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Short Communication

A new cell-permeable calpain inhibitor

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Abstract: The ubiquitous calpains, μ- and m-calpain, are implicated in a variety of vital (patho)physiological processes and therefore cell-permeable specific inhibitors represent important tools for defining the role of calpains in cells and animal models. A synthetic *N*-acetylated 27-mer peptide derived from exon B of the human calpastatin inhibitory domain 1 is known to be the most potent and selective reversible inhibitor of calpains. To improve the membrane permeability of this peptidic inhibitor, it was *N*-terminally extended with or disulfide-linked to the *C*-terminal 7-mer fragment of penetratin, a well-established vector for cell membrane translocation of bioactive compounds. Despite the shorter penetratin sequence, both constructs showed increased cell permeability and retained their full calpain inhibitory potency. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: calpains; peptidic inhibitors; cellular uptake; penetratin

INTRODUCTION

The calpains, μ- and m-calpain, are calcium-dependent cysteine proteases, which coexist with their specific endogenous inhibitor calpastatin in the cytosol of almost all mammalian cells. There are strong indications that the ubiquitous calpains are involved in signalling pathways, in the control of cell proliferation, differentiation, and motility as well as in cell death. But the assignment of clear physiological functions and pathophysiological roles to calpains has proved to be elusive [1–3]. Most of the information on cellular processes involving calpains has been collected by the use of more or less selective synthetic calpain inhibitors that are sufficiently cell-permeable [4]. Among the known calpain inhibitors, an *N*-acetylated and *C*-amidated 27-mer peptide (Figure 1, **1**) derived from exon B of the human calpastatin inhibitory domain 1 [5] is known to bind reversibly to the catalytic subunit of μ- and m-calpain with high affinity and selectivity [6]. But high concentrations of this peptidic inhibitor are required to achieve intracellular effects [6,7], and, in isolated organs, the peptidic inhibitor is practically ineffective [8]. Penetratin is a 16-mer peptide derived from the third helix of the homeodomain of the Antennapedia protein [9,10], which has found widespread use as vector for intracellular delivery of bioactive molecules by receptor-independent mechanisms [11–13]. In order to increase the cell-permeability of the calpastatin peptide **1**, we have previously reported the synthesis of a construct of **1** disulfide conjugated with penetratin [14]. It was found to not only inhibit intracellular calpain [14] but also exert its effect on isolated organs [8] when applied in submicromolar concentrations. Inherent drawbacks of this relatively large construct were the synthetic effort required and the complex chemistry for regioselective disulfide linkage to the penetratin peptide.

Since the significantly shorter *C*-terminal penetratin sequence Arg-Arg-Met-Lys-Trp-Lys-Lys is known to retain an efficient cell membrane translocation potency [15], in the present communication we have analyzed the effect of this short cell-penetrating peptide on the cellular uptake of the large peptidic calpain inhibitor **1**.

RESULTS AND DISCUSSION

Generally, cellular vectors such as the cell-penetrating peptides are combined with the bioactive cargo molecules by conjugation via cleavable linkers such as disulfides or by colinear synthesis into tandem constructs with or without more or less extended flexible linkers. In previous attempts to increase the cell permeability of epoxysuccinyl-based cathepsin B inhibitors,

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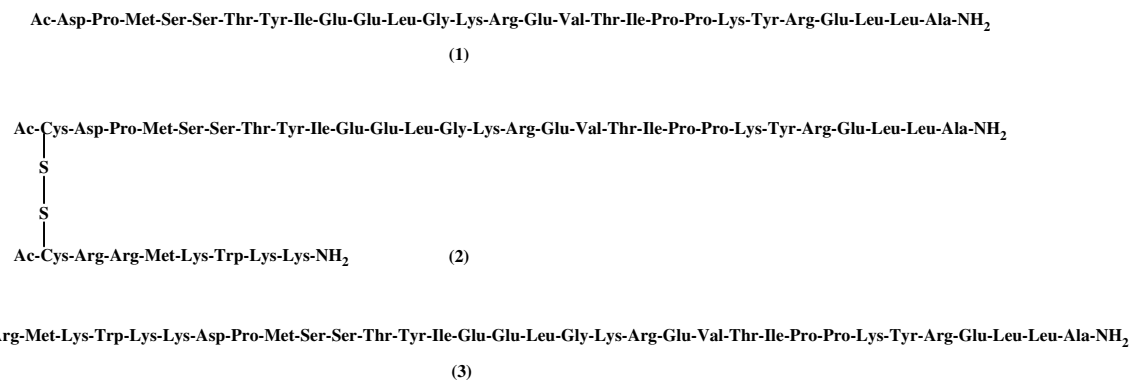

