Levente Endre Dókus

Synthesis and characterisation of calpain inhibitor peptidomimetics

Theses of Doctoral (Ph.D.) Dissertation

Supervisor:
Dr. Zoltán Bánóczi assistant professor
ELTE TTK Institute of Chemistry

Ph.D. School of Chemistry, Eötvös Loránd University
School leader: Dr. Attila Császár professor

Synthetic Chemistry, Materials Science and Biomolecular Chemistry Ph.D. Program
Head of the Program: Dr. András Perczel professor

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1. Introduction

The proteases have very important role in physiological processes, they regulate the function of many biologically active proteins (1). The members of calpain family, 15 members were identified, are intracellular cystein proteases (2). The m- and μ-calpains (calpain 1 and 2) can be found in all tissues of mammals. Their names come from the Ca^{2+}-ion concentration (milli- or micromolar) which necessary for their activation (3). Calpains take part in regulation and signal transduction processes (4).

For studying their function inhibition (5) or altered expression (6) of them is needed. Because of their different physiological functions their overregulation may cause various pathological disorders. Over-activated calpains could be involved in the wide range of neurodegenerative disorders such as Alzheimer's, Huntington's, and Parkinson's diseases and multiple sclerosis (7). Some data indicate that calpains have important role in cancerous diseases too (8).

More than 100 natural substrates of calpain were identified, such as membrane receptors, enzymes, cytoskeletal and structural proteins (9). Calpastatin protein is the only one specific calpain inhibitor (10) but because of its large size its applications are limited in calpain research. Because of the potency of specific inhibitors in the basic calpain research or in medicinal use high effort is done to develop effective and selective calpain inhibitors. Based on the literature it can be seen, that the cell-penetrating peptides may be potentially useful to deliver drug molecules into various cell types. It was observed, that the internalization ability of these peptides depends on the number of positively-charged amino acid sidechains. In case of oligoarginines (11) at least six arginines are required for the cell penetrating ability. Furthermore more arginines improve the cell-penetrating potential, but above 8 arginines this increase is not so significant (12).

2. Aims

During my PhD work our aim was to design and synthesis selective calpain inhibitor peptidomimetics, which contain various reactive groups in P_1 position. Among of the very different kind of functionality that were selected which allow the insertion of amino acids in the P_n and P_n' positions. The amino-acids were selected based on the preference matrix (13). Peptides based on the sequence of calpastatin containing the GERDD or GKREV sequence between the P_2 and P_1' positions were planned too.

1) In case of azapeptide inhibitors peptides containing azaglycine (Agly) in P_1 position were designed (for example: ***Ac*-Thr-Pro-Leu-Agly-Ser-Pro-Pro-Pro-Ser-*NH*$_2$**). The effect of different amino acids in P_3, P_2, P_1' and P_2' positions on the inhibitory activity was planned to examined.

2) Epoxysuccinyl-peptide derivatives were planned with epoxysuccinyl-group containing oxirane-ring in P_1 position. The effect of configuration of reactive group on the inhibitory activity was planned to study (*cis*- and D- or L-*trans*-derivatives; (for example: ***NH*$_2$-Thr-›Pro-›Leu-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$** and ***NH*$_2$-Thr-›Pro-›Leu-›(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH*$_2$**). The synthesis of cell-penetrating octaarginine conjugates of effective epoxysuccinyl-peptide inhibitors was planned. For the characterisation of cell-penetrating ability the synthesis of fluorescently labelled cell-penetrating derivatives of effective epoxysuccinyl-peptide inhibitors (for example: ***H*$_2$*N*-Thr-›Pro-›Leu-›(D-Eps)-Thr-Pro-Pro-Pro-Ser-(Arg)$_8$-Lys(Cf)-*NH*$_2$**) was planned. The cellular uptake of these labelled conjugates was planned to measure by flowing cytometry (FACS).

3) The synthesis of compounds containing ketomethylene group instead of -CO-NH- bond between P_1 and P_1' positions was planned. The effect of amino-acids in P_n position on inhibitor potential was planned to study.

4) Calpastatin based inhibitors were designed. These peptides contain GKREV or GERDD fragment which are opposite to the active center of calpain after binding and therefor could have inhibitory effect. These fragments were planned to build in between the P_4-P_2 and P_1'-P_5' segments of the most effective azapeptide inhibitor (***Ac*-Thr-Ser-Leu-Agly-Ser-Pro-Pro-Pro-Ser-*NH*$_2$**). Two derivatives containing Ac-TSL segment next to P_1 position at the N-terminus and TIPPEYR segment in P_1'-P_5' positions, as high conserved sequence of calpastatin (for example: ***Ac*-Thr-Ser-Leu-Gly-Lys-Arg-Glu-Val-Ser-Pro-Pro-Pro-Ser-*NH*$_2$** és ***Ac*-Thr-Ser-Leu-Gly-Lys-Arg-Glu-Val-Thr-Ile-Pro-Pro-Glu-Tyr-Arg-*NH*$_2$**).

