase Synthesis of the Epoxysuccinyl Peptide/Penetratin HP Conjugate **4**.
Reaction conditions: step (i), a. piperidine/NMP (1:5), b. Fmoc-AA/HBTU/HOBt/DIEA (4:4:4:8), NMP; 8 cycles of double coupling; step (ii), a. piperidine/NMP (1:5), b. HO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu/HBTU/HOBt/DIEA (1.5:1.5:3), NMP; step (iii), 95% aq. TFA/TIS (98.5:1.5). The synthesis was performed using a rink amide MBHA resin.

derived from inhibitor **1** following the concept of effector functionalisation outlined above.

The 16-mer peptide (amino acids 43 – 58, RQIKIWFQ-NRRMKWKK) derived from the third helix of the homeodomain of the Antennapedia protein, also called penetratin, is widely used for the intracellular delivery of pep-

Table 1 Second-Order Rate Constants for the Inactivation of Cysteine Proteases by the Penetratin Pentapeptide (Penetratin HP)-Functionalised Epoxysuccinyl Peptide.

Inhibitor	Cathepsin B	Cathepsin L	Ratio CB/CL
	k_2/K_i [$M^{-1}s^{-1}$]	k_2/K_i [$M^{-1}s^{-1}$]	
MeO-Gly-Gly-Leu(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**1**)	1 520 000±88 800	1 204±29	1 262
Rhodamine B-NH-$(CH_2)_6$-NH-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**2**)	1 530 000±83 500	323±30	4 736
Biotin-NH-$(CH_2)_6$-NH-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**3**)	1 726 000±40 900	256±14	6 742
Penetratin HP-CO-$(CH_2)_5$-NH-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**4**)	6 100 000±270 000	3 590±154	1 699

The k_2/K_i-value for compound **4** was determined in 250 mM sodium acetate buffer pH 5.5 as described previously (Schaschke *et al.*, 1998) and is the mean ± SD from 7 experiments after correction for substrate competition. The inhibitory potencies of compounds **1**, **2** and **3** (Schaschke *et al.*, 1998, 2000a) are listed for comparison.

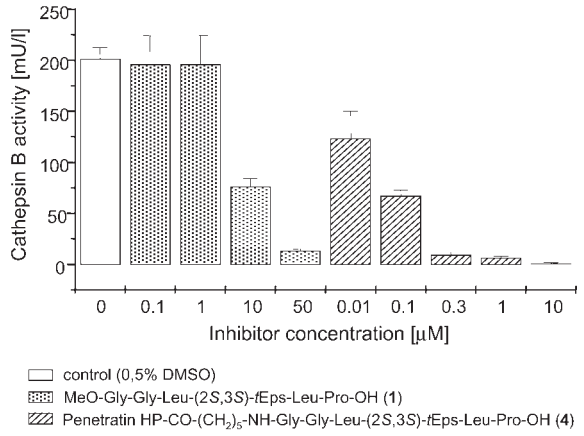

Fig. 3 Cell Permeability of Epoxsuccinyl-Peptides.
Cell permeability was determined with MCF-7 breast cancer cells essentially as described previously (Schaschke *et al.*, 2000a). Confluent cells grown in 24-well plates were incubated for 30 min at 37 °C with 300 µl of serum-free medium containing 0.5% DMSO without or with increasing concentrations of inhibitor **1** (0.1, 1.0, 10, and 50 µM) or penetratin-functionalised inhibitor **4** (0.01, 0.1, 0.3, 1.0 and 10 µM). Thereafter, the cells were washed five times with PBS and lysed in 200 µl of 50 mM sodium acetate, pH 5.5, 0.5% Triton X-100, 2 mM EDTA for 30 min at room temperature. Residual cathepsin B activity in the lysates was measured with the substrate Z-Arg-Arg-NHMec (50 µM) followed by inhibition with compound **1**. The columns represent mean values of three experiments with standard deviations (bars). Incubation of the cells with the penetratin heptapeptide itself (at concentrations ≤10 µM) had no effect on residual cathepsin B activity (data not shown).

tides and oligonucleotides (Derossi *et al.*, 1998). Recently, by an extensive SAR study it was shown that the C-terminal heptapeptide segment of penetratin (amino acids 52–58, RRMKWKK, penetratin HP) is sufficient for efficient cell membrane translocation (Fischer *et al.*, 2000). Furthermore, it was shown that Met-54 is freely exchangeable with either Leu or Nle (Fischer *et al.*, 2000). The truncated penetratin, in which Met-54 is exchanged against Nle, is very attractive because the synthetical effort is drastically reduced and the resulting peptide is oxidation-insensitive. Therefore we have chosen this particular new vector for our study.

The penetratin heptapeptide was synthesised by standard Fmoc/*t*Bu chemistry on solid support (Figure 2). After introducing ε-amino hexanoic acid as an additional spacer, the suitably protected inhibitor [HO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu] obtained by solution synthesis as described previously (Schaschke *et al.*, 2000a) was coupled as fragment using HBTU/HOBt. The final cleavage/deprotection was performed with 95% aq TFA containing 1.5% triisopropylsilane yielding conjugate **4,** as characterised by RP-HPLC, ESI-MS and amino acid analysis.

The second-order rate constants of inactivation of cathepsin B and L by conjugate **4** as well as by inhibitor **1** and the affinity labels **2** and **3** are summarised in Table 1. Comparison of the rate constants for cathepsin B inactivation by the parent inhibitor **1** and the labels **2** and **3** clearly reveals that the functionalisation with reporter groups has no effect on the inhibitory potency. In the case of the new conjugate **4** the inhibitory potency is even slightly increased upon covalent attachment of the penetratin segment, presumably due to additional interactions of the highly positively charged penetratin heptapeptide portion with the protein surface. The heptapeptide itself has no inhibitory activity against cathepsin B (K_i ≥240 µM; data not shown). The selectivity for cathepsin B *versus* cathepsin L is not affected by the conjugation. The data obtained with the new conjugate **4** support our design concept and characterise the parent inhibitor **1** as a structure privileged for modifications due to its particular binding mode.

Membrane permeability studies were performed with MCF-7 breast cancer cells as described previously (Schaschke *et al.*, 2000a). The results are summarised in Figure 3. The covalent functionalisation of the parent inhibitor with the penetratin segment leads to an efficient cell membrane translocation of the cargo molecule and fully confirms the reported data on the potential of the penetratin heptapeptide (Fischer *et al.*, 2000). Whereas incubation (30 min) of MCF-7 cells with 1 µM inhibitor **1** does not effect inactivation of intracellular cathepsin B, a

concentration of 0.3 μM of conjugate **4** results in almost complete inactivation. A concentration as low as 0.01 μM is sufficient to block approx. 60% of the intracellular cathepsin B activity. As expected, the heptapeptide itself has no effect on intracellular cathepsin B activity (data not shown). In contrast to prodrug-like epoxysuccinyl peptides, such as E64d and CA074Me, conjugate **4** permeates the membrane as an active inhibitor, thus circumventing an intracellular activation step by deesterification.

In conclusion, the data obtained from inhibition kinetics and cell permeability experiments classify the new epoxysuccinyl peptide-penetratin conjugate **4** as a potent tool for inactivation of intracellular cathepsin B. In combination with inhibitor **1** and the affinity labels **2** and **3** this set of compounds provides a promising tool kit to selectively investigate the roles of intracellular and pericellular cathepsin B.

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