Figure 1 Peptidic calpain inhibitors: (**1**) The 27-mer peptide of calpastatin, (**2**) the disulfide crosslinked construct of the calpastatin peptide with the *C*-terminal 7-mer fragment of penetratin, and (**3**) the linear construct of the penetratin fragment and the calpastatin peptide.

we used an ε-amino-hexanoic acid as linker in the tandem construct, which showed a markedly enhanced cellular uptake [16]. To analyze the efficiency of the penetratin 7-mer fragment, it was attached to the *N*-acetyl-cysteine-calpastatin peptide amide via a disulfide bridge (**2**) or linearly cosynthesized as an *N*-terminal extension of the calpastatin peptide (**3**) without linkers, as shown in Figure 1. Disulfide conjugation of the calpastatin peptide with the short penetratin sequence was performed following the synthetic route reported previously [14]. For this purpose, both peptides were appropriately modified by *N*-terminal elongation with an *N*-acetyl-cysteine residue. Selective formation of the disulfide bond was achieved by reacting the *S*-nitropyridylsulfanyl-activated calpastatin peptide with the free thiol of the short penetratin peptide. For the linear tandem construct **3**, the 27-mer calpastatin peptide was extended *N*-terminally with the *N*-acetylated 7-mer fragment of penetratin. Purification of the relatively large tandem peptide **3** was facilitated by the clustering of positive charges in the penetratin fragment.

As shown in Table 1, the constructs **2** and **3** retain the full inhibitory potency and selectivity against μ-calpain when compared with the parent calpastatin 27-mer peptide (**1**), while, expectedly, the short penetratin peptide is inactive as an inhibitor of this enzyme.

In order to comparatively evaluate the cellular uptake of compounds **2** and **3**, LCLC 103H cells derived from a human large cell lung carcinoma [17] were used and the inhibitory effects on the intracellular calpains were compared with those of the parent calpastatin peptide **1**. Ionomycin-induced hydrolysis of the cell-permeable fluorogenic calpain substrate Suc-Leu-Leu-Val-Tyr-AMC was measured in nontreated control cells (100%) and after preincubation with increasing concentrations of the inhibitors **1**–**3** (Figure 2). The IC_{50} values clearly demonstrate an increase in the cellular uptake of peptides **2** and **3,** which is similar to that obtained with the full penetratin sequence [14]. Thus, even the highly basic *C*-terminal 7-mer sequence of the penetratin peptide acts as an efficient vector for intracellular delivery, independently, whether it is attached via a disulfide to the cargo or constitutes a linear extension of the inhibitory peptide. From a synthetic point of view, these results significantly simplify the synthesis of cell-permeable calpain inhibitors as required for biochemical studies of the (patho)physiological functions of calpains using living cells and isolated organs as model systems.

Table 1 Inhibition potencies of compounds **1**–**3** for μ-calpain and cathepsin L; the K_i values (μM) are means from 5–10 experiments with different inhibitor concentrations (S.D. <10%)

Inhibitor	μ-Calpain	Cathepsin L
1	0.00020[a]	6[a]
2	0.00018	n.d.
3	0.00019	5
Short penetratin fragment	≥400	43

[a] Values taken from [14].

