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Original article

Probing of primed and unprimed sites of calpains: Design, synthesis and evaluation of epoxysuccinyl-peptide derivatives as selective inhibitors

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ABSTRACT

Calpains are intracellular cysteine proteases with important physiological functions. Up- or down-regulation of their expression can be responsible for several diseases, therefore specific calpain inhibitors may be considered as promising candidates for drug discovery. In this paper we describe the synthesis and characterization of a new class of inhibitors derived from the analysis of amino acid preferences in primed and unprimed sites of calpains by incorporation of L- or D-epoxysuccinyl group (Eps). Amino acids for replacement were chosen by considering the substrate preference of calpain 1 and 2 enzymes. The compounds were characterized by RP-HPLC, amino acid analysis and ESI-MS. Selectivity of the compounds was studied by using calpain 1 and 2; and cathepsin B. We have identified five calpain specific inhibitors with different extent of selectivity. Two of these also exhibited isoform selectivity. Compound NH_2-Thr-Pro-Leu-(D-Eps)-Thr-Pro-Pro-Pro-Ser-NH_2 proved to be a calpain 2 enzyme inhibitor with at least 11.8-fold selectivity, while compound NH_2-Thr-Pro-Leu-(L-Eps)-Ser-Pro-Pro-Pro-Ser-NH_2 possesses calpain 1 enzyme inhibition with at least 4-fold selectivity. The results of molecular modeling calculations suggest that the orientation of the bound inhibitor in the substrate binding cleft is markedly dependent on the stereochemistry of the epoxysuccinyl group.

1. Introduction

The members of calpain family are intracellular Ca^{2+}-ion dependent cysteine proteases [1,2] which are present in almost all eukaryotes and also in some bacteria. The two ubiquitous forms are the calpain 1 (μ-calpain) and calpain 2 (m-calpain). Calpains have important role in various cellular activities (cell adhesion and motility, signal transduction, exocytosis and regulation of gene expression etc.) [3–7]. Over-activated calpains could be involved in the pathogenesis of a wide range of disorders such as Alzheimer's, Huntington's and Parkinson's diseases, multiple sclerosis, amyotrophic lateral sclerosis or ischemic and traumatic brain injury [8,9].

Most of the peptide- and non-peptide-based calpain inhibitors interact only with S_n positions of the active site [10], although expanding the binding surface towards S_n' sites may result in more selective inhibitors. Design of inhibitors by studying the preference of amino acids in certain positions of calpain substrates can be considered as a promising strategy. Tompa et al. analyzed numerous cleavage sites in calpain substrates, and established a matrix containing preferences of amino acids in positions P_4–P_7' [11]. Based on this matrix, a FRET calpain substrate with excellent characteristics, and also its cell-penetrating derivative was successfully prepared and applied to monitor intracellular calpain activity [12,13].

Based on this substrate sequence, we have designed a group of azaglycine (Agly) containing analogues as potential novel calpain inhibitors [14]. In these compounds Lys in position P_1 has been changed to azaglycine.

The epoxysuccinyl group (Eps) can be used to design inhibitors with different amino acids at P_n and P_n' sites. Some synthetic and

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natural peptide epoxides have already been described as proven cysteine protease inhibitors [15].

The *trans*-L-epoxylsuccinyl-L-leucylamido-4-guanidino-butane (E-64) was first published as an effective and selective cysteine protease inhibitor possessing epoxy group [16]. This compound and its derivatives are irreversible inactivators of cysteine proteases. Different parts of E-64 were varied to improve its inhibitory effect on calpains [17].

Using L- or D-epoxysuccinyl analogues the effect of changes in positions P_4–P_2 was studied on calpain 1 and 2 inhibition [18]. As a result of this systematic study a peptide analogue, WRH(D-Eps)-OEt was identified as a selective and irreversible calpain inhibitor.

Pfizer et al. attached different dipeptides to the free carboxylic group of Ep-460 (HO-(L)Eps-Leu-NH-$[CH_2]_4$-NH-Z; e.g. HO-AA_1-AA_2-(L)Eps-Leu-NH-$[CH_2]_4$-NH-Z), and studied the inhibitory activity on calpain 1, cathepsin B and cathepsin L [19]. Ep-460 was supposed to direct the dipeptide part to the primed position in the substrate binding pocket. The amino acid preference was examined using amino acid libraries of positions P_1' and P_2'.

Recently, Schiefer et al. reported E-64 analogues as potent calpain 1 selective inhibitors [20]. The capping group of E-64 (4-guanidino-butane) at position P_3/P_4 and Leu at position P_2 were changed. The selectivity of these compounds was characterized using papain.