The inhibitory effect of all compounds was planned to characterize on μ- and m-calpains and in case of inhibition on cathepsin B- and L enzymes.

3. Methods

3.1. Solid-phase peptide synthesis

The synthesis of peptides and peptide-derivatives was done by solid-phase peptide synthesis (SPPS). The azapeptides was prepared bby using Boc/Bzl strategy while in other cases Fmoc/^{t}Bu strategy was used.

3.2. Reversed-phase high performance liquid chromatography (RP-HPLC)

The homogeneity of peptides and peptide derivatives were characterised by analytical RP-HPLC, on Agilent Zorbax SB-C18 4.6mm x150mm, 100Å column using linear gradient elution. Gradient: 0 min-0% B, 2 min-0% B, 22 min-90% B; Eluents: 0,1 V/V% TFA/H_2O (A); 0,1 V/V% TFA/80V/V% acetonitrile/20 V/V% H_2O (B); flowing rate: 1ml/min; λ_{det}=220 nm. The compounds were purified by semi-preparative RP-HPLC, using the previous eluents and linear gradient elution. Flow rate was 4 ml/min as well as λ_{det}=220 nm.

3.3. Amino acid analysis

The amino acid composition of 14 epoxysuccinyl-peptides were checked by amino acid analysis on Beckman (Fullerton, CA, USA) Model 6300 automatic amino acid analysator. The peptide derivatives were hydrolysed in 6 mol/dm^3 HCl-solution at 110°C with 24h reaction time.

3.4. Mass-spectrometry

The qualitative characterisation of compounds was achieved by mass-spectrometry. The spectra were recorded in positive mode in 50-2500 m/z range. Samples were dissolved in acetonitrile-water solvent (50:50, V/V), wich contained 0,1 V% acetic-acid.

3.5. Spectrofluorimetry measurements

The inhibitory potency of compounds was examined on isolated μ- and m-calpain and cathepsin B and L enzymes with 96 well-plate by spectrofluorimetry at 37 °C. The measurements on μ- and m-calpain was done in calpain-buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 5 mM), on cathepsin B and L enzymes in MES-buffer (0,25 mM-os MES, pH=5).

3.6. Enzyme inhibition assay

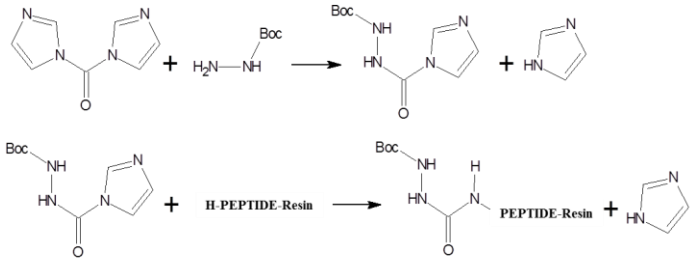
In the case of μ- and m-calpains Suc-LY-Amc (AMC=7-amino-4-methyl-coumarin) substrate and in case of cathepsin B and L enzyme Z-Arg-Arg-Amc substrate was used in the enzyme assay measurements. The concentration of the enzymes, substrate and $CaCl_2$ in case of calapains was constant while the inhibitor was applied in various concentration in each measurements. The experience was started by the addition of enzyme solution and was recorded for 1 min. The K_i values of reversible azapeptide inhibitors were calculated with Linewear-Burk plot (14). The K_i values of irreversible epoxysuccinyl-peptide derivatives were calculated based on literature analogue (15). For calculations Origin 6.5 software was used.

4. Results and conclusions

4.1. Azapeptides

Azapeptide derivatives contained azaglycine in P_1 position and the first, second and third most preferred amino-acids (based on the data of the preferece matrix) in P_3, P_2, P_1' and P_2' positions (Table 1).

4.1.1. Synthesis of azapeptides



Nine new azapeptide derivatives were synthesized by (SPPS) using Boc/Bzl technique (Figure 1). The azaglycin was formed in *in situ* reaction (Figure 2).

Figure 1.

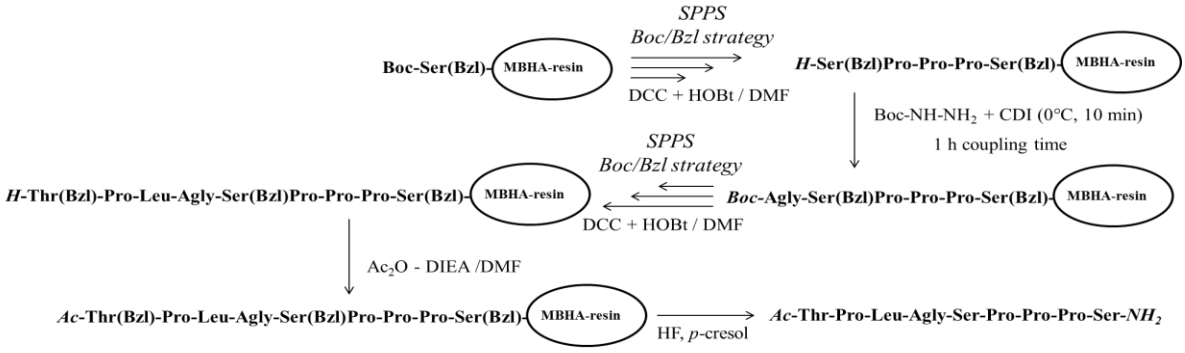


Figure 2.

4.1.2. Inhibitory activity of azapeptides

Table 1.

Azapeptides		$K_{i(\mu M)}$ m-calpain	$K_{i(\mu M)}$ μ-calpain	$K_{i(\mu M)}$ cathepsin B
1	***Ac*-Thr-Pro-Leu-Agly-Ser-Pro-Pro-Pro-Ser-NH_2**	8.7(0.1)	16.0(7.8)	57.0(8.5)
2	***Ac*-Thr-Trp-Leu-Agly-Ser-Pro-Pro-Pro-Ser-NH_2**	5.8(0.4)	3.9(1.9)	42.9(13.5)
3	***Ac*-Thr-Ser-Leu-Agly-Ser-Pro-Pro-Pro-Ser-NH_2**	3.5(0.1)	14.0(5.3)	69.9(20.5)
4	***Ac*-Thr-Pro-Thr-Agly-Ser-Pro-Pro-Pro-Ser-NH_2**	>50	no data	no data
5	***Ac*-Thr-Pro-Val-Agly-Ser-Pro-Pro-Pro-Ser-NH_2**	>50	no data	no data
6	***Ac*-Thr-Pro-Leu-Agly-Thr-Pro-Pro-Pro-Ser-NH_2**	>50	no data	no data
7	***Ac*-Thr-Pro-Leu-Agly-Arg-Pro-Pro-Pro-Ser-NH_2**	>50	no data	no data
8	***Ac*-Thr-Pro-Leu-Agly-Ser-Ser-Pro-Pro-Ser-NH_2**	>50	no data	no data
9	***Ac*-Thr-Pro-Leu-Agly-Ser-Gln-Pro-Pro-Ser-NH_2**	>50	no data	no data

The inhibitor activity of azapeptides was charachterised on m-calpain (Table 1). Three compounds containing the first, second and third most preferred amino acids in P_3 position (**1** K_i(m-kalpain)=8.7 μM, **2** K_i(m-kalpain)=5.8 μM , **3** K_i(m-kalpain)=3.5 μM) were effective m-calpain inhibitors. To examine the calpain isoform selectivity, the inhibitor potential of this three compounds was characterized on μ-calpain also. All of compounds were effective μ-calpain inhibitor (**1** K_i(μ-kalpain)=16.0 μM, **2** K_i(μ-kalpain)=3.9 μM, **3** K_i(μ-kalpain)=14.0 μM). These calpain inhibitor azapeptides showed selectivity against cathepsin B. Based on the results we can say that the P_3 modification results in the conservation of inhibitor property, but modification in other positions causes loss of the inhibitory effect.

4.2. Epoxysuccinyl-peptide derivatives

4.2.1. Synthesis of epoxysuccinyl-peptide derivatives

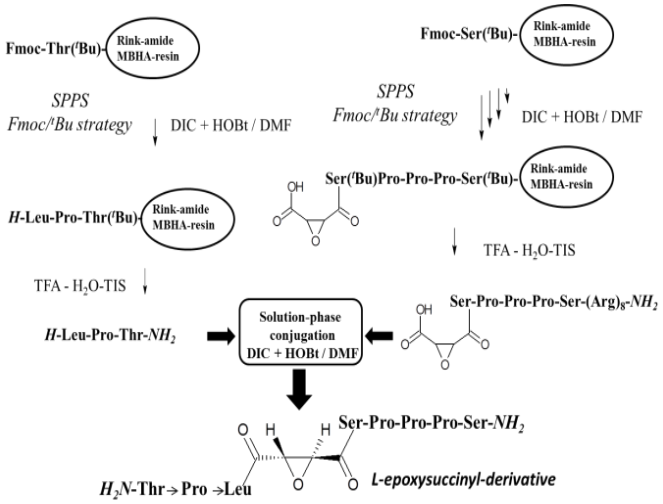


Figure 3.

