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Substrate/propeptide-derived endo-epoxysuccinyl peptides as highly potent and selective cathepsin B inhibitors

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Abstract Based on recent information about the anti-substrate binding mode of the propeptide portion of procathepsin B and the well established substrate-like binding of epoxysuccinyl-dipeptide carboxylates to the S′ subsites of cathepsin B a new endo-*trans*-epoxysuccinyl peptide was synthesized that contains the dipeptide moiety Leu-Pro-OH for the P1′–P2′ substrate positions and the tripeptide moiety Leu-Gly-Gly-OMe (sequence portion 46–48 of the propeptide) for the P2–P4 positions in anti-substrate orientation. With an unequivocal (2*S*,3*S*) configuration this new *trans*-epoxysuccinyl peptide derivative was found to inhibit cathepsin B with an apparent second-order rate constant of 1 520 000 M^{-1} s^{-1} which represents so far the most potent inhibitor among E-64-derived compounds. Conversely, the (2*R*,3*R*) diastereomer exhibited a significantly lower inhibition potency. This observation fully agrees with our previous findings that inhibitor/enzyme interactions at the S subsites are favored by the (2*S*,3*S*) and reverse interactions at the S′ subsites by the (2*R*,3*R*) configuration of the *trans*-epoxysuccinyl moiety.

Key words: Epoxysuccinyl peptide; Configuration; Inhibitor; Cysteine proteinase; Cathepsin B; Cathepsin L; Selective inhibition

1. Introduction

Lysosomal cysteine proteases are known to play important roles in intracellular protein degradation [1] and antigen processing [2], but have also been implicated in various pathological processes like bone resorption [3], tumor invasion and metastasis [4], cartilage degradation in arthritis [5] and muscular dystrophy [6]. This major role in pathological conditions makes them promising targets for inhibitor development. Among these lysosomal enzymes cathepsin B represents a major component that exhibits both endopeptidase and exopeptidase activities. The design of highly selective inhibitors of the cathepsin class of cysteine proteases was found to be difficult because of their broad substrate specificity [7]. Nevertheless, some success was obtained with synthetic analogs of the natural product (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucyl-agmatine (E-64), which is a very powerful irreversible inhibitor of cysteine proteases although of limited selectivity [8,9].

Valuable information on the mode of binding of these *trans*-epoxysuccinyl-based inhibitors is meanwhile available from the X-ray structures of their adducts to actinidin [10], papain [11–13] and cathepsin B [14,15], thus enabling a structure-based design of new inhibitors of enhanced affinity and selectivity. In this context we have recently synthesized a series of *trans*-epoxysuccinyl peptide derivatives both in the (2*R*,3*R*) and (2*S*,3*S*) configuration in order to analyze the role of the stereochemistry of this residue in dictating inhibitory potency and selectivity for cysteine proteases [16]. We confirmed that binding of E-64-type *trans*-epoxysuccinyl-based inhibitors to the S subsites of the enzymes is highly favored by the (2*S*,3*S*) configuration, whereas CA030-type inhibitors that exploit interactions with the occluding loop of cathepsin B for preferential binding to the S′ subsite [14,15] are stronger inhibitors in the (2*R*,3*R*) configuration than the related (2*S*,3*S*) diastereomers. Consequently, the design of bis-peptidyl derivatives of the *trans*-epoxysuccinic acid to possibly increase the selectivity of this class of inhibitors is hampered by the non-additivity of the configurational requirements for optimal interaction with both the S and S′ subsites [16].