In the present paper we describe the design, synthesis and functional characterization of novel potential peptide inhibitors with epoxysuccinyl group of cysteine proteases calpain 1 and 2, and cathepsin B. Molecular modeling was also performed to understand the binding of inhibitors with L- or D-epoxysuccinyl group. The presented peptide analogues are based on the sequence of calpain substrate peptide (TPLKSPPPSPR), containing different amino acids at positions P and P′, derived from the preference matrix [11]. By incorporation of L- or D-epoxysuccinyl group, the amino acid preference at positions P and P′ was scanned to identify novel calpain inhibitors.

2. Materials and methods

All amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland) and Reanal (Budapest, Hungary); whereas DIEA, HOBt, DIC, TFA were FLUKA (Buchs, Switzerland) products. Rink-amide MBHA resin was from Iris Biotech GmbH (Marktredwitz, Germany). Racemic *trans*-L/D epoxysuccinic acid was obtained from TCI (Tokyo, Japan). Solvents for synthesis and purification were obtained from Molar Chemicals Kft (Budapest, Hungary). The fluorescent substrates Suc-Leu-Tyr-AMC (catalog no. S 1153) and all other chemicals used in biochemical experiments were purchased from Sigma. The calpain 1 enzyme from human erythrocytes (EC 3.4.22.52) and cathepsin B from human liver (EC 3.4.22.1), as well as the substrate III of cathepsin B (Z-Arg-Arg-AMC × 2 HCl) were obtained from Calbiochem/Merck (Darmstadt, Germany). All buffers were prepared with ion exchanged distilled water.

2.1. Synthesis of tripeptides and L- or D-epoxysuccinyl-pentapeptides

The resolution of *trans*-L- and D-epoxysuccinic acid isomers using racemic starting material was performed as described by Tamai et al. [21] without modification. Yield of resolution was 41% for the D-enantiomer and 46% for the L-enantiomer. The tripeptide segments were synthesized manually by solid phase peptide synthesis on Rink-amide MBHA resin (0.2 g, 0.52 mmol/g) using Fmoc/^{t}Bu strategy. The amino acid side-chain protecting groups were trityl for Gln and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg. The side chain of Ser and Thr was protected with *tert*-butyl group. The N^{α}-Fmoc group was removed with 2% DBU in the presence of 2% piperidine in DMF (2, 2, 5, 10 min) followed by washing with DMF (8 × 0.5 min). For coupling, amino acid derivatives, DIC and HOBt dissolved in DMF were used in 3-fold molar excess for the resin capacity. The reaction proceeded at RT for 60 min, then the resin was washed with DMF.

The epoxysuccinyl-pentapeptides were built up also on Rink-amide MBHA resin (0.2 g, 0.52 mmol/g) by the same coupling strategy. After the removal of the terminal N^{α}-Fmoc protection, the *trans*-epoxysuccinyl group was attached to the pentapeptide chain by L- or D-*trans*-epoxysuccinic acid using DIC and HOBt. The tripeptides as well as the epoxysuccinyl-pentapeptides were removed from the resin by cleavage with 5 mL TFA containing 0.25 mL distilled water. In case of Trp containing tripeptide segments the cleavage was performed by TFA in the presence of scavengers (TFA, water, thioanisole, EDT, crystalline phenol = 5 mL, 0.25 mL, 0.25 mL, 0.125 mL, 0.375 g). The crude product was precipitated by dry diethyl ether, dissolved in 10% acetic acid, freeze-dried and separated by RP-HPLC. Yield of peptide synthesis was 35–40%.

2.2. Synthesis of epoxysuccinyl-nonapeptide analogues

The purified epoxysuccinyl-pentapeptides and the tripeptides were coupled in solution (DMF). 10 mg (0.01 mmol) epoxysuccinyl-pentapeptide was reacted with 1.2 equivalent tripeptide in the presence of 1.2 equivalent DIC and HOBt. The reaction proceeded at RT overnight, then the DMF was evaporated and the components of the reaction mixture were separated by RP-HPLC. Yield of the conjugation reactions was 28–35%.

2.3. RP-HPLC

Analytical RP-HPLC was performed on a Zorbax SB C18 column (150 × 4.6 mm I.D.) with 5 μm silica (100 Å pore size) (Torrance, CA USA) as a stationary phase. A linear gradient elution was developed: 0 min 0% B; 2 min 0% B; 22 min 90% B with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20, v/v)). A flow rate of 1 mL/min was used at ambient temperature. Samples were dissolved in eluent B, injection volume: 20 μL. Peaks were detected at $\lambda = 220$ nm. The purification of the crude products was carried out by RP-HPLC using Phenomenex Jupiter C18 column (250 × 10 mm I.D.) with 10 μm silica (300 Å pore size) (Torrance, CA, USA), flow rate: 4 mL/min, room temperature. Linear gradient elution was applied. Gradient I: 0 min 5% B, 5 min 5% B, 50 min 50% B. Gradient II: 0 min 10% B, 5 min 10% B, 50 min 70% B.

2.4. Amino acid analysis

The amino acid composition of the peptides was determined by amino acid analysis performed on a Sykam Amino Acid S433H analyser (Eresing, Germany). Prior to analysis, samples were hydrolyzed with 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h.

2.5. Mass spectrometry

The peptides and peptide analogues were identified by electrospray ionization mass spectrometry (ESI-MS) on a Bruker Daltonics Esquire 3000 Plus (Bremen, Germany) ion trap mass spectrometer, operating in continuous sample injection at 4 μL/min flow rate. Samples were dissolved in ACN–water (50:50 v/v%) mixture containing 0.1 v/v% AcOH. Mass spectra were recorded in positive ion mode in the m/z 50–2000 range.

2.6. Enzyme purification

The 80-kDa large subunit and the 21-kDa truncated small subunit of rat calpain 2 were expressed in *Escherichia coli* as described

earlier [22] and purified with Akta Explorer (GE Healthcare) fast protein liquid chromatography (FPLC) system, on a HisTrap HP column using step elution. Purified calpain 2 was dialyzed against 10 volume of calpain buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH = 7.5). The enzyme preparation in solution was stored at 4 °C until use.

2.7. Enzyme inhibition assay

Activity of the enzymes (calpain 1 and 2, cathepsin B) was measured using a Synergy microplate reader, on a 96-well plate. In case of the calpains, the reaction mixture contained 100 μM Suc-LY-AMC (Sigma) substrate, 3 mM Ca^{2+} and the inhibitor at various concentrations in 50 μL of calpain buffer. The reaction was initiated by adding and mixing rapidly 0.2 μM calpain 2 or 0.5 μM calpain 1. The cathepsin B activity was measured in 50 μL of 0.25 mM MES buffer (pH = 5.0) containing 100 μM cathepsin B substrate III and inhibitor at various concentrations. The reaction was started by adding and mixing rapidly 0.7 nM cathepsin B. Cathepsin B was activated in 0.25 mM MES buffer containing 10 mM DTE. The enzyme activity was followed by the change of fluorescence intensity measured at excitation/emission wavelengths of λ = 380/460 nm. Three replicates of each experiment were performed. The kinetic data were obtained from the pre-steady-state phase of the progress curve as described previously, accepting the method of previous authors for calculation of their epoxy-dipeptide inhibitors [19,23,24].

2.8. Molecular modeling

A model structure for the calpain 2 complex with inhibitor **2** and **11** was built. Starting structure was derived from the crystal structure of the calpain 2–calpastatin complex [25], using domains II and III of the large catalytic unit, fitting both inhibitors to the calpastatin active region (612–613) in two opposing orientations, resulting in 4 different arrangements. The mutated catalytic residue (Ser105) was re-mutated *in silico* to its wild type equivalent of Cys. Monte Carlo Multiple Minimum (MCMM) searches were carried out by involving the random variation (within the range of 0–180°) of a randomly selected subset of all torsional angles of the inhibitor, and the random translation (0–5 Å) and rotation (0–180°) of the inhibitor within the enzyme matrix in a Monte Carlo step. The perturbed structures were energy-minimized and the unique structures were stored within a 40 kJ/mol energy window above the global minimum. Calculations were carried out using the OPLS 2005 forcefield [26]. Solvent-effect was modeled by the GB/SA algorithm (using water solvent). The first search of 3000 steps was restrained, where the distance of the SG atom of Cys105 and C2 and C3 of the epoxy-ring was held between 2.9 and 5.5 Å using a flat-bottom constraint, while energy minimization was carried out on all inhibitor atoms and the side chain atoms of all residues reaching within 6 Å of the inhibitor. In a second calculation, 1000 steps of constraint free MCMM search (Monte Carlo steps involving the inhibitor, but energy minimization carried out on the entire structure) was conducted on the lowest energy arrangement of each restrained search. The unbound inhibitors were also subject to 10,000 steps constraint-free MCMM search to be able to estimate the binding energy.

3. Results

3.1. Synthesis and chemical characterization of epoxysuccinyl-peptide analogues

We demonstrated earlier that oligopeptides with 9–11 amino acid residues as calpain inhibitors can be identified based on an 11-mer calpain substrate sequence (TPLKSPPPSPR) [11]. In the group of previously published inhibitory peptides, lysine at position P_1 was replaced with azaglycine [26]. Here we report on the design and synthesis of a novel group of oligopeptide analogues containing D- or L-*trans*-epoxysuccinyl-group (D-Eps, L-Eps) (Table 1). In these 9-mer compounds of TPLKSPPPS sequence the Lys residue at position P_1 was replaced by the epoxysuccinyl moiety.

The synthesis of epoxysuccinyl-nonapeptide analogues, as outlined in Scheme 1, was carried out by the combination of the separate solid phase peptide synthesis of the two segments, and their conjugation in solution. The *N*-terminal tripeptide amide (P_4–P_2) as well as the *N*-epoxysuccinyl-pentapeptide amide (P_1'–P_5') were prepared on Rink-amide MBHA solid phase using Fmoc/^{t}Bu strategy. The epoxysuccinyl group was attached to the *N*-terminal amino group of the pentapeptide on solid phase using L- or D-*trans*-epoxysuccinic acid. After removal from the resin, the purified free tripeptide amide and the purified epoxysuccinyl-pentapeptide amide were conjugated in solution by *in situ* activation using DIC–HOBt coupling reagents. The epoxysuccinyl-nonapeptides were purified by semipreparative RP-HPLC. It should be noted that no significant ring opening was observed in case of epoxysuccinyl-pentapeptide analogues during the cleavage from the resin by TFA in the presence of a small amount of water. The homogeneity of the purified compounds was characterized by analytical RP-HPLC (in Supplementary information), while the amino acid composition and primary structure were verified by ESI-MS (Table 1) and amino acid analysis (in Supplementary information).

The common feature of this group of nonapeptide analogues is that the P_4–P_2 segment (e.g. *H*-Leu-Pro-Thr-*NH*$_2$) as well as the pentapeptide unit (e.g. *H*-Ser-Pro-Pro-Pro-Ser-*NH*$_2$) are attached with their *N*-terminal amino group to the epoxysuccinyl group, so all sequences have a C–N (epoxysuccinyl) N–C orientation in their structure.

3.2. Inhibition of the activity of calpain 1 and 2

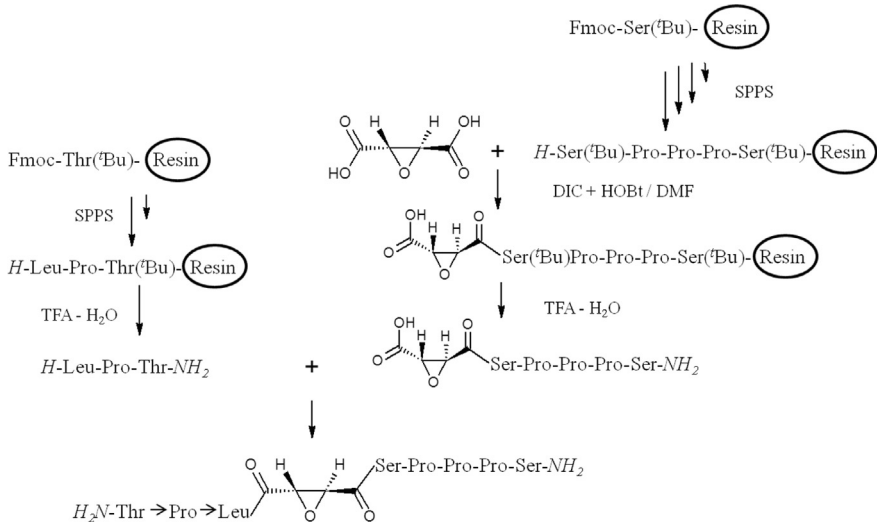
The inhibitory potential of epoxysuccinyl-peptides was studied on calpain 1 and 2 using Suc-LY-AMC as substrate, and it was

Table 1
Chemical characteristics of epoxysuccinyl-nonapeptides.

Compound	R_t[a] (min.)	*M* (cal.)	*M*[b] (meas.)
1. *NH*$_2$-Thr-Pro-Leu-(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	12.5	906.5	906.5
2. *NH*$_2$-Thr-Trp-Leu-(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	12.6	995.8	995.6
3. *NH*$_2$-Thr-Ser-Leu-(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	10.3	884.4	884.7
4. *NH*$_2$-Thr-Pro-Thr-(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	9.7	882.5	882.4
5. *NH*$_2$-Thr-Pro-Val-(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	10.7	892.7	892.6
6. *NH*$_2$-Thr-Pro-Leu-(L-Eps)-Arg-Pro-Pro-Pro-Ser-*NH*$_2$	11.1	976.0	976.7
7. *NH*$_2$-Thr-Pro-Leu-(L-Eps)-Thr-Pro-Pro-Pro-Ser-*NH*$_2$	11.0	921.8	921.5
8. *NH*$_2$-Thr-Pro-Leu-(L-Eps)-Ser-Ser-Pro-Pro-Ser-*NH*$_2$	12.0	896.5	896.5
9. *NH*$_2$-Thr-Pro-Leu-(L-Eps)-Ser-Gln-Pro-Pro-Ser-*NH*$_2$	10.8	937.5	937.5
10. *NH*$_2$-Thr-Pro-Leu-(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	10.9	906.5	906.7
11. *NH*$_2$-Thr-Trp-Leu-(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	13.2	995.8	995.7
12. *NH*$_2$-Thr-Pro-Thr-(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	9.1	882.5	882.7
13. *NH*$_2$-Thr-Pro-Val-(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	10.2	892.5	892.5
14. *NH*$_2$-Thr-Ser-Leu-(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$	10.2	884.4	884.7
15. *NH*$_2$-Thr-Pro-Leu-(D-Eps)-Arg-Pro-Pro-Pro-Ser-*NH*$_2$	11.1	976.0	975.9
16. *NH*$_2$-Thr-Pro-Leu-(D-Eps)-Thr-Pro-Pro-Pro-Ser-*NH*$_2$	12.3	921.8	921.8
17. *NH*$_2$-Thr-Pro-Leu-(D-Eps)-Ser-Ser-Pro-Pro-Ser-*NH*$_2$	11.9	896.5	896.5
18. *NH*$_2$-Thr-Pro-Leu-(D-Eps)-Ser-Gln-Pro-Pro-Ser-*NH*$_2$	10.7	937.5	937.5

[a] HPLC retention time; column: Zorbax SB C18 column (150 × 4.6 mm I.D.) with 5 μm silica (100 Å pore size). Linear gradient elution: 0 min 0% B; 2 min 0% B; 22 min 90% B with eluent A (0.1% TFA in water), and eluent B (0.1% TFA in acetonitrile–water (80:20, v/v)); flow rate: 1 mL/min, ambient temperature. Peaks were detected at λ = 220 nm.

[b] ESI-MS analysis was carried out on a Bruker Esquire 3000 plus (Germany). The sample was dissolved in acetonitrile–water (50:50, v/v), 0.1% acetic acid.



Scheme 1. Outline of the synthesis strategy of epoxysuccinyl-peptide analogue.

characterized by K_i values (Table 2). If there was no inhibition at 50 μM concentration, the K_i value was not calculated.

Concerning the activity of epoxysuccinyl-peptides with L-Eps isomer on calpains, we found that compound **1** with the L-trans-epoxysuccinyl group had no effect on calpain 2 even at $c = 50$ μM, but had moderate effect on calpain 1 ($K_i = 12.50$ μM). Substitution of Pro at position P_3 with Trp (**2**) or Ser (**3**) resulted in compounds with inhibitory activity on both calpain 2 ($K_i = 4.05$ and 14.3 μM, respectively) and calpain 1 ($K_i = 17.14$ and 21.92 μM, respectively). The effect of replacement of Leu residue at position P_2 with Thr (**4**) had not effect on the activity, while the presence of Val (**5**) resulted in a peptide analogue with moderate inhibitory effect only on calpain 2 ($K_i = 29.23$ μM). Moderate activity on calpain 2 ($K_i = 17.86$ μM) and also on calpain 1 ($K_i = 23.73$ μM) was observed after incubation of the enzymes with compound **6** with Arg residue at position P_1'. Compound **7** with Thr at position P_1' exhibited good, but not excellent effect on calpain 1 ($K_i = 8.38$ μM). Compound with Ser or Thr at position P_2' (**8** or **9**) did not exhibit any activity against calpains.

Table 2
The inhibitory effect of epoxysuccinyl-peptides.

Compound	K_i (μM) (SD)[a]		
	Calpain 2	Calpain 1	Cathepsin B
1	>50[b]	12.50 (3.54)	>50
2.	4.05 (2.10)	17.14 (3.29)	5.00 (2.06)
3.	14.30 (2.70)	21.92 (1.29)	>50
4.	>100	>100	>100
5.	29.23 (5.00)	>50	>100
6.	17.86 (2.45)	23.73 (7.28)	>50
7.	>50	8.38 (0.06)	12.07 (3.02)
8.	>100	>100	>100
9.	>100	>100	>100
10.	>50	>100	>50
11.	21.43 (1.07)	3.70 (0.60)	4.45 (1.79)
12.	no data	>100	no data
13.	>100	>50	>100
14.	no data	30.84 (1.05)	no data
15.	>50	26.33 (1.44)	>100
16.	4.24 (1.85)	>50	>50
17.	>100	>100	>100
18.	>100	>100	>100

[a] Data represent the mean S.D. value of triplicate experiments.
[b] >50, >100: the epoxysuccinyl-peptide did not inhibit the enzyme at 50 or 100 μM concentration.

In order to study the effect of stereochemical orientation related to the presence of D-epoxysuccinic group instead of the L-isomer, nonapeptides with D-isomer were also prepared and examined. Among this group of analogues only compounds **11** and **16** inhibited calpain 2 ($K_i = 21.43$ and 4.24 μM), and compounds **11, 14** and **15** inhibited calpain 1 ($K_i = 3.70$, 30.84 and 26.33 μM).