Nine new D- and nine L-trans-epoxysuccinyl-group containing peptide derivatives were prepared. The reactive group was in the P_1 position. Into P_3, P_2, P_1' and P_2' positions the first, second and third most preferred amino acids were incorporated. The eopxysuccinyl-pentapeptide and tripeptide-fragments of derivatives were synthesised by SPPS, using Fmoc/^{t}Bu strategy. These peptides were conjugated in solution phase (Figure 3).

4.2.2. Inhibitory effect of epoxysuccinyl-peptide derivatives

The inhibitory activity of derivatives were characterised on m- and μ-calpains, furthermore their selectivity was examined opposite to cathepsin B and L (Table 2).

Table 2.

	Epoxysuccinyl-peptides	$K_{i(\mu M)}$ m-calpain	$K_{i(\mu M)}$ μ-calpain	$K_{i(\mu M)}$ cathepsin B	$K_{i(\mu M)}$ cathepsin L
10	***NH_2*-Thr-›Trp-›Leu-›(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	21.8(1.1)	2.95(0.6)	4.5(1.8)	2.3(1.9)
11	***NH_2*-Thr-›Pro-›Val-›(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	>50	>50	>50	>50
12	***NH_2*-Thr-›Ser-›Leu-›(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	>50	39.1(1.1)	>50	6.3(5.3)
13	***NH_2*-Thr-›Pro-›Thr-›(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	>50	>50	>50	>50
14	***NH_2*-Thr-›Pro-›Leu-›(D-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	>50	>50	>50	3.4(2.7)
15	***NH_2*-Thr-›Pro-›Leu-›(D-Eps)-Thr-Pro-Pro-Pro-Ser-*NH_2***	4.2(1.9)	>50	>50	10.9(9.1)
16	***NH_2*-Thr-›Pro-›Leu-›(D-Eps)-Arg-Pro-Pro-Pro-Ser-*NH_2***	>50	41.9(1.4)	>50	0.5(0.4)
17	***NH_2*-Thr-›Pro-›Leu-›(D-Eps)-Ser-Gln-Pro-Pro-Ser-*NH_2***	>50	>50	>50	>50
18	***NH_2*-Thr-›Pro-›Leu-›(D-Eps)-Ser-Ser-Pro-Pro-Ser-*NH_2***	>50	>50	>50	4.5(3.8)
19	***NH_2*-Thr-›Trp-›Leu-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	4.1(2.1)	14.9(3.3)	6.6(2.1)	0.4(0.3)
20	***NH_2*-Thr-›Pro-›Val-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	29.2(5.0)	>50	>50	35.9(28.3)
21	***NH_2*-Thr-›Ser-›Leu-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	14.3(2.7)	21.9(1.3)	>50	7.0(4.5)
22	***NH_2*-Thr-›Pro-›Thr-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	>50	>50	>50	>50
23	***NH_2*-Thr-›Pro-›Leu-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	>50	12.5(3.5)	>50	8.1(6.2)
24	***NH_2*-Thr-›Pro-›Leu-›(L-Eps)-Thr-Pro-Pro-Pro-Ser-*NH_2***	>50	8.4(0.1)	12.1(3.0)	1.3(0.8)
25	***NH_2*-Thr-›Pro-›Leu-›(L-Eps)-Arg-Pro-Pro-Pro-Ser-*NH_2***	40.9(2.5)	23.7(7.3)	>50	1.6(1.2)
26	***NH_2*-Thr-›Pro-›Leu-›(L-Eps)-Ser-Gln-Pro-Pro-Ser-*NH_2***	>50	>50	>50	13.7(11.5)
27	***NH_2*-Thr-›Pro-›Leu-›(L-Eps)-Ser-Ser-Pro-Pro-Ser-*NH_2***	>50	>50	>50	5.4(2.8)
28	***H_2N*-Thr-›Trp-›Leu-›(L-Eps)-Thr-Pro-Pro-Pro-Ser-*NH_2***	1.9(1.2)	3.6(3.1)	1.5(0.8)	>100
29	***H_2N*-Thr-›Trp-›Leu-›(D-Eps)-Thr-Pro-Pro-Pro-Ser-*NH_2***	2.9(2.1)	2.2(1.8)	0.8(0.6)	0.3(0.2)
30	***H_2N*-Thr-›Trp-›Val-›(L-Eps)-Arg-Pro-Pro-Pro-Ser-*NH_2***	5.6(2.6)	3.7(4.9)	56.9(7.7)	0.5(0.2)
31	***H_2N*-Thr-›Trp-›Leu-›(L-Eps)-Gly-Pro-Pro-Pro-Ser-*NH_2***	2.4(1.9)	no data	>50	17.7(18.6)
32	***H_2N*-Thr-›Trp-›Leu-›(*cis*-Eps)-Ser-Pro-Pro-Pro-Ser-*NH_2***	>100	>100	>100	>100
33	***H_2N*-Thr-›Pro-›Leu-›(*cis*-Eps)-Thr-Pro-Pro-Pro-Ser-*NH_2***	>100	>100	>100	>100

In case of L-epoxysuccinyl-group containing derivatives the compound from the most preferred amino acids was moderate inhibitor of μ-calpain (**23** K_i(μ-calpain)=12.5 μM), but did not inhibit m-calpain. The amino acid substitution in P_3 position increased the inhibitory activity, the tryptophan had the strongest effect (**19** K_i(m-calpain)=4.1; K_i(μ-calpain)=14.9 μM, and **21** K_i(m-calpain)=14.3; K_i(μ-calpain)=21.9 μM). Threonine in the P_2 position diminished the inhibitory effect (**22**), but valine in this position resulted in low m-calpain inhibition (**20**). Insertion of threonine into P_1' position resulted in selective and effective inhibitor of μ-calpain (**24** K_i(μ-calpain)=8.4 μM), while arginine in this position gave moderate m- and μ-calpain inhibitory activity of compound. (**25** K_i(m-calpain)=4.9 μM; K_i(μ-calpain)=23.7 μM). Modification in the P_2' position caused the loss of the inhibitory effect (**26, 27**).

For studying the effect of the configurations of epoxysuccinyl moeity on the inhibitory activity the analogues with D-epoxysuccinyl-group were synthesised. Derivative with the most preferred amino acids did not have any inhibitory effect on m- and μ-calpains (**10**). The compound

containing tryptophan in P_3 position was effective inhibitor of m- and μ-calpains also (**11** K_i(m-calpain)=21.8; K_i(μ-calpain)=2.9 μM) as the L-isomer, but the presence of serine in this position caused moderate and selective μ-calpain inhibitory activity (**12** K_i(μ-calpain)=39.1 μM). The exchange of amino acid in P_2 position to threonine or valine resulted in the loss of calpain inhibitory effect (**11, 13**). The presence of threonine in P_1' position resulted in m-calpain selective and effective inhibitor (**15** K_i(m-calpain)=4.2 μM), but compound with arginine in this position showed only low μ-calpain inhibition (**25** K_i(μ-calpain)=41.9 μM/). All of the P_2' modifications diminished the inhibitory effect (**17, 18**).

The selectivity of the epoxysuccinyl-peptide derivatives was studied on cathepsin B and L enzymes. Among the L-epoxysuccinyl-peptides only compound **19** and **24** were inhibitor of cathepsin B and in case of D-isomers only compound **10** inhibited this enzyme. All of the calpain inhibitor compounds were good cathepsin L inhibitors too.

In summary partially similar results were observed as in case of azapeptides. Similar to azapeptides the P_3 modification increased the inhibitor activity, but P_2' modification diminished that. The derivatives with D- and L-epoxysuccinyl-group and the identical amino acid had different inhibitory effect on m- and μ-calpains (for example: **14** K_i(μ-calpain)>50 μM; opposite: **23** K_i(μ-calpain)=12.5 μM), in some cases reverse isoform-selectivity was observed (**15** K_i(m-calpain)=4.2 μM; K_i(μ-calpain)>50 μM, **24** K_i(m-calpain)>50 μM; K_i(μ-calpain)=8.4 μM).

In contrast to azapeptides the P_2 modification did not cause the loss of inhibitory effect (***NH₂*-Thr-›Pro-›Val-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-*NH₂*** K_i(m-calpain)=29.2 μM), and P_1' modification increased the inhibitory activity (for example: ***NH₂*-Thr-›Pro-›Leu-›(D-Eps)-Thr-Pro-Pro-Pro-Ser-*NH₂*** K_i(m-calpain)=4.2 μM). Any compound was not selective for calpains against cathepsin B and L. The tryptophan in P_3 impair the cathepsin B selectivity. We also noticed that all of the calpain inhibitors are effective cathepsin L inhibitors also.

Based on the previous results new inhibitors were designed. D- and L- epoxysuccinyl-peptide derivatives, which contain tryptophan in P_3 position and serine in P_1' position (**28**, **29**) were synthesized. L-epoxysuccinyl-peptide containing tryptophane in P_3, valin in P_2 and arginine in P_1' position was also prepared (**30**). To examine the effect of the configuration of epoxysuccinyl-group on the inhibitory effect *cis*-epoxysuccinyl-analogues of the two most effective inhibitors were designed (**32**, **33**). Finally, a L-epoxysuccunyl-peptide derivative was synthesized to examine the effect of glycine in P_1' position on the inhibitory activity (**31**).