The cysteine proteases are produced in vivo as inactive precursors that contain an additional N-terminal propeptide extension. This propeptide has been shown to act as a potent and highly selective inhibitor of the mature enzymes [17,18]. Recently the X-ray structures of rat [19] and human procathepsin B [20] have been reported which revealed binding of the propeptide segment in anti-substrate mode to the active-site cleft of the enzyme. A superposition of E-64, as structured in the actinidin [10] and papain [11,12] adducts, to the propeptide segment bound to the substrate binding site of the enzyme revealed a shift of the E-64 relative to the propeptide within the binding site by approximately one bond length, a fact which results from the four-carbon backbone of the succinyl moiety vs. the three-atom backbone of an amino acid residue [19]. While in E-64 the leucine side chain occupies the S2 subsite near Leu-46 of the propeptide, the related P2 residue of the propeptide, i.e. Thr-44, is shifted towards the edge of the S2 pocket in the direction of the active site. Moreover, it is well established that the peptidyl dipeptidase activity of cathepsin B is dependent on the presence of the occluding loop with the two His residues 110 and 111 accepting the negative charge of the P2′ carboxylate [14,15]. Therefore, to mimic the reverse propeptide binding to the S subsites as well as the normal substrate binding to the S′ subsites the endo-*trans*-epoxysuccinyl peptide, shown in Fig. 1, was designed where (i) the dipeptide moiety Leu-Pro-OH was selected for the P1′–P2 substrate positions in agreement with previous positive experiences with related *trans*-epoxysuccinyl derivatives [8,9,16] and (ii) the tripeptide moiety Leu-Gly-Gly-OMe (sequence portion 46–48 of the propeptide) was selected for the P2–P4 positions in anti-substrate orientation.

2. Materials and methods

2.1. Materials

Solvents and reagents for the synthesis were of the highest quality

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commercially available. Papain (EC 3.4.22.2) from Boehringer (Mannheim) was rechromatographed on S-Sepharose as described [21]. Human cathepsin B (EC 3.4.22.1) and cathepsin L (EC 3.4.22.15) were purchased from Calbiochem (Bad Soden/Taunus), Z-Phe-Arg-NH-Mec from Bachem (Heidelberg), E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (**2a**) and all other chemicals from Sigma. NMR spectra were recorded on a Bruker AMX500 spectrometer and FAB-MS spectra on a Finnigan MAT 900. TLC was carried out on silica gel 60 plates (Merck AG, Darmstadt) and analytical HPLC with Waters equipment on Nucleosil 300/C18 (Machery and Nagel, Düren) using a linear gradient of acetonitrile/2% H_3PO_4 from 95:5 to 20:80 in 30 min.

2.2. Synthesis of the trans-epoxysuccinyl-peptides

The *trans*-epoxysuccinyl (*t*Eps) peptides HO-(2*R*,3*R*)-*t*Eps-Leu-Agm (**2b**), EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**3a**) and EtO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**3b**) were synthesized as described previously [16].

2.2.1. MeO-Gly-Gly-Leu-(2S,3S)-tEps-Leu-Pro-OH (**1a**). HO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OtBu [16] was reacted overnight at room temperature with H-Leu-Gly-Gly-OMe (1.2-fold excess) in chloroform by ECD/HOBt. The solvent was evaporated and the residue was washed in ethyl acetate successively with 5% $KHSO_4$, 5% $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$ and evaporated to dryness; yield: 95%; homogeneous on TLC ($CHCl_3$/MeOH, 4:1; R_f 0.75) and HPLC (t_R 21.0 min); ^{1}H-NMR (DMSO-d_6) spectrum: consistent with the assigned structure.

The product was deprotected by exposure to 20% trifluoroacetic acid in $CHCl_3$ for 24 h at room temperature. The solvent was evaporated and the residue precipitated from ethyl acetate with petroleum ether; yield: 83%; homogeneous on HPLC (t_R 16.5 min); ^{1}H-NMR (DMSO-d_6) δ = 0.82–0.95 (m, 12H, 2×δ_1CH_3 Leu, 2×δ_2CH_3 Leu), 1.40–1.70, 1.84, 1.93, 2.15 (4 m, 10H, 2×βCH_2 Leu, 2×γCH Leu, βCH_2 Pro, γCH_2 Pro), 3.63 (s, 3H, OCH_3), 3.48–3.79 (br. m, 6H, δ_1CH_2 Pro, δ_2CH_2 Pro, 2×CH *t*Eps, αCH_2 Gly), 3.86 (m, 2H, (αCH_2 Gly), 4.26 (m, 1H, αCH Pro), 4.38 (m, 1H, αCH Leu), 4.62 (m, 1H, αCH Leu), 8.18 (m, 1H, NH Gly), 8.36 (m, 1H, NH Gly), 8.55 (d, 1H, J = 8.1 Hz, NH Leu), 8.71 (d, 1H, J = 8.1 Hz, NH Leu); FAB-MS: *m/z* 584.2 $[M+H]^+$; calcd. for $C_{26}H_{41}N_5O_{10}$: 583.