3.3. Selectivity: inhibition of cathepsin B activity

Compounds with D- or L-epoxysuccinyl-group were also studied for their inhibitory effect on cathepsin B. In both groups of nonapeptide analogues, we found compounds with ability to inhibit the activity of cathepsin B. Epoxysuccinyl-peptides (**2**) and (**7**) with L-Eps ($K_i = 5.00$ and 12.07 μM, respectively), and also compound **11** with D-Eps ($K_i = 4.45$ μM) were active on cathepsin B (Table 2).

3.4. Molecular modeling

Epoxysuccinyl inhibitors have been co-crystallized with calpain 1 (2nqg [18], 2nqi, 2nqg [18], 1tlo [27]), papain (1cvz [28]) and cathepsin B (1ito [29]) previously. These crystal structures show the covalently linked product of the cleavage – the epoxy-ring open and relaxed into an open-chain conformation. However, no small molecular complexes of calpain 2 have been, to date, determined. The calculations carried out here depict the pre-cleavage state of calpain 2 in complex with compound **2** (the most effective inhibitor identified) and compound **11** (its markedly less effective, stereochemical counterpart), in the rate limiting docked state, prior to the nucleophilic attack of Cys.

Peptide NH_2-Thr-Trp-Leu-(L-Eps)-Ser-Pro-Pro-Pro-Ser-NH_2 (**2**) is oriented within the binding grove by 11 intermolecular H-bonds (see Fig. 1): residues P_2–P_4 are linked to Lys61, Gly103, Glu193, Ser196 of domain II of the enzyme, the epoxysuccinyl part is bound by the amide nitrogen of Gly198, P_1' Ser by the carbonyl oxygen of Gly261 of domain II and the P_5' Ser by Asp425 and Met426 of domain III – it is thus bound in a conformation where not the P_2 Leu, but the P_1' Ser is immersed in the S_2 pocket. This is not so surprising, since the S_2 pocket is lined by polar residues (Thr200, Thr201, Ser241, Glu339), whereby it is far from being an ideal docking site for Leu, and although the P_1' Ser is too short to reach these residues, it benefits from the polarized environment created by them. P_2 Leu is stabilized by favorable contacts from Leu102 of the enzyme.

