DOI: 10.1002/cbic.200800103

# Inhibition of Cathepsin L by Epoxysuccinyl Peptides Simultaneously Addressing Active-Site and Remote-Site Regions

Norbert Schaschke,*[a] Irmgard Assfalg-Machleidt,[b] and Werner Machleidt[c]

Cathepsin L is one of the eleven known human cysteine cathepsins belonging to the papain family (C1) of the clan CA of cysteine proteases.[1] The enzyme is ubiquitously expressed and shows endopeptidase activity with a preference for hydrophobic amino acid residues in subsites S2 and S3.[1] This lack of a clearly defined sequence specificity reflects its well known contribution to the unspecific bulk proteolysis in the lysosomal and related compartments.[2] Newer data, however, strongly support the notion that cathepsin L plays specific roles in important physiological processes[1,3] including antigen presentation,[4] epidermal homeostasis,[5] and endothelial progenitor cell-induced neovascularization.[6] Moreover, it is well accepted that the proteolytic activity of cathepsin L plays a causal role in the malignant progression of human tumours.[7]

Generally, the assignment of specific functions to individual members of the human cysteine cathepsins is a challenging task. This is mainly due to their high degree of structural homology, which results in overlapping cleavage patterns and thus functional redundancy. Accordingly, many data concerning specific roles of cathepsin L have been assessed by the gene knock-out approach by using cathepsin L-deficient mice.[4–6] Affinity probes that specifically block the activity of its target protease in an irreversible fashion on different levels of complexity, including cells, tissues and even whole organisms, would represent a powerful alternative.

Epoxysuccinyl peptides of the E-64 type (Scheme 1), which contain (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid as a thiol-reactive group, are irreversible broad-spectrum inhibitors of human cysteine cathepsins.[8] Therefore, these compounds have received much attention as a privileged platform[9] for the design of probes which allow the proteolytic activity of either the whole group of human cysteine cathepsins[10] or individual members to be detected. Addressing the dipepdidylcarboxypeptidase activity of cathepsin B, which is unique amongst the human cysteine cathepsins, we succeeded in designing and synthesising the highly potent and selective inhibitor NS-134 (**2**; Scheme 1).[11] The X-ray structure of this inhibitor in complex with cathepsin B has elucidated the binding mode in detail. The peptide portion -Leu(P2)-Gly(P3)-Gly(P4)- binds along the S sites in a *non*-substrate-like manner whereas -Leu(P1′)-Pro(P2′)- addresses the S′ sites in a substrate-like manner.[12] Based on inhibitor **2**, the affinity probe NS-196 (**3**, Scheme 1) has been developed[13] and already successfully applied to detect novel physiological functions of this enzyme.[14,15]

Due to the lack of unique structural features, like in the case of cathepsin B, it is generally difficult to gain reliable selectivity for cathepsin L by only addressing interactions along the active-site cleft based solely on a E-64-type structural core. Therefore, access to additional peptide–protein interactions beyond the active-site cleft on the surface of the protease would represent an interesting approach. Without a structural guide, however, it is very difficult to identify suitable regions on the surface of cathepsin L. In this context, it is important that human cysteine cathepsins are expressed as catalytically inactive proenzymes that are activated proteolytically at their physiological destinations.[16] In the case of cathepsin L, the binding mode of its propeptide domain has been elucidated at the atomic level by X-ray crystallography (Figure 1).[17] The propeptide domain binds along the active-site cleft in a *non*-substrate-like manner. Phe78p (in the X-ray structure this residue is substituted by Leu) occupies the S2 and Gln79p the S3 pocket of the protease. Beyond the S sites of the active-site cleft, the remaining C-terminal part of the propeptide (residues 80p–96p) interacts with regions on the surface of the enzyme. In particular, the residues Lys87p–Phe89p are constituents of an antiparallel β-sheet motif and thus represent a clearly defined remote site.

To investigate whether the α-amino function of Phe78p is able to covalently connect portions of increasing length of the C-terminal part of the propeptide domain with the thiol-reactive group (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid, the X-ray structures of procathepsin L and cathepsin B in complex with the inhibitor NS-134 (**2**) were superposed (Figure 2). It turned out that the peptide backbone of the section Leu78p(P2)-Gln79p(P3)-Asn80p(P4), including the side chain of Leu78p of the propeptide, can be superposed well with the corresponding region of the inhibitor (that is, Leu(P2)-Gly(P3)-Gly(P4)).