2.2.2. MeO-Gly-Gly-Leu-(2R,3R)-tEps-Leu-Pro-OH (**1b**). The title compound was prepared from HO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OtBu and H-Leu-Gly-Gly-OMe as described for **1a**; yield: 90% over the two steps; homogeneous on HPLC (t_R 16.5 min); ^{1}H-NMR (DMSO-d_6) δ = 0.81–0.94 (m, 12H, 2×δ_1CH_3 Leu, 2×δ_2CH_3 Leu), 1.40–1.70, 1.84, 1.93, 2.16 (4 m, 10H, 2×βCH_2 Leu, 2×γCH Leu, βCH_2 Pro, γCH_2 Pro), 3.51 (m, 1H, δ_2CH_2 Pro), 3.63 (m, 5H, 2×CH *t*Eps, OCH_3) 3.71 (m, 1H, δ_1CH_2 Pro), 3.73 (d, 2H, J = 5.8 Hz, αCH_2 Gly), 3.85 (m, 2H, αCH_2 Gly), 4.25 (m, 1H, αCH Pro), 4.33 (m, 1H, αCH Leu), 4.57 (m, 1H, αCH Leu), 8.18 (m, 1H, NH Gly), 8.29 (m, 1H, NH Gly), 8.58 (d, 1H, J = 8.1 Hz, NH Leu), 8.66 (d, 1H, J = 8.0 Hz, NH Leu); FAB-MS: *m/z* 584.2 $[M+H]^+$; calcd. for $C_{26}H_{41}N_5O_{10}$: 583.

2.3. Enzyme inhibition assays

Continuous fluorometric inhibition assays were performed and evaluated as described in detail elsewhere [21,22]. Inhibition of papain (10 pM), cathepsin B (27 pM) and cathepsin L (5 pM) were assayed at 30°C with the substrate Z-Phe-Arg-NH-Mec (for cathepsin B 10 μM, K_m = 220 μM; for papain 10 μM, K_m = 52 μM; for cathepsin L 4 μM, K_m = 2.9 μM) in 1.2 ml of 0.25 M sodium acetate buffer pH 5.5, containing 2 mM EDTA, 0.015% Brij-35, 1 mM dithiothreitol (added fresh), 1% DMSO (from added substrate solution). The inhibitors were dissolved in DMSO or water (10 mM), diluted in buffer or DMSO and added in 1–10 μl to the preactivated enzymes after a constant substrate release was observed. The final inhibitor concentrations in the assay were in the range of 0.6 nM to 30 nM in order to achieve almost complete inhibition within 15–90 min.

The apparent pseudo-first-order rate constant k_{obs} was obtained from the pre-steady-state phase of the progress curve as described by Morrison [23] and Knight [24] by non-linear regression analysis of the collected data using the commercial program FigP (Biosoft, Cambridge). The apparent second-order rate constants k_2/K_i were then calculated as $k_2/K_i = k_{obs}/[I]$ when k_{obs} increased linearly with increasing inhibitor concentration ($[I] \ll K_i$). Substrate depletion was < 5% within the duration of the experiments; a correction was made for competition of the inhibitors with the substrate.

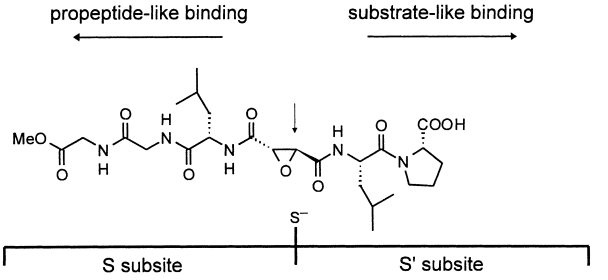

Fig. 1. Schematic presentation of the proposed binding mode of **1a**; S^- symbolizes the side chain thiolate of Cys-29, and the arrow indicates the oxirane carbon where the nucleophilic attack of Cys-29 should occur according to Yamamoto et al. [15].