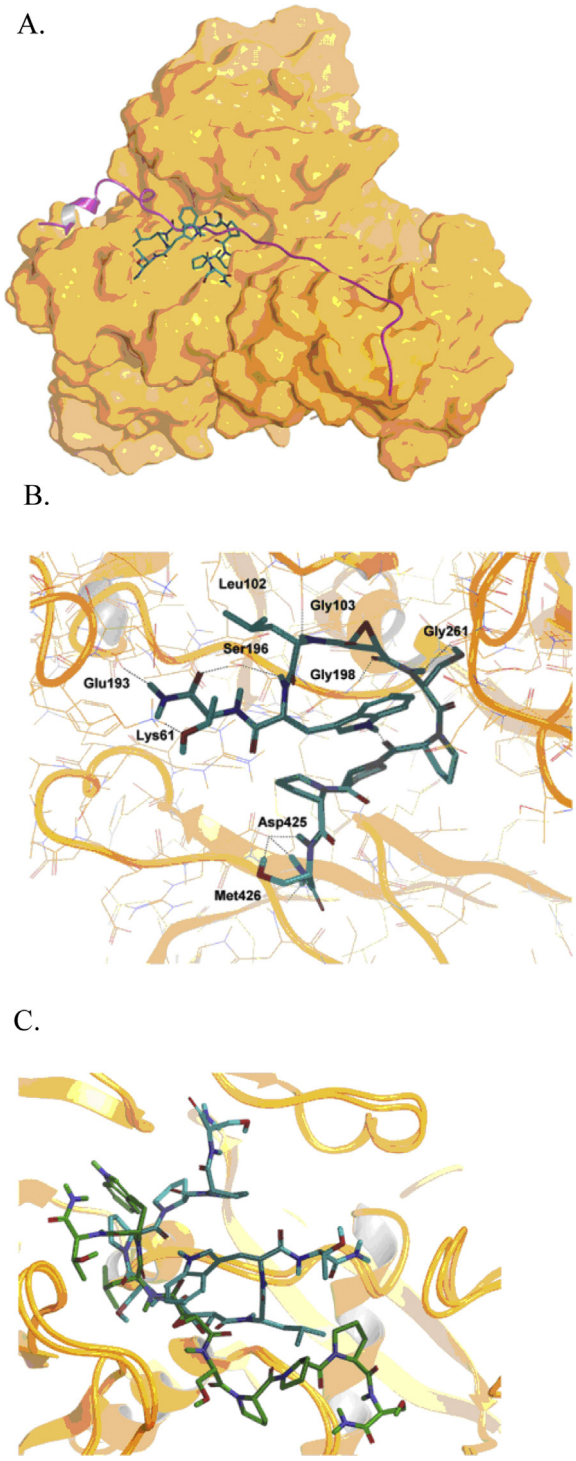


Fig. 1. A. Binding of compound **2** (NH_2-Thr-Trp-Leu-(L-Eps)-Ser-Pro-Pro-Pro-Ser-NH_2) (shown in blue with non-polar hydrogen atoms omitted for clarity) to the substrate binding cleft of calpain 2 (orange surface). The corresponding segment of calpastatin is also shown as a ribbon (purple). B. H-bond network formed by the binding of compound **2**. C. Overlay of compound **2** and compound **11** bound in opposing orientation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The energetically most favorable conformation of compound **11** is of reversed orientation. While the epoxysuccinyl part of the peptide analogue is stabilized by an H-bond from the backbone amide group of Gly198, just as in case of compound **2**, the reversed orientation places the Leu residue in P_2 position in the S_2 pocket. The estimated binding energy for this complex, however, is 31 kJ/mol weaker than that of the calpain 2–compound **2** complex.

Binding mode of both inhibitors is canonical in the sense that the central, epoxysuccinyl part of the molecules is anchored in both cases by an H-bond between the carbonyl oxygen of the inhibitor and the backbone amide of Gly198. Gly198 (and the corresponding Gly208 of calpain 1, Gly66 of papain and Gly74 of cathepsin B) forms a similar H-bond with the carbonyl oxygen atom of the Leu at position P_2 of calpastatin (3df0 [30], ltl9 [27]), or the epoxysuccinyl carbonyl oxygen of E64 (1tlo [27]), E64C (1ito [29]), WR18 (2nqg [18]), WR13 (2nqi [18]) or also of CLIK148 [28].

4. Discussion and conclusions

Over-activated calpain enzymes are involved in several pathological processes in cells. Identification of appropriate inhibitors (effective and selective) may lead compounds for the treatment of diseases associated with over-activation. Such inhibitors could also be useful in studying the function of calpains even in normal cells. Inhibitors capable to distinguish not only between different cysteine proteases, but calpain isoforms could be even more valuable. Our approach to develop selective calpain inhibitors is based on the analysis of the sequences of relevant protein substrates [11].

The most examined part of calpain inhibitors is the P_4–P_1 preference region [10]. Only few studies were reported on the preference of the primed site (P_4'–P_1') of inhibitors. We have selected the epoxysuccinyl functional group as a suitable moiety to examine the effect of amino acids at primed and non-primed sites on the inhibitory activity and selectivity. This group has an important contribution to the inhibitory potency of a cysteine protease selective inhibitor, E-64 [16]. Therefore the incorporation of this functional moiety could result in compounds with selectivity against cysteine proteases.

According to our knowledge, no reports were published so far in which the effect of simultaneous substitutions at both non-primed and primed binding sites were analyzed. We have designed and synthesized compounds with amino acid substitutions considering the substrate preference matrix [11]. Amino acids with the first, the second and the third highest score were incorporated into the substrate sequence, TPLKSPPPSPR. Guided by the results of our earlier study with a group of azaglycine substituted substrate peptides exhibiting inhibitory properties [14], we have prepared a limited set of the analogues for the present investigations.

4.1. The effect of the replacement of Lys at position P_1 by L/D-Eps, and the amino acid substitution on inhibitory activity

As our data suggest, the replacement of Lys residue at position P_1 with L-Eps in the substrate sequence resulted in selective calpain 1 inhibition, while the analogue containing D-Eps did not have any activity. These findings are in agreement with the literature describing that the stereochemistry of the epoxy-group could also have an effect on the activity. The analysis of inhibitory activity of compounds with L- or D-Eps modified sequences showed difference between their potency and selectivity against enzymes, although compounds **2** and **11** with the same amino acid composition possessed similar potency against calpain 1 and 2 as well as cathepsin B. It seems that the binding mode of these compounds makes inhibitory potency insensitive for the stereochemistry of the epoxysuccinyl group. While compound **16** with D-Eps had a marked inhibitory activity exclusively against calpain 2, its analogue with L-Eps, compound **7**, inhibited both calpain 1 and cathepsin B, but not calpain 2.

Our results confirmed the findings of Courrier et al. [18] that at position P_2 the Leu residue was preferred. Although its substitution to Val (**5**) resulted in a moderate calpain 2 inhibitory activity, without inhibiting the other enzymes. Thr (**4**) did not result in inhibition in this position.