Based on these structural observations, four peptide portions of the C-terminal part of the cathepsin L propeptide (ProCL) were selected for the inhibitor design: 1) ProCL-[78p–80p] interact only with the active site; 2) ProCL-[78p–85p] starts to addresses regions on the surface of the protease; 3) ProCL-[78p–91p] includes the amino acid residues constituting the antiparallel β-sheet motif and 4) ProCL-[78p–96p] the entire C-terminal propeptide domain (Scheme 2).

The inhibitors **4**–**7** were synthesisised on solid support based on the Fmoc/*t*Bu protecting-group scheme (Scheme 3).

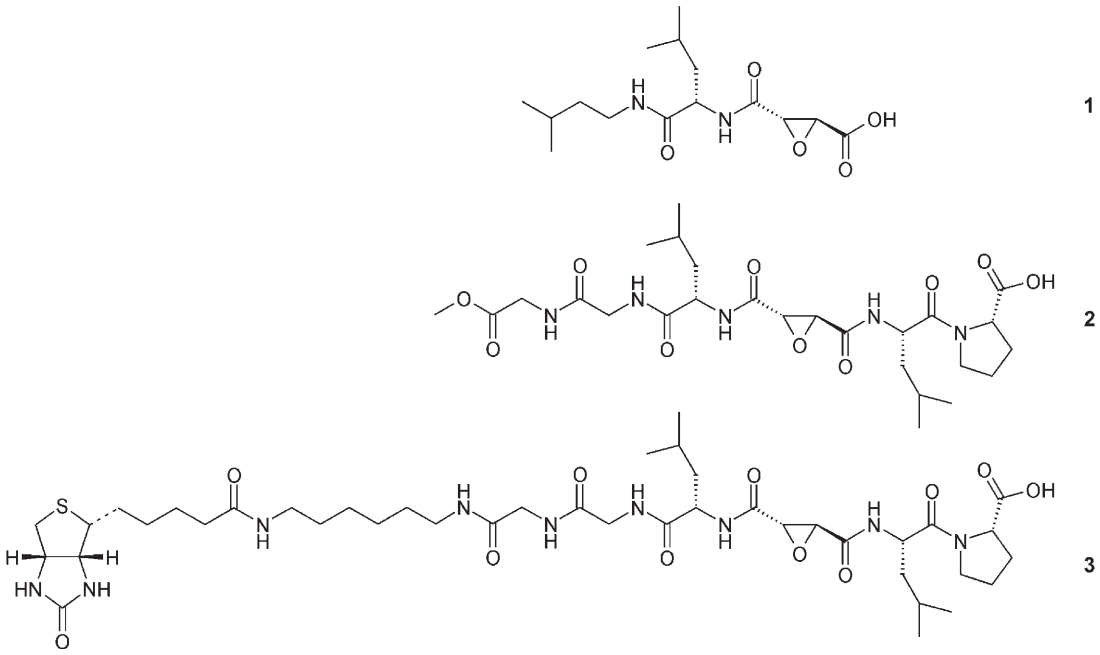
[a] *Dr. N. Schaschke*
*Fakultät für Chemie, Universität Bielefeld*
*Universitätsstrasse 25, 33615 Bielefeld (Germany)*
*Fax: (+49) 521-106-8094*
*E-mail: norbert.schaschke@uni-bielefeld.de*

[b] *Dr. I. Assfalg-Machleidt*
*Abteilung für Klinische Chemie und Klinische Biochemie,*
*Chirurgische Klinik, Ludwig-Maximilians-Universität*
*Nussbaumstrasse 20, 80336 München (Germany)*

[c] *Prof. Dr. W. Machleidt*
*Adolf-Butenandt-Institut, Ludwig-Maximilians-Universität*
*Schillerstrasse 42, 80336 München (Germany)*

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




**Scheme 1.** Chemical structures of E-**64c** (**1**), as a representative example of an E-64-type inhibitor, NS-134 (**2**) and NS-196 (**3**).