The D- and L-epoxysuccinyl-peptide derivatives, contain the tryptophan in P_3 and serine in P_1' position were effective calpain inhibitors but the isoform selectivity of the basic compounds

could not be observed (**28**, **29**). These two compounds are not selective against cathepsin B, but compound **28** does not inhibit cathepsin L enzyme. The L-epoxysuccinyl-peptide derivative that contains tryptophan in P_3, valine in P_2 and arginine in P_1' position was effective calpain inhibitor, and was selective against cathepsin B, although did not have selectivity against cathepsin L. The *cis*-epoxysuccinyl-group containing derivatives were not inhibitors of the calpain and cathepsin enzymes too. It turned out that glycine in P_1' position resulted in good inhibitory effect.

These new derivatives showed that using earlier results of the structure-activity relationship may increase the inhibitory potential. Modification at two position at once resulted in effective inhibitors, but these compounds did not show isoform and cathepsin B selectivity. This property may be related to the tryptophan in P_3 position, but one of the new compounds (**28**) had cathepsin L selectivity. The *cis*-conformation of the epoxysuccinyl-group resulted in the loss of inhibitory effect of both derivatives. Modification in three positions also increased the inhibitory effect and at the same time resulted in cathepsin B selectivity.

4.3. Cell-penetrating epoxysuccinyl-peptide derivatives

The cell-penetrating derivatives of the effective epoxysuccinyl-peptide derivatives were synthesised (Table 3) to study the effect of octaarginine on the inhibitory activity.

Table 3.

Cell-penetrating epoxysuccinil-peptide conjugates		$K_{i(\mu M)}$ m-calpain	$K_{i(\mu M)}$ μ-calpain	$K_{i(\mu M)}$ cathepsin B	$K_{i(\mu M)}$ cathepsin L
42	***H*$_2$*N*-Thr-›Trp-›Leu-›(L-Eps)Ser-Pro-Pro-Pro-Ser-(Arg)$_8$-*NH*$_2$**	0.9(0.2)	0.7(0.5)	3.3(2.6)	3nM(1nM)
43	***H*$_2$*N*-Thr-›Pro-›Leu-›(D-Eps)Thr-Pro-Pro-Pro-Ser-(Arg)$_8$-*NH*$_2$**	1.0(0.5)	5.0(4.1)	11.1(4.6)	2nM(0.8nM)
44	***H*$_2$*N*-Thr-›Trp-›Leu-›(L-Eps)Thr-Pro-Pro-Pro-Ser-(Arg)$_8$-*NH*$_2$**	0.2(0.1)	0.6(0.6)	0.4(0.1)	3nM(0.5nM)
45	***H*$_2$*N*-Thr-›Trp-›Leu-›(D-Eps)Thr-Pro-Pro-Pro-Ser-(Arg)$_8$-*NH*$_2$**	>100	>100	>100	>100

4.3.1. Synthesis of cell-penetrating epoxysuccinyl-peptide conjugates

The octaarginine was built into the C-terminal of the derivatives. The synthesis of conjugates was done on the same way than in case of epoxysuccinyl-derivatives, but the synthesis was begun with the building up of the octaarginine at the C-terminal.

4.3.2. Inhibitory effect of cell-penetrating epoxysuccinyl-peptide conjugates

The inhibitory potential of the derivatives was characterized on m- and μ-calpain enzymes and their selectivity was examined againstcathepsin B and L enzymes (Table 3). It can be observed, that the presence of octaargininein generally increased approximately one order of magnitude the calpain inhibitor property, only the compound **45** did not show any inhibitory effect. The conjugates did not have isoform selectivity. All of the effective calpain inhibitor derivatives

inhibited cathepsin B and L enzymes too. These compounds inhibit cathepsin L three order of magnitude better, than calpains. Probably, the octaarginine increases significantly the affinity of derivatives to the binding-site of calpains and cathepsins too.

4.4. Fluorescent labeled cell-penetrating epoxysuccinyl-peptide conjugates

Two fluorescent labelled derivatives of the most effective epoxysuccinyl-peptide inhibitors were prepared. The cellular-uptake was planned to measure using these constructs.

4.4.1. Synthesis of the fluorescent-labeled cell-penetrating epoxysuccinyl-peptide conjugates

The synthesis of the fluorescent labelled conjugates was begun by coupling of lysine to the resin. This amino-acid will be found on the C-terminal end of the peptide derivative. The fluorescent dye (5(6)-carboxyfluorescein) was coupled to the ε-amino-group of this lysine. The synthesis was continued with building of octaarginine- and epoxysuccinyl-segments. The made derivative were cleaved from the resin and it was coupled in solution phase with the adequate tripeptide.