MATERIALS AND METHODS

μ-Calpain was isolated from human erythrocytes [18]. Cathepsin L and calpastatin peptide **1** were purchased from Calbiochem (Bad Soden, Germany), the substrates Suc-Leu-Leu-Val-Tyr-AMC and Suc-Leu-Tyr-AMC from Bachem (Heidelberg, Germany), and ionomycin from Calbiochem. All amino acid derivatives, resins, reagents, and solvents were of the highest quality commercially available. The LCLC 103H cell line (ACC 384) was supplied by DSMZ-GmbH (Braunschweig, Germany).

For peptide characterization, analytical HPLC was performed on Waters equipment (Eschhborn, Germany) with Nucleosil C_8 (5 μm, 100 Å, 4 × 125 mm, Macherey-Nagel, Düren, Germany) columns and a linear gradient from

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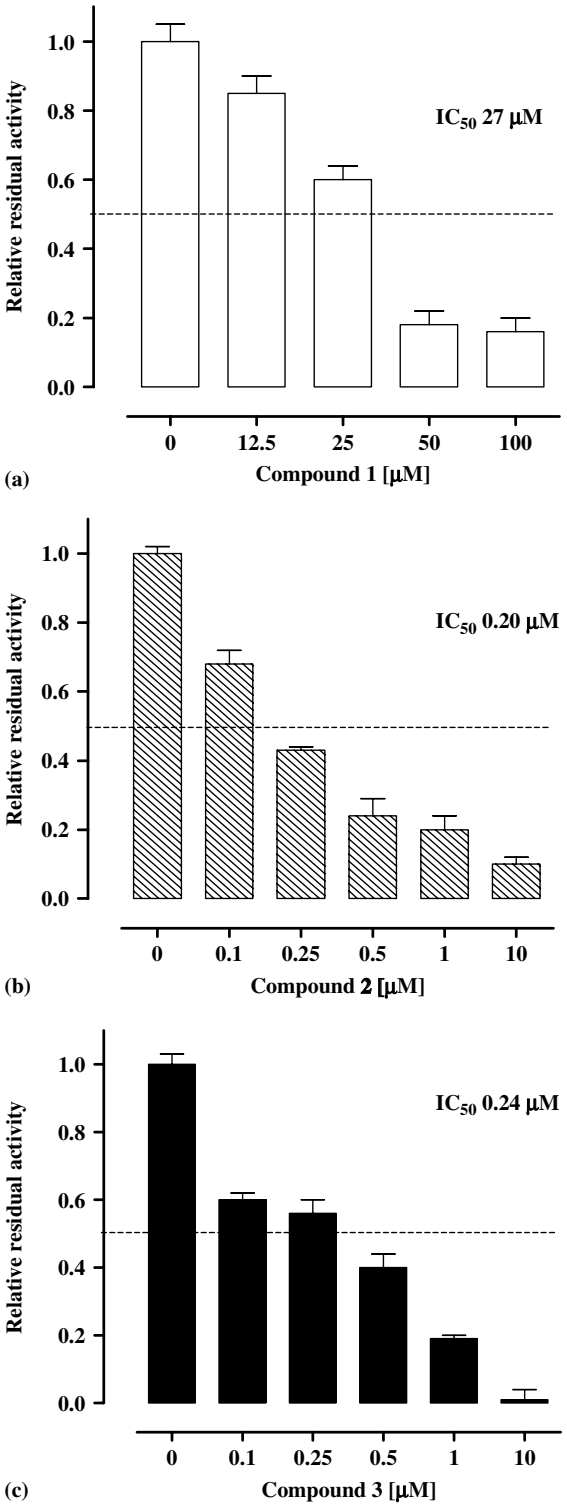

Figure 2 Inhibition of calpains in LCLC 103H cells by peptides **1**–**3**.