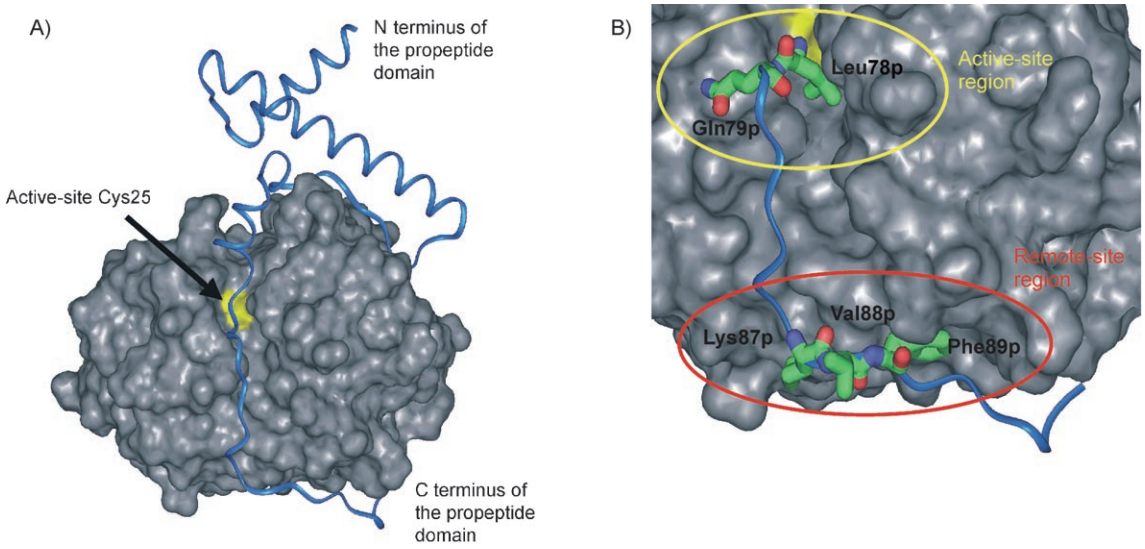



**Figure 1.** X-ray structure of procathepsin L (PDB ID: 1CJL).[17] A) Entire propeptide domain; B) Expanded C-terminal part of the propeptide domain. The active-site and remote-site regions are indicated. Cathepsin L is shown in grey Connolly surface representation, the region of the active-site Cys25 is highlighted in yellow. The propeptide domain is shown in blue ribbon representation, and individual amino acid residues are indicated in stick representation (colour code: C atoms: green, O atoms: red, and N atoms: blue; H atoms are omitted).

Upon final Fmoc-cleavage, (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid mono *tert*-butylester was coupled manually as the sodium salt by using only HBTU. Finally, the inhibitors were cleaved from the resin by TFA/$H_2O$/TIS (95:2.5:2.5, *v/v/v*).

The second-order rate constants ($k_2/K_i$) of inhibitors **4**–**7** for irreversible inhibition of cathepsin L are summarised in Table 1. Additionally, to assess information about the selectivity, $k_2/K_i$ values for the inhibition of cathepsin B were also determined. Inhibitor **4,** which exclusively interacts with the active site, is a weak inhibitor of cathepsin L with only moderate selectivity ($k_2/K_i = 17\,200\ M^{-1}s^{-1}$; ratio CL/CB = 16.2). However, in the case of the entire C-terminal propeptide (inhibitor **7**), the picture changes fundamentally. Compared to inhibitor **4**, the affinity increases by a factor of almost 60 ($k_2/K_i = 992\,000\ M^{-1}s^{-1}$) and the selectivity against cathepsin B reaches approximately two orders of magnitude. The entire C-terminal propeptide sequence can even be truncated substantially without influencing the inhibitory profile (inhibitor **6**). Further truncation, including the amino acid residues constituting the antiparallel β-sheet motif (inhibitor **5**), is accompanied by a moderate loss of affinity and selectivity ($k_2/K_i = 577\,000\ M^{-1}s^{-1}$; ratio CL/CB = 49.7).