4.4.2 Cellular-uptake of fluorescent labelled cell-penetrating epoxysuccinyl-peptide conjugates

The internalization ability of the fluorescent labelled conjugates was characterised by flow cytometry, on HL-60 cell-line. Based on the results it can be seen, that both compounds (***H_2N*-Thr-›Trp-›Leu-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-$(Arg)_8$-Lys(Cf)-*NH_2***, ***H_2N*-Thr-›Pro-›Leu-›(D-Eps)-Thr-Pro-Pro-Pro-Ser-$(Arg)_8$-Lys(Cf)-*NH_2***) could effectivly internalize into cells. Furthermore it can be observed, that the presence of tryptophan in P_3 position resulted in much more effective internalization ability (***H_2N*-Thr-›Trp-›Leu-›(L-Eps)-Ser-Pro-Pro-Pro-Ser-$(Arg)_8$-Lys(Cf)-*NH_2***).

4.5. Ketomethylene-group containing peptide-derivatives

Pseudopeptide derivatives were prepared which contain ketomethylene-group (-CO-CH_2-) instead of amide-bond between P_1 and P_1' positions. The cysteine-proteases cannot cleave this bond, therefore this compounds may be inhibitors of calpains. The derivatives contained the first, second and third most preferred amino-acids in P_3 and P_2 positions. The P_1' amino acid could be modified, because it is a part of the ketomethylene-group. Furthermore I did not plane P_2' modification also, because this change caused the loss of inhibitory activity in all cases of aza- and epoxysuccinyl-peptide derivatives.

4.5.1. Synthesis of the pseudopeptide-derivatives

Four new ketomethylene-group containing peptide derivatives were synthesised. The synthesis of the pseudopeptides was done by SPPS, with Fmoc/^{t}Bu strategy. The Fmoc-Gly-Ψ(CO-CH_2)-Gly-OH building-block, which is compatible with Fmoc/^{t}Bu strategy (Figure 4) was synthesized. Then this building-block was coupled to the α-amino-group of serine in the P_2' position and the

synthesis of derivatives were continued from its α-amino-group, with Fmoc/^{t}Bu strategy (Figure 5.).

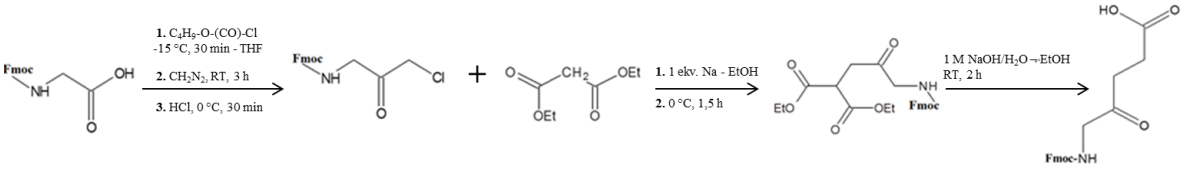


Figure 4.

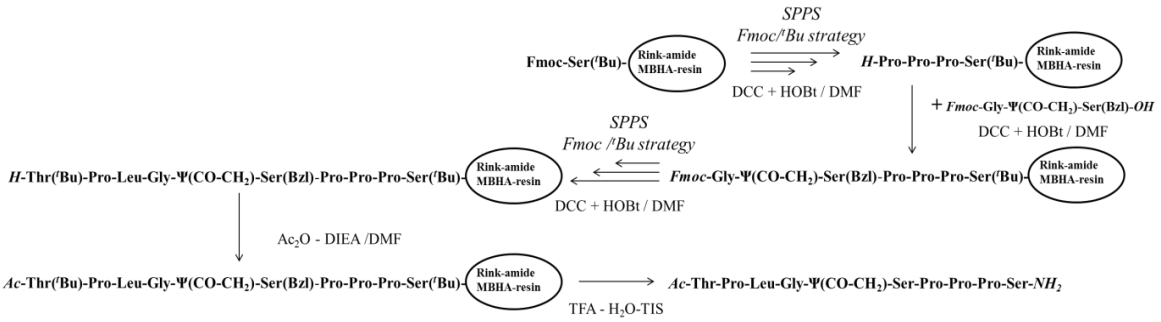


Figure 5,

4.5.2. Inhibitory effect of the pseudopeptide-derivatives

Table 4.

Pseudopeptides		$K_{i(\mu M)}$ m-calpain	$K_{i(\mu M)}$ μ-calpain
34	***Ac*-Thr-Pro-Leu-Gly-Ψ(CO-CH$_2$)-Gly-Pro-Pro-Pro-Ser-*NH$_2$***	>100	>100
35	***Ac*-ThrSer-Leu-Gly-Ψ(CO-CH$_2$)-Gly-Pro-Pro-Pro-Ser-*NH$_2$***	>100	>100
36	***Ac*-Thr-Trp-Leu-Gly-Ψ(CO-CH$_2$)-Gly-Pro-Pro-Pro-Ser-*NH$_2$***	>100	>100
37	***Ac*-Thr-Pro-Val-Gly-Ψ(CO-CH$_2$)-Gly-Pro-Pro-Pro-Ser-*NH$_2$***	>100	>100

The inhibitory effect of pseudopeptides was characterised on m- and μ-calpains (Table 4). Unfortunately the compounds did not have inhibitory effect on any enzymes. Probably, the ketomethylene-group decrease the affinity of compounds to the binding-site of calpain. For example it may induce unfavorable structure for the derivatives.

4.6. Peptide inhibitors based on the calpastatin sequence

Four new peptides were synthesised, which contained the P_4-P_2 region of the most effective azapeptide calpain inhibitor. Based on the literature, GKREV and GERDD segment were built in into two-two derivatives. These segments localised in front of the substrate cleavage site of calpain, but they form a loop-structure, which results in inhibitory activity for the calpastatin protein. In my constructs they were built in between the P_2 and P_1' positions. The C-terminal

region (from P_1' position to the C-terminal) of these construct was SPPPS (which was selected based on the results with aza- and epoxysuccinyl-peptide derivatives) andTIPPEYR (conserved region of calpastain) segments The synthesis of these peptides was done by SPPS, with Fmoc/^{t}Bu strategy.

4.6.1. Inhibitory effect of the peptides are based on the sequence of calpastatin

Table 5.

Peptides being based on the sequence of calpasatatin		**$K_{i(\mu M)}$ m-calpain**
38	***Ac*-Thr-Ser-Leu-Gly-Lys-Arg-Glu-Val-Ser-Pro-Pro-Pro-Ser-*NH_2***	>100
39	***Ac*- Thr-Ser-Leu-Gly-Glu-Arg-Asp-Asp-Ser-Pro-Pro-Pro-Ser-*NH_2***	>100
40	***Ac*- Thr-Ser-Leu-Gly-Lys-Arg-Glu-Val-Thr-Ile-Pro-Pro-Glu-Tyr-Arg-*NH_2***	>100
41	***Ac*- Thr-Ser-Leu-Gly-Glu-Arg-Asp-Asp-Thr-Ile-Pro-Pro-Glu-Tyr-Arg-*NH_2***	>100

The inhibitory effect of peptides was characterised on isolated μ-calpain. Any of the compounds has μ-calpain inhibitory activity (Table 5). Probably, the peptides have low affinity to the binding-site of calpain, furthermore it is possible that the GKREV and GERDD do not assume the loop structure.

5. Summary

1. During my PhD work I designed and synthesised nine azapeptide derivatives. I found, that compound with substitution in P_3 position were effective inhibitors of m- and μ-calpains, and serine in this position resulted inm-calpain selectivity. The modification in all other positions caused decreased inhibitory activity. All of azapeptide compounds which inhibited calpains were selective did not inhibited cathepsin B.

2. I prepared 9-9 new D- and L-epoxysuccinyl-peptide derivatives. Among them 10 compounds were effective calpain inhibitors and three derivatives had calpain isoform selectivity. Compound **23** and **24** had selectivity to μ-calpain whereas compound **15** is m-calpain selective inhibitor. I characterised the selectivity of epoxysuccinyl-peptides on cathepsin B and L enzymes (as other cysteine-proteases). 3. Using of the effective epoxysuccinyl inhibitors I designed and synthesised four new cell-penetrating conjugates. These compounds not only preserved the μ- and m-calpain inhibitory activity of the short peptide derivatives, but the presence of octaarginin increased it, except of compound **45**, which did not behave as calpain or cathepsin inhibitor. Unfortunately this increase was noticed in case of cathepsins too, so the selectivity of inhibitorsagainst cathepsin B and L enzymes was decreased. Probably, the presence of octaarginine increases the affinity to the binding site of enzyme, but not only to calpains, but to cathepsins too.

4. I designed fluorescent labelled cell-penetrating derivatives of effective calpain inhibitor epoxysuccinyl-peptides, to examine their internalization activity. The two synthesised conjugates had effective cell-penetrating ability. It can be observed that the presence of tryptophan in P_3 position causes higher internalization of the compound, than those of the other derivative.

5. I synthesised ketomethylene-group containing peptide derivatives. These pseudopeptides did not show any inhibitory activity on μ- and m-calpains. Probably the ketomethylene-group causes this results, because this may cause lower affinity of compounds to the binding-site of the enzyme.

6. Based on the literature analogue and the results of azapeptides and epoxysuccinyl-peptides I designed peptide inhibitors based on calpastatin. I founded that this peptides did not have any inhibitory effect on μ-calpain. Probably these compounds have low affinity to binding site of calpain furthermore it is possible that the GKREV and GERDD do not assume loop structure trough the binding process.

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s1. Banoczi, Z., Tantos, A,, Farkas A., Majer, Zs., Dókus, E. L., Tompa, P., Hudecz, F. (2013) New mcalpain substrate-based azapeptide inhibitors. *J. Pept. Sci.***19**, 370-376.

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