The replacement of Pro in position P_3 had dramatic effect on the inhibitory potency. In the L-series, substitution with Trp or Ser, compound **2** and **3** resulted in calpain 2 inhibitors. It should be noted that compound **2** with Trp was found to be the most active nonapeptide analogue. These changes at the same time decreased the inhibitory activity towards the calpain 1 enzyme, and only the Trp analogue (**2**) expressed cathepsin B inhibitory activity. Our observation about the preference of Trp residue at this position is also in harmony with findings in the literature [18]. Trp was the most preferred amino acid next to the Arg at this position, and Pro was one of the most unfavorable. In the D-series, the substitutions had little effect on the inhibitory potency of the original sequence. Only the presence of Trp residue at position P_3 (**11**) and of Thr at position P_1' (**16**) resulted in inhibitory activity. The former compound was active on all three enzymes (calpain 1 and 2, cathepsin B), while the latter was selective on calpain 2. The appearance of Thr residue at position P_1' in the L-Eps analogue (**7**) exhibited an opposite effect. This compound inhibited calpain 1 and cathepsin B, but not calpain 2.

4.2. Comparison of the sequences of inhibitory peptide analogues with azaGly or L/D-Eps

When the sequence of the peptide TPLKSPPPS was modified by incorporation of azaglycine instead of Lys, the compound inhibited both calpain 1 and 2 [14]; while the presence of L/D-Eps in this position resulted in no activity. These observations suggest that the binding mode of two the structures can be different. While the azaglycine modification preserved the manner of substrate binding, the incorporation of L- or D-Eps moiety had no such influence. There are two main differences between the structures of molecules with azaamino acid or with epoxysuccinyl group. Incorporation of an azaamino acid does not alter the length of the peptide backbone, while the presence of an epoxysuccinyl moiety increases the distance with an additional methylene group. The second (major) difference is in the orientation of amino acid side chains originating from the reverse order of amino acid residues. In case of azapeptides only N to C direction occurs in the peptide sequence. In sharp contrast, in compounds with epoxysuccinyl group the orientation of the amide bonds in the "N-terminal" (P_4—P_2 direction) part is reverse. These differences between the azaGly and L-Eps substituted analogues may result in markedly changed inhibitory activity even in case of an "identical" peptide sequence.

4.3. Comparison of selectivity of inhibitory peptide analogues with L/D-Eps

In order to study the selectivity of compounds described in this and earlier published papers [14], the inhibitory activity was studied not only on calpain 1 and 2, but also on cathepsin B. The calpain 1 and 2 enzymes are major, ubiquitous isoforms of calpain with similar substrate preferences [31]. In fact, as our data suggest, we have identified a specific calpain 2 inhibitor, compound *NH_2*-Thr-Pro-Leu-(D-Eps)-Thr-Pro-Pro-Pro-Ser-*NH_2* (**16**) which exhibits at least 11.8-fold selectivity over calpain 1. Another nonapeptide analogue, *NH_2*-Thr-Pro-Leu-(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2* (**1**) could inhibit calpain 1 with at least 4-fold selectivity.

Here we also describe that the incorporation of the epoxysuccinyl moiety could result in compounds with cathepsin B selectivity. The above mentioned calpain 1 and 2 selective inhibitors (**1** and **16,** respectively) are selective also over cathepsin B (4- and 11.8-fold, respectively). It is interesting to note that compounds with inhibitory activity against cathepsin B have also inhibited calpain 1 (compounds **2**, **7**, **11**).

Taken together, the results presented in this communication demonstrate that it is possible to design novel calpain inhibitory peptide analogues based on a substrate sequence by insertion of epoxysuccinyl moiety at position P_1 even into a long, 9-mer sequence. It is also described that the inhibitory activity does not depend only on the amino acid sequence, but also on the stereochemical arrangement of the epoxysuccinyl group. This finding was further supported by the results of the molecular modeling studies suggesting that a change in the stereochemistry of the epoxysuccinyl group might reverse the orientation of the inhibitor within the substrate binding groove of calpain 2. Calculations also showed that specifically bound inhibitors could form a number of well-positioned H-bonds with the protein matrix including both side chain and main-chain contacts, originating from an extensive surface of the protein.

Furthermore, with compounds **1** and **16** we could demonstrate that there is a possibility of developing selective calpain 1 and 2 inhibitors by the appropriate combination of epoxysuccinyl moiety, simultaneous extension of peptide parts at both sides, and topological considerations. Further studies should be performed to investigate the effect of these inhibitors on intracellular calpains. Based on our preliminary studies, these inhibitors are not taken up by cells, therefore in order to study their effect on intracellular enzymes cell-penetrating conjugate derivatives will be prepared.

Author contributions

Designed the experiments: ZB, AT, FH, DKM.
Performed the experiments: ZB, LED, AT, DKM.
Analyzed and interpreted the data: ZB, LED, AT, DKM, FH.
Wrote the paper: LED, ZB, AT, DKM, FH.
Revised critically the paper: all authors.
All authors read and approved the final version of the manuscript.

Conflict of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.05.058.

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