3. Results and discussion

Using diethyl L-(+)- and D-(−)-tartrate as starting compounds for the synthesis of the (2*R*,3*R*)- and (2*S*,3*S*)-*trans*-epoxysuccinic acid intermediates [16,25] the two epoxysuccinyl peptides **1a** and **1b** were obtained as homogeneous compounds and well defined in the configuration at the C2 and C3 atoms of the *trans*-epoxysuccinyl group as the (2*S*,3*S*) and (2*R*,3*R*) diastereomer, respectively. The inhibition of papain, cathepsin B and cathepsin L by these two new compounds was compared to the inhibitory potencies of (2*S*,3*S*)-E-64 (natural compound) (**2a**) and (2*R*,3*R*)-E-64 (**2b**), and of (2*S*,3*S*)-EtO-*t*Eps-Leu-Pro-OH (**3a**) and (2*R*,3*R*)-EtO-*t*Eps-Leu-Pro-OH (**3b**), determined previously [16].

As shown in Table 1, extension of the inhibitors **3a,b** which most probably bind to the enzymes in the CA030 mode, i.e. to the S′ subsites [14], with the propeptide portion Leu-Gly-Gly-OMe, expected to bind to the S subsites, leads to strongly differentiated effects depending upon the configuration of the *trans*-epoxysuccinyl moiety. Compound **1a** with the (2*S*,3*S*) configuration was found to be the most potent epoxysuccinyl-based inhibitor of cathepsin B known so far, whereas

Table 1
Second-order rate constants of inactivation of cysteine proteases by (2*S*,3*S*)- and (2*R*,3*R*)-*trans*-epoxysuccinyl peptides

Inhibitor	Papain k_2/K_i (M^{-1} s^{-1})	Cathepsin B k_2/K_i (M^{-1} s^{-1})	Cathepsin L k_2/K_i (M^{-1} s^{-1})	Ratio CB/PA	Ratio CB/CL
MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**1a**)	14 800 ± 1 077	1 520 000 ± 88 800	1 204 ± 29	103	1 262
MeO-Gly-Gly-Leu-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**1b**)	870 ± 65	214 600 ± 11 980	269 ± 23	247	798
HO-(2*S*,3*S*)-*t*Eps-Leu-Agm, E-64 (**2a**)	869 000 ± 23 500	81 400 ± 2 760	43 800 ± 4 390	0.09	1.9
HO-(2*R*,3*R*)-*t*Eps-Leu-Agm (**2b**)	86 000 ± 3 260	1 170 ± 40	4 930 ± 183	0.01	0.2
EtO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**3a**)	6 130 ± 55	44 400 ± 1 690	170 ± 17	7.2	260
EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**3b**)	56	567 000 ± 21 500	26	10 100	21 800

k_2/K_i for compounds **1a** and **1b** were determined at pH 5.5 in 250 mM sodium acetate buffer ± S.E.M. from 5–10 experiments and are corrected for substrate competition. The inhibitory potencies of compounds **2a**, **2b**, **3a** and **3b** reported previously [16] are listed for comparison.

the related (2*R*,3*R*) diastereomer **1b** exhibited a significantly lower inhibitory potency than the S′ sites binding **3b**. This would strongly support our previous observation that inhibitor-enzyme interactions at the S subsites are favored by the (2*S*,3*S*) and reverse interactions at the S′ subsites by the (2*R*,3*R*) configuration [16]. Thus, in the case of both inhibitors **1a** and **1b** the binding contributions to the S subsites apparently prevail over those to the S′ subsites. This is further supported by the lower selectivity of **1a** and **1b** for cathepsin B vs. cathepsin L than **3b**. However, only X-ray analysis of the complexes of cathepsin B with the two new diastereomers could confirm whether a loss or weaker interaction of the dipeptide carboxylate moiety with the occluding loop, the characteristic structural feature of cathepsin B, is responsible for the observed lower selectivity.

Because of the extremely high inhibitory potency of the new *trans*-epoxysuccinyl compound **1a** with its k_2/K_i value of 1 520 000 M^{-1} s^{-1} and selectivity ratio of 1260 vs. cathepsin it represents a promising tool for selective inhibition of cathepsin B both in vitro and in vivo.

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