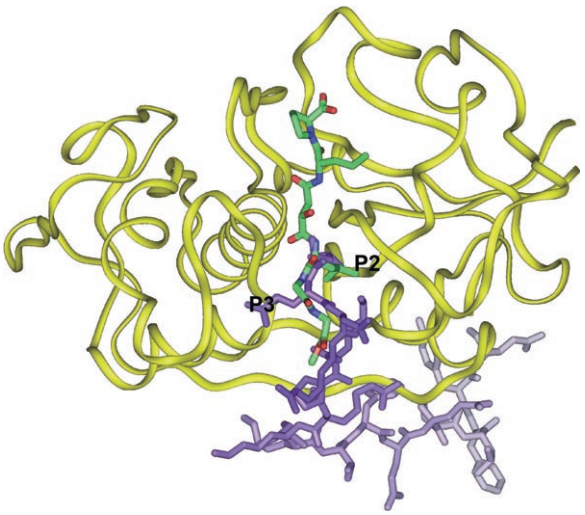
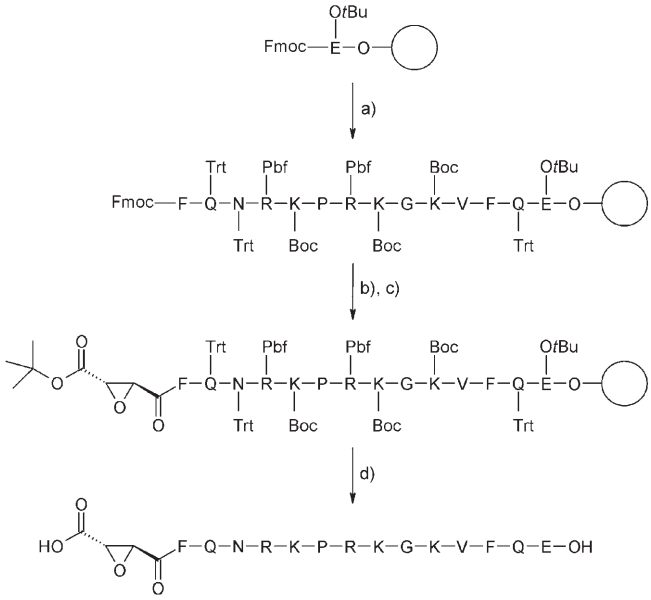
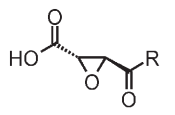



**Figure 2.** Superposition of the X-ray structures of procathepsin L (PDB ID: 1CJL)[17] and cathepsin B in complex with the inhibitor NS-134 (PDB ID: 1SP4).[12] Cathepsin B is shown in yellow ribbon representation, NS-134 in stick representation (colour code: C atoms: green, O atoms: red, and N atoms: blue; H atoms are omitted). From procathepsin L only the C-terminal part of the propeptide domain starting with Leu78p is shown in violet stick representation (H atoms are omitted). The position of the P2 and the P3 residues are indicated, respectively.




**Scheme 3.** Synthesis of inhibitor **6** as a representative example for all inhibitors. Reaction conditions: a) i: piperidine/NMP (1:4, *v/v*), ii: Fmoc-Xaa-OH/HBTU/HOBt/DIEA (1:1:1:2, 4 equiv), NMP, 13 cycles of automated double coupling; b) piperidine/NMP (1:4, *v/v*); c) NaO-*t*Eps-O*t*Bu/HBTU (1:1, 4 equiv), DMF; d) TFA/$H_2O$/TIS (95:2.5:2.5, *v/v/v*).




**4**: R= FQN-OH, [78p-80p]
**5**: R= FQNRKPRK-OH, [78p-85p]
**6**: R= FQNRKPRKG**KVF**QE-OH, [78p-91p]
**7**: R= FQNRKPRKG**KVF**QEPLFYE-OH, [78p-96p]

**Scheme 2.** Chemical structures of propeptide-derived irreversible inhibitors of cathepsin L. The amino acid residues interacting with the remote-site region are highlighted in bold.

**Table 1.** Irreversible inhibition of cathepsin L (CL) and cathepsin B (CB) by Pro-CL-derived inhibitors.

| Inhibitor | $k_2/K_i$ for CL [$M^{-1}s^{-1}$][a] | $k_2/K_i$ for CB [$M^{-1}s^{-1}$][a] | Ratio CL/CB |
|---|---|---|---|
| **4** | 17200 | 1060 | 16.2 |
| **5** | 577000 | 11600 | 49.7 |
| **6** | 1000000 | 13600 | 73.5 |
| **7** | 992000 | 13700 | 72.4 |

[a] Mean of 5–10 experiments with different inhibitor concentrations ($SD_{n-1} \leq 10\%$).

The kinetic data obtained clearly show that inhibitor **6** represents an optimum of affinity and selectivity to which the remote-site interaction contributes substantially. This finding correlates well with the H-bond network that has been observed in the crystal structure of procathepsin L between the C-terminal part of the propeptide domain (ProCL-[78p–96p]) and the surface of the protease, which is already fully established in the section ProCL-[78p–91p].