CH_3CN/2% H_3PO_4 (5 : 95) to CH_3CN/2% H_3PO_4 (90 : 10) in 13 min (flow rate, 1.5 ml min^{-1}; UV detection at 210 nm). Preparative HPLC was carried out on Abimed equipment (Langenfeld, Germany) with Nucleosil C_{18} (5 μm, 300 A, 21 × 250 mm, Macherey-Nagel, Düren, Germany) columns and linear gradients from 10%–90% of eluent A (0.08% TFA in CH_3CN) in B (0.1% TFA in H_2O) in 90 min; flow rate: 10 ml min^{-1}. ESI-MS spectra were recorded on a Perkin-Elmer SCIEX API 165 triple quadrupole spectrometer.

Peptide Synthesis

Peptides were synthesized on an Applied Biosystems peptide synthesizer (model 431A) on a 0.25 mmol scale by standard Fmoc/tBu chemistry on Rink amide (MBHA) resin using double coupling of Fmoc-amino acids/HBTU/HOBt/DIEA (1 : 1 : 1 : 2; 4 equiv.) in NMP/CH_2Cl_2 (4 : 1). The Fmoc group was cleaved with 20% piperidine in NMP/CH_2Cl_2 (4 : 1) in two cycles of 3 and 12 min, respectively. *N*-terminal acetylation was carried out with acetic anhydride/DIEA (1 : 1.5; 10 equiv.) and final cleavage/deprotection was performed with TFA/CH_2Cl_2/TIPS (95 : 5 : 11.5) for 2 h at room temperature. The crude products were purified by RP-HPLC.

Ac-RRMKWKKDPMSSTYIEELGKREVTIPPKYRELLA-NH_2 (**3**): 2% yield; homogeneous on HPLC (t_R 6.3 min); ESI-MS: m/z = 1398.0 $[M + 3H]^{3+}$, 1048.8 $[M + 4H]^{4+}$, 839.4 $[M + 5H]^{5+}$, 699.8 $[M + 6H]^{6+}$, 600.0 $[M + 7H]^{7+}$; calcd. 4192.02.

Ac-CDPMSSTYIEELGKREVTIPPKYRELLA-NH_2: 5% yield; homogenous on HPLC (t_R 6.8 min); ESI-MS: m/z = 1094.8 $[M + 3H]^{3+}$, 820.8 $[M + 4H]^{4+}$; calcd. 3280.83;

Ac-CRRMKWKK-NH_2: 12% yield; homogeneous on HPLC (t_R 4.0 min); ESI-MS: m/z 589.0 $[M + 2H]^{2+}$, 393.0 $[M + 3H]^{3+}$; calcd. 1175.52.

Ac-C(Npys)DPMSSTYIEELGKREVTIPPKYRELLA-NH_2: A 1 mM solution of Ac-CDPMSSTYIE-ELGKREVTIPPKYRELLA-NH_2 (37 mg; 1.13×10^{-5} mole) in degassed and argon-saturated DMF/AcOH 95 : 5 (11.3 ml) was added dropwise to a 10 mM solution (35 mg; 1.13×10^{-5} mole) of 10 equivalents of di(5-nitro-2-pyridyl)disulfide in DMF/AcOH (95 : 5; 11.3 ml) under exclusion of air oxygen. The reaction was monitored spectroscopically at 430 nm and after the completion (1–2 h), the solvent was removed under reduced pressure; the residue was dissolved in 50 mM of AcOH and lyophilized. The crude compound was purified by preparative RP-HPLC: 20% yield; homogeneous on HPLC (t_R 7.4 min); ESI-MS: m/z = 1718.0 $[M + 2H]^{2+}$, 1145.6 $[M + 3H]^{3+}$, 859.8 $[M + 4H]^{4+}$; calcd. 3434.99.

Compound **2**: a 1 mM solution of Ac-CRRMKWKK-NH_2 (2.43 mg; 2.06×10^{-6} mole) in 2.06 ml of 50 mM NH_4OAc (pH 5.5) degassed and argon-saturated was added dropwise to a 5 mM solution of 1.1 equiv. of Ac-C(Npys)DPMSSTYIEELGKREVTIPPKYRELLA-NH_2 (7.8 mg; 2.27 μmole) in 454 μl of 50 mM NH_4OAc (pH 5.5) under exclusion of air oxygen. The reaