Epoxysuccinyl peptide-derived affinity labels for cathepsin B

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Abstract Extracellular cysteine proteases, in particular cathepsin B, have been implicated in a variety of pathological processes. Selectively targeting labels of this enzyme are important tools to gain more detailed understanding of its specific roles. Starting from our recently developed irreversible epoxysuccinyl-based inhibitor (R-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH, R = OMe), we have synthesized two affinity labels, R = NH-$(CH_2)_6$-NH-rhodamine B and R = NH-$(CH_2)_6$-NH-biotin. Using MCF-7 cells, the labeled inhibitors were shown to be virtually non-cell-permeant. Moreover, affinity blot analysis with the biotinylated inhibitor allowed a highly sensitive and selective non-radioactive detection of active cathepsin B.

Key words: Cathepsin B; Affinity label; Inhibitor; Epoxysuccinyl peptide

1. Introduction

The lysosomal cysteine proteinase cathepsin B has been implicated in various pathological processes like inflammatory airway diseases [1], bone and joint disorders [2], as well as cancer progression and metastasis [3–5]. In most of these processes, cathepsin B has been found in the extracellular space, frequently associated with the plasma membrane [6–8]. To understand the role of this enzyme in pathological processes in more detail, irreversible inhibitors are desirable that selectively recognize and label extracellular, active cathepsin B. Until now, biotinylated [9] and radioactive iodinated [10] peptidyl diazomethanes have been described as affinity labels for active cathepsin B. The iodinated diazomethanes are cell-permeant; no cell permeation studies have been reported for the biotinylated compounds. However, not all diazomethane inhibitors achieve the selectivity of epoxysuccinyl peptides of the CA030/CA074 type.

Already in 1991 Katunuma's group showed that the CA030/CA074 derivatives are strictly specific inhibitors for cathepsin B in vitro and in vivo [11–13]. This high degree of selectivity was explained later by crystal structures of cathepsin B complexed with CA030 [14] and CA074 [15] revealing an opposite binding mode compared to that of E-64 [16] and a strong interaction of the Pro carboxylate with the two His residues of the 'occluding loop' of cathepsin B.

Attempting to combine the structural and binding characteristics of E-64- and CA074-type inhibitors in 'chimeric' constructs we found that the requirements for optimal interaction with the S and S′ subsites are not simply additive and are strongly dependent on the absolute configuration of the epoxysuccinyl group [17]. By elongation of the CA030-like fragment HO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH with the cathepsin B propeptide-derived fragment Leu-Gly-Gly (amino acids 46–48 of the propeptide), a very potent and selective cathepsin B inhibitor was obtained [18]. According to modeling studies, the propeptide portion of the inhibitor MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**8**) bridges the whole non-primed subsite whereas the terminal glycine residue is located on the surface of the enzyme and thus should allow further functionalization with effector groups without affecting its inhibitory potency (Fig. 1). This working assumption was fully confirmed by the conjugate of β-cyclodextrin with MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**8**) as a cathepsin B-selective drug carrier system [19]. Indeed, the observed second order rate constant for the conjugate was only slightly decreased in comparison to the parent inhibitor. These results were the incentive to use this type of irreversible cathepsin B inhibitor as a basis for the design of affinity labels.

In the present communication the synthesis and functional characterization of a rhodamine B-labeled as well as a biotinylated *endo*-epoxysuccinyl peptide is described. Both compounds proved to be potent, highly selective and non-cell-permeable cathepsin B inhibitors.

2. Materials and methods

2.1. Materials and instruments

All reagents and solvents were of the highest quality commercially available. Rhodamine B isothiocyanate (mixed isomers) was purchased from Sigma (Munich, Germany), Z-OSu and DBSI from Fluka (Buchs, Switzerland).

TLC was carried out on silica gel 60 plates (Merck AG, Darmstadt, Germany) and compounds were visualized by chlorine/*o*-toluidine. Analytical HPLC was performed with Waters equipment (Eschborn, Germany) on Nucleosil 300/C18 (Macherey and Nagel, Düren, Germany) using a linear gradient of MeCN/2% H_3PO_4 from 5:95 to 80:20 in 12 min unless otherwise stated, preparative HPLC on Nucleosil 100-5 C18 (Macherey and Nagel), and medium pressure reverse phase chromatography on Lichroprep RP-8 (Merck). NMR spectra were recorded on Bruker AMX500, FAB-MS spectra on Finnigan MAT 900 and ESI-MS on PE SICEX API 165.

Human cathepsin B (EC 3.4.22.1) and cathepsin L (EC 3.4.22.15) were purchased from Calbiochem (Bad Soden/Taunus, Germany),

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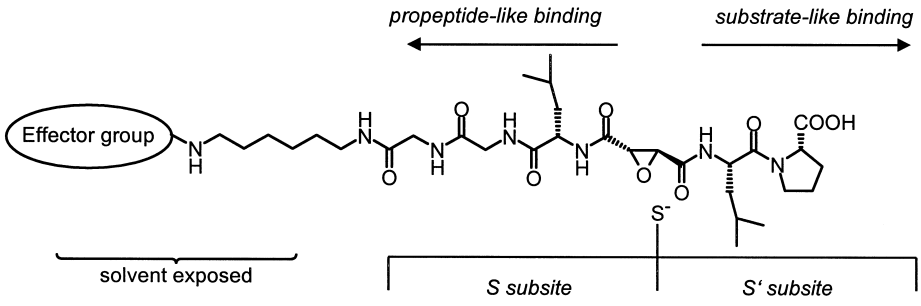


Fig. 1. Design concept of *endo*-epoxysuccinyl peptide-derived affinity labels. Schematic representation of the proposed binding mode of the labels (the effector group is rhodamine B (**6**) or biotin (**7**)).

Z-Phe-Arg-NHMec from Bachem (Heidelberg, Germany), DMEM medium from Biochrom KG (Berlin, Germany) and MCF-7 cells from the European Collection of Cell Cultures (Salisbury, UK).

2.2. Synthesis of endo-epoxysuccinyl peptide-derived active site labels

*2.2.1. HO-Gly-Gly-Leu-(2S,3S)-tEps-Leu-Pro-OtBu (**2**).* To a solution of MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OtBu (**1**) [14] (0.20 g, 0.312 mmol) in THF (12 ml) at 0°C, 0.1 N NaOH (3.5 ml) was added. After 3 h the solvent was removed in vacuo. The residue was dissolved in water, acidified with 5% $KHSO_4$ and extracted with AcOEt. The combined organic layers were washed with brine, dried (Na_2SO_4) and evaporated. Compound **2** was isolated by precipitation from AcOEt/petroleum ether; yield: 0.15 g (75%); TLC ($CHCl_3$/MeOH, 4:1) R_f 0.30; FAB-MS: *m/z* 626.2 $[M+H]^+$; calcd. for $C_{29}H_{47}N_5O_{10}$: 625.3.

*2.2.2. Z-NH-(CH$_2$)$_6$-NH$_2$×DBSI (**3**).* To a solution of 1,6-diaminohexane (4.66 g, 40.1 mmol) in dioxane (750 ml), Z-OSu (1.00 g, 4.01 mmol) dissolved in dioxane (200 ml) was added within 4 h. The precipitate was filtered off, and the solvent evaporated. The residue was dissolved in water (100 ml) and extracted with AcOEt (5×50 ml). The combined organic layers were washed with brine (3×50 ml), dried (Na_2SO_4) and evaporated. The residual oil was dissolved in a small amount of MeOH, and DBSI (1.13 g, 3.80 mmol) was added. The salt was precipitated with diisopropyl ether; yield: 1.70 g (73%); TLC (*n*-BuOH/AcOH/H_2O/AcOEt, 3:1:1:5) R_f 0.33; ESI-MS: *m/z* 251.0 $[M+H]^+$; calcd. for $C_{14}H_{22}N_2O_2$: 250.1.

*2.2.3. Z-NH-(CH$_2$)$_6$-NH-Gly-Gly-Leu-(2S,3S)-tEps-Leu-Pro-OtBu (**4**).* To a solution of compound **3** (0.60 g, 1.03 mmol) in $CHCl_3$ (10 ml) neutralized with NMM (114 μl, 1.03 mmol) and compound **2** (0.50 g, 0.79 mmol), EDC (0.20 g, 1.03 mmol)/HOBt (0.11 g, 0.79 mmol) were added. After 12 h, the solvent was evaporated and the residual oil dissolved in AcOEt. The organic layer was washed with 5% $KHSO_4$, 5% $NaHCO_3$ and brine, dried (Na_2SO_4) and evaporated. The crude product was purified by flash chromatography (silica gel 60, eluent $CHCl_3$/MeOH, 9:1). Compound **4** was isolated by precipitation from AcOEt/petroleum ether; yield: 0.42 g (62%); TLC ($CHCl_3$/MeOH, 9:1) R_f 0.43; TLC (*n*-BuOH/AcOH/H_2O/AcOEt, 3:1:1:5) R_f 0.84; ESI-MS: *m/z* 858.4 $[M+H]^+$; calcd. for $C_{43}H_{67}N_7O_{11}$: 857.4.

*2.2.4. H$_2$N-(CH$_2$)$_6$-NH-Gly-Gly-Leu-(2S,3S)-tEps-Leu-Pro-OtBu×HOAc (**5**).* Compound **4** (0.10 g, 0.117 mmol) was hydrogenated over 10% Pd-C in MeOH (50 ml) in the presence of AcOH (75 μl). After 30 min, the catalyst was filtered off and the solvent evaporated. The title compound was isolated by precipitation from MeOH/diisopropyl ether as a colorless solid; yield: 0.072 g (78%); TLC (*n*-BuOH/AcOH/H_2O/AcOEt, 3:1:1:5) R_f 0.17; HPLC t_R 8.2 min; ESI-MS: *m/z* 724.2 $[M+H]^+$; calcd. for $C_{35}H_{61}N_7O_9$: 723.4.

*2.2.5. Rhodamine B-NH-(CH$_2$)$_6$-NH-Gly-Gly-Leu-(2S,3S)-tEps-Leu-Pro-OH×TFA (**6**).* To a suspension of **5** (28.3 mg, 0.036 mmol) in dioxane (1 ml), 0.1 N $NaHCO_3$ (0.36 ml) was added followed by rhodamine B isothiocyanate (28.9 mg, 0.054 mmol) suspended in a mixture of dioxane (2 ml) and 0.1 N $NaHCO_3$ (1.08 ml). After stirring overnight at room temperature, the solvent was evaporated and the residue dissolved in $CHCl_3$. The organic layer was washed with water, dried (Na_2SO_4) and evaporated. The crude product was dissolved in ice-cold 90% TFA (10 ml) and stirred for 3 h. The solvent was removed in vacuo and the residual oil was treated twice with toluene. Upon precipitation from MeOH/diisopropyl ether, the crude product was purified by medium pressure reverse phase chromatography (isocratic elution with MeCN/water, 20:80 (0–10 min), followed by a linear gradient of MeCN/water from 20:80 to 80:20 (10–130 min), and finally isocratic elution with MeCN/water, 80:20 (130–230 min)). Chromatographically homogeneous fractions were pooled and lyophilized; yield: 10.8 mg (23%) as a 1:1 mixture of the rhodamine B regioisomers; HPLC (Nucleosil 300/C8, linear gradient of MeCN/2% H_3PO_4 from 5:95 to 80:20 in 30 min): t_R 23.6 min/24.1 min; ESI-MS: *m/z* 1168.0 $[M+H]^+$; calcd. for $C_{60}H_{82}N_{10}O_{12}S$: 1166.5.

*2.2.6. Biotinyl-NH-(CH$_2$)$_6$-NH-Gly-Gly-Leu-(2S,3S)-tEps-Leu-Pro-OH (**7**).* To a solution of **5** (72.0 mg, 0.091 mmol) in DMF (3 ml), DIEA (16 μl, 0.091 mmol) and biotin *N*-hydroxysuccinimide ester [20] (46.6 mg, 0.136 mmol) in DMF (2 ml) were added. After stirring overnight at room temperature, the solvent was removed in vacuo, and the residual oil treated with AcOEt. The precipitate was collected by centrifugation and washed successively with *tert*.-butyl methyl ether and petroleum ether. The crude product was dissolved in ice-cold 95% TFA (5 ml), after 3.5 h, the acid was evaporated and the residual oil treated twice with toluene. The crude **7**, isolated by precipitation from AcOEt/petroleum ether, was purified by preparative HPLC (isocratic elution with 0.1% aqueous TFA/MeCN, 95:5 (0–5 min), followed by a linear gradient of 0.1% aqueous TFA/MeCN from 95:5 to 82:18 (5–10 min), finally followed by a linear gradient of 0.1% aqueous TFA/MeCN from 82:18 to 40:60 (10–90 min)). The homogeneous fractions were pooled and lyophilized; yield: 43.0 mg (58%); amino acid analysis of the acid hydrolysate (6 M HCl, 110°C, 72 h): Gly 1.97 (2), Leu 2.03 (2), Pro 1.00 (1), peptide content: 80.3%; HPLC: t_R 7.0 min; ESI-MS: *m/z* 894.8 $[M+H]^+$; calcd. for $C_{41}H_{67}N_9O_{11}S$: 893.4.

2.3. NMR spectroscopy

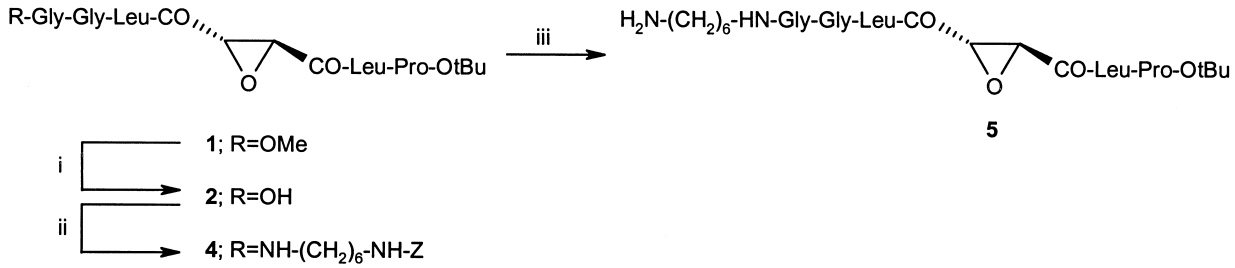
NMR spectra of biotinyl-NH-$(CH_2)_6$-NH-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**7**) were recorded at 500 MHz in DMSO-d_6 (5 mM). For 1D and 2D proton spectra, the following parameters were used: 1D-^{1}H-NMR: 64 acquisitions, size 16 K, sweep width 7002.8 Hz; TOCSY [21,22]: mixing time for MLEV17 76 ms, trim pulse 2.5 ms, size 2 K, sweep width 7002.8 Hz in t_1 and t_2, 64 acquisitions, 512 increments [23]; NOESY [24]: mixing time 200 ms, size 2 K, sweep width 7002.8 Hz in t_1 and t_2, 64 acquisitions, 512 increments [23]; prior to transformation of the TOCSY and NOESY spectra, gaussian window function in t_2 and shifted sine-bell function in t_1 were used.

2.4. Enzyme inhibition assays

Continuous fluorimetric assays were performed and evaluated as described in detail elsewhere [18].

2.5. Cell permeability assay

MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with essential amino acids, 1 mM pyruvate, 10 μg/ml insulin and 10% calf serum, in a humidified CO_2 incubator at 37°C and 5% CO_2. For the experiments cells were grown in 24-well plates where they reached confluence within a week (from initial 2×10^4 cells/well to approx. 2×10^6 cells/well). After washing the cells three times with serum-free medium (SFM) at 37°C, they were incubated at 37°C with 300 μl of SFM containing 1% DMSO without or with inhibitors for 30 min. Thereafter, cells were washed



Scheme 1. Synthesis of the spacer-functionalized *endo*-epoxysuccinyl peptide. Reaction conditions: (i) NaOH/THF/H_2O; (ii) Z-NH-$(CH_2)_6$-NH_2 × DBSI (**3**)/NMM/EDC/HOBt/$CHCl_3$; (iii) 10% Pd-C/H_2/MeOH/AcOH.

five times with phosphate-buffered saline at room temperature, and lysed by treatment with 200 μl lysis buffer (0.5% Triton X-100, 50 mM sodium acetate, pH 5.5, 2 mM EDTA) for 30 min at room temperature. The residual cathepsin B activity in the lysates was determined with the fluorogenic substrate Z-Phe-Arg-NHMec followed by inhibition with the cathepsin B-specific inhibitor **8** (50 nM).

2.6. Fluorimetric determination of rhodamine

The fluorescence of the rhodamine B-labeled inhibitor **6** was measured at 554 nm excitation and 571 nm emission in a spectrofluorimeter Fluoro Max 1 (Instruments S.A.). The fluorescence was linear with inhibitor concentration after blank subtraction. After calibration with 1–30 nM inhibitor in 1.5 ml of 0.25 M sodium acetate pH 5.5, the rhodamine B concentration was determined in 0.1 ml of the lysates (diluted with 1.4 ml sodium acetate buffer, pH 5.5) of MCF-7 cells that had been incubated with inhibitor **6** before lysis (0–50 μM, see Section 2.5).

2.7. Affinity blots

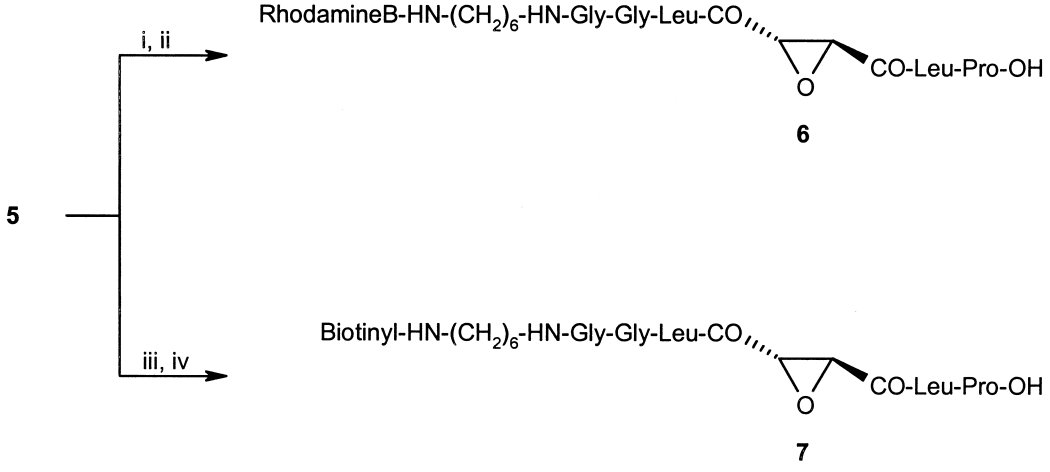
Lysates from MCF-7 cells (200 μl) incubated with biotinylated inhibitor **7** were precipitated with 3.3% TCA, washed twice with acetone and dissolved in Laemmli buffer containing 2-mercaptoethanol. Purified human liver cathepsin B labeled with the biotinylated inhibitor **7** was used as control. After electrophoresis in 12.5% (w/v) polyacrylamide gel slabs, the proteins were transferred to nitrocellulose membranes (Protran, Schleicher and Schuell) by a semidry electrotransfer procedure [25] in a MilliBlot-SDE System (Millipore) for 45 min at 30 mA. The biotin-labeled proteins were visualized by the avidin/biotinylated peroxidase system, Vectastain ABC Elite (Vector Laboratories) using diaminobenzidine as substrate. In control blots, the bands were stained additionally with anti-cathepsin B, a polyclonal IgG from rabbit (Calbiochem), and a second biotinylated antibody from goat (Vector Laboratories) before incubation with avidin/biotinylated peroxidase.

3. Results and discussion

3.1. Synthesis and characterization of the functionalized inhibitors

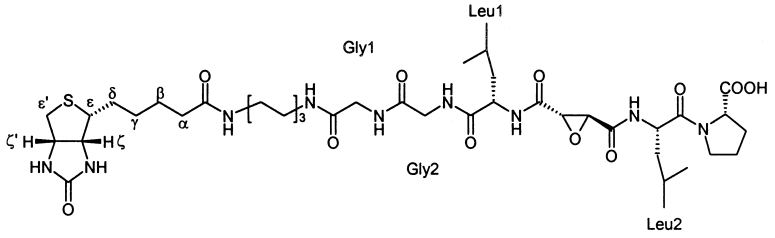
Starting point for the synthesis was the orthogonally protected inhibitor **1** [18] (Scheme 1). Upon saponification of the methyl ester, the free carboxylic acid **2** was coupled with mono-Z-protected 1,6-diaminohexane as an additional flexible spacer to avoid steric clashes of the effector group with the surface of the enzyme. The hydrogenolytic cleavage of the benzyloxycarbonyl group was carried out under strictly controlled conditions to avoid opening of the oxirane ring that we had observed in contrast to reports of Huang et al. [26] in the case of prolonged hydrogenation. The resulting spacer-functionalized inhibitor **5** was then reacted with rhodamine B isothiocyanate (1:1 mixture of both regioisomers) or with biotin activated as *N*-hydroxysuccinimide ester (Scheme 2). The final acidolytic cleavage of the *tert*.-butyl ester protection yielded the labeled *endo*-epoxysuccinyl peptides **6** and **7** as homogeneous compounds as assessed by HPLC and ESI-MS.

The biotinylated *endo*-epoxysuccinyl peptide **7** was further analyzed by 2D-NMR spectroscopy. The TOCSY and NOESY spectra recorded in DMSO-d_6 allowed the full assignment of the peptide portion as well as of the biotin label (Table 1). The observed intra- and interresidual NOEs within the spacer and/or biotin region displayed a negative sign with respect to the diagonal indicating a high degree of flexibility of this part of the molecule.



Scheme 2. Synthesis of the labeled *endo*-epoxysuccinyl peptides. Reaction conditions: (i) rhodamine B isothiocyanate/$NaHCO_3$/dioxane/H_2O; (ii) 90% TFA; (iii) biotin *N*-hydroxysuccinimide ester/DIEA/DMF; (iv) 95% TFA.

Table 1
Chemical shifts [ppm] of the biotin-functionalized *endo*-epoxysuccinyl peptide **7** in DMSO-d_6



Amino acid residue	NH	CαH	Others
Gly1	7.51	3.13	
Gly2	7.88	3.18	
Leu1	8.06	3.85	CβH/CγH: 0.99, 1.08; CδH: 0.35
Leu2	8.23	4.10	CβH/CγH: 0.98, 1.14; CδH: 0.39
Pro		3.73	$C\beta_1H$: 1.62; $C\beta_2H$: 1.33; CγH: 1.42; $C\delta_1H$: 3.18; $C\delta_2H$: 3.00, COO*H*: 11.90
CH-*t*Eps			3.08, 3.10
Spacer	7.20		CH_2NH: 2.50; $-(CH_2)_4-$: 0.86, 0.72
Biotin	5.83, 5.90		CαH: 1.53; CβH/CγH/CδH: 0.76, 0.96, 1.10; CεH: 2.57, CεH′: 2.06, 2.30; CζH: 3.61; CζH′: 3.79

3.2. Inhibitory properties

A comparison of the second order rate constants for inhibition of cathepsin B by the parent inhibitor **8** and the conjugates **6** and **7** (Table 2) shows that the inhibitory potency is not affected by the functionalization of the inhibitor. The selectivity of the affinity labels **6** and **7** vs. cathepsin L is even slightly increased, and reaches a value of 6742 in the case of the biotinylated inhibitor. These data indicate that the terminal glycine is an optimal site for attaching effector groups via a spacer and confirm our design concept (Fig. 1).

3.3. Cell permeability

The cell permeability of the parent inhibitor **8** as well as of the conjugates **6** and **7** was assessed using MCF-7 breast cancer cells (Fig. 2). No or only slight reduction of cathepsin B activity was found in the lysates after incubation of the cells with 0.1 and 1.0 μM of the *endo*-epoxysuccinyl peptides **6**, **7** and **8**. Not more than 50–70% of the cathepsin B activity was blocked in cells incubated with an inhibitor concentration of 10 μM, and even at 50 μM inhibitor approx. 10% of the cathepsin B activity remained detectable in the cell lysates (Fig. 2A). In comparison, despite its lower specificity and reactivity [13] E-64d (1 μM) blocked intracellular cathepsin B activity by approx. 90% (data not shown). Taking into account that a low nM concentration of inhibitors **6**, **7** and **8** is sufficient to completely inhibit isolated cathepsin B as well as the cathepsin B activity in lysates of MCF-7 cells within minutes (as shown in Fig. 2B for inhibitor **8**), both the parent inhibitor **8** and the affinity labels **6** and **7** can be considered to be virtually non-cell-permeant. This was confirmed by direct measurements of rhodamine fluorescence in the lysates: after calibration with the rhodamine-labeled inhibitor **6** as a standard, the concentration of free plus enzyme-bound inhibitor **6** in the cell lysates was calculated (Table 3). These concentrations were less than 0.5% of those in the cell culture medium and correlate well with the observed degree of cathepsin B inhibition (see Fig. 2A).

3.4. Affinity blots with the biotinylated inhibitor

The applicability of the biotinylated inhibitor **7** for affinity blotting was investigated with isolated human liver cathepsin B and with the cell lysates from the permeability studies. Lanes 3–5 in the blot (Fig. 3) show two bands with molecular masses of approximately 31 and 5 kDa, respectively. Both bands were colocalized with those detected by immunoblot with a polyclonal cathepsin B antibody (data not shown). As expected for an affinity label that covalently binds to the active site Cys29 of cathepsin B, both the single-chain form (31 kDa) as well as the light chain (5 kDa) of the double-chain form were detected [27,28]. Once covalently attached via a thioester bond to the active site cysteine of cathepsin B, the biotinylated inhibitor **7** apparently is able to interact with avidin without significant steric hindrance. This is in agreement with the results from the NOESY spectrum of the conjugate **7** (see Section 3.1) indicating that the portion of the molecule containing the spacer and the biotin moiety is fully flexible in comparison to the epoxysuccinyl peptide portion. The sensitivity of the affinity label was assessed with decreasing amounts of isolated human liver cathepsin B (Fig. 3, lanes 3–5). Considering the composition of the cathepsin B prepa-

Table 2
Second order rate constants of inactivation of cysteine proteases by epoxysuccinyl peptide-derived affinity labels

Inhibitor	Cathepsin B (CB) k_2/K_i [M^{-1} s^{-1}]	Cathepsin L (CL) k_2/K_i [M^{-1} s^{-1}]	Ratio CB/CL
Rhodamine B-NH-$(CH_2)_6$-NH-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**6**)	1 530 000 ± 83 500	323 ± 30	4736
Biotin-NH-$(CH_2)_6$-NH-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**7**)	1 726 000 ± 40 900	256 ± 14	6742
MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**8**)	1 520 000 ± 88 800	1204 ± 29	1262

k_2/K_i values were determined in 250 mM sodium acetate buffer pH 5.5, ± S.D. from 7–10 experiments and are corrected for substrate competition; data for compound **8** are from [18].

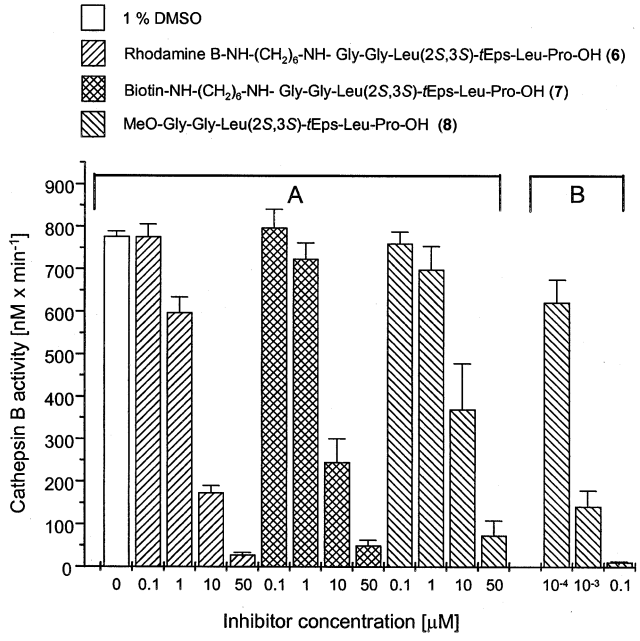


Fig. 2. Cell permeability of epoxysuccinyl peptides. A: Residual cathepsin B activity was measured in lysates of MCF-7 breast cancer cells that had been incubated without or with increasing concentrations (0.1, 1.0, 10, and 50 μM) of the inhibitors **6** (rhodamine B-labeled), **7** (biotinylated), and **8** (parent). B: In a control experiment, lysates of cells pre-incubated without inhibitor were reacted with increasing concentrations (10^{-4}, 10^{-3}, and 0.1 μM) of inhibitor **8**. The columns represent mean values of three experiments with standard deviations (bars).

ration (ca. 15% single-chain and 85% double-chain form), about 3 ng of the single-chain form (31 kDa) was detectable in the blot (Fig. 3, lane 5). This sensitivity of active site labeling was similar to the sensitivity of immunoblots performed with a polyclonal cathepsin B antibody and a biotinylated second antibody using the same detection system (data not shown).

In the lysates from the cell permeability experiments (lanes 6–10), the 31 kDa band as well as the light chain (5 kDa) were detected under reducing conditions. The observed staining is in good agreement with the cathepsin B activity in the lysates (see Section 3.3). Furthermore, the blotting results with the lysates of the cell penetration studies confirm the high degree of selectivity anticipated from inhibition kinetics. No additional bands were found in the affinity blots. However, this does not definitely exclude the possibility that the cathepsin B inhibitors can bind to one of the new thiol cathepsins that

Table 3
Concentrations of the rhodamine B-labeled inhibitor **6** in lysates of MCF-7 cells

Concentration of **6** in the incubation solution (μM)	Concentration of **6** in the lysate (nM)
0.1	0.47 ± 0.31
1.0	5.23 ± 0.45
10	23.15 ± 1.13
50	91.87 ± 0.18

Cells were incubated with increasing concentrations of inhibitor **6** for 30 min at 37°C, washed, lysed, and the concentration of the inhibitor in the lysates was measured using its rhodamine fluorescence. Results are presented as mean of three experiments with standard deviations.

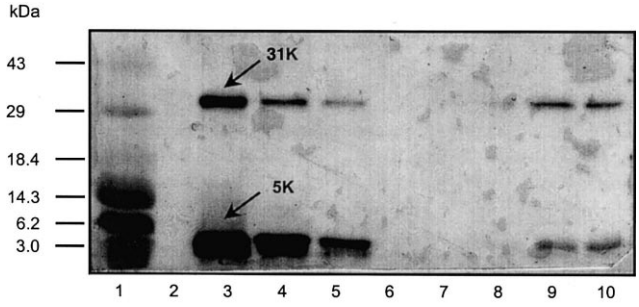


Fig. 3. Affinity blot with the biotinylated inhibitor **7**. Lane 1: prestained low molecular weight standards; lane 2: biotinylated inhibitor **7** (same amount as used in lane 3); lanes 3–5: purified human cathepsin B labeled with biotinylated inhibitor **7** (200, 50, and 20 ng); lanes 6–10: labeled proteins precipitated by TCA from lysates of MCF-7 cells that had been incubated with different concentrations of the biotinylated inhibitor **7** (0, 0.1, 1, 10 and 50 μM).

have been discovered more recently [29] and might not be expressed in MCF-7 cells. A putative target might be cathepsin X which has been shown to contain a mini-loop with a single histidine residue similar to the occluding loop of cathepsin B with two histidines [30]. Inhibitors **6**, **7**, and **8** completely abolished the peptidase activity of the cell lysates measurable with the substrate Z-Phe-Arg-NHMec, but cathepsin X would not have been detected with this substrate due to its extremely weak endopeptidase activity [31].

3.5. Conclusions

In conclusion, the parent inhibitor **8** as well as the affinity labels **6** and **7** represent a novel tool kit that facilitates studies of extracellular cathepsin B activity in inflammation, tumor invasion and metastasis. During preparation of this article, work on targeting of lysosomal cysteine proteinases by radioiodo-labeled epoxysuccinyl peptides derived from CA074 has been published by Bogyo et al. [32]. By replacement of the *n*-propyl moiety by a tryptamine moiety to allow subsequent labeling with ^{125}I they obtained a non-cell-permeable epoxysuccinyl peptide (MB-074) with relatively poor affinity in comparison with our *endo*-epoxysuccinyl peptide **8** (MB-074: $k_2/K_i = 2750 \pm 450$; peptide **8**: $k_2/K_i = 1\,520\,000 \pm 88\,800$). In contrast, our novel design concept (Fig. 1) allows functionalization with a wide variety of non-radioactive labels or effector molecules [19] without loss of inhibitory potency and selectivity, because interaction of the inhibitor with the active site is virtually unaffected by the label that is located outside. In future, this concept of double-headed epoxysuccinyl peptide inhibitors may be further exploited to meet the specificity requirements of other cathepsins, particularly cathepsin X.

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