In conclusion, by addressing simultaneously active-site and remote-site interactions, the inhibitory profile of irreversible inhibitors of cathepsin L, based on the thiol-reactive group (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid, was improved significantly. Moreover, the concept of using structural information provided by the cathepsin L propeptide domain as a guide to access peptide–protein interactions beyond the active site was confirmed.

## *Acknowledgements*

*Funding of N.S. by a Heisenberg Fellowship from the Deutsche Forschungsgemeinschaft is gratefully acknowledged.*

**Keywords:** cysteine protease · E-64 · inhibitors · peptide–protein interactions · propeptides

[1] a) H. Kirschke in *Handbook of Proteolytic Enzymes, Vol. 2*, 2nd ed. (Eds.: A. J. Barrett, N. D. Rawlings, J. F. Woessner), Elsevier, London, **2004**, pp. 1097–1102; b) F. Lecaille, J. Kaleta, D. Brömme, *Chem. Rev.* **2002**, *102*, 4459–4488; c) M. E. McGrath, *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 181–204.
[2] A. J. Barrett, *Ann. N. Y. Acad. Sci.* **1992**, *674*, 1–15.
[3] B. Turk, D. an Turk, V. Turk, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **2000**, *1477*, 98–111.
[4] T. Nakagawa, W. Roth, P. Wong, A. Nelson, A. Farr, J. Deussing, J. A. Villadangos, H. Ploegh, C. Peters, A. Y. Rudensky, *Science* **1998**, *280*, 450–453.
[5] W. Roth, J. Deussing, V. A. Botchkarev, M. Pauly-Evers, P. Saftig, A. Hafner, P. Schmidt, W. Schmahl, J. Scherer, I. Anton-Lamprecht, K. von Figura, R. Paus, C. Peters, *FASEB J.* **2000**, *14*, 2075–2086.
[6] C. Urbich, C. Heeschen, A. Aicher, K. Sasaki, T. Bruhl, M. R. Farhadi, P. Vajkoczy, W. K. Hofmann, C. Peters, L. A. Pennacchio, N. D. Abolmaali, E. Chavakis, T. Reinheckel, A. M. Zeiher, S. Dimmeler, *Nat. Medicine* **2005**, *11*, 206–213.
[7] C. Jedeszko, B. F. Sloane, *Biol. Chem.* **2004**, *385*, 1017–1027.
[8] J. C. Powers, J. L. Asgian, Ö. D. Ekici, K. E. James, *Chem. Rev.* **2002**, *102*, 4639–4750.
[9] N. Schaschke, *J. Biotechnol.* **2007**, *129*, 308–315.

[10] a) D. Greenbaum, K. F. Medzihradszky, A. Burlingame, M. Bogyo, *Chem. Biol.* **2000**, *7*, 569–581; b) D. Greenbaum, A. Baruch, L. Hayrapetian, Z. Darula, A. Burlingame, K. F. Medzihradszky, M. Bogyo, *Mol. Cell. Proteomics* **2002**, *1*, 60–68.
[11] N. Schaschke, I. Assfalg-Machleidt, W. Machleidt, L. Moroder, *FEBS Lett.* **1998**, *421*, 80–82.
[12] I. Štern, N. Schaschke, L. Moroder, D. Turk, *Biochem. J.* **2004**, *381*, 511–517.
[13] N. Schaschke, I. Assfalg-Machleidt, T. Laßleben, C. P. Sommerhoff, L. Moroder, W. Machleidt, *FEBS Lett.* **2000**, *482*, 91–96.
[14] K. N. Balaji, N. Schaschke, W. Machleidt, M. Catalfamo, P. A. Henkart, *J. Exp. Med.* **2002**, *196*, 493–503.
[15] H. Büth, B. Wolters, B. Hartwig, R. Meier-Bornheim, H. Veith, M. Hansen, C. P. Sommerhoff, N. Schaschke, W. Machleidt, N. E. Fusenig, P. Boukamp, K. Brix, *Eur. J. Cell Biol.* **2004**, *83*, 781–795.
[16] B. Wiederanders, *Acta Biochim. Pol.* **2003**, *50*, 691–713.
[17] R. Coulombe, P. Grochulski, J. Sivaraman, R. Ménard, J. S. Mort, M. Cygler, *EMBO J.* **1996**, *15*, 5492–5503.

Received: February 18, 2008
Published online on June 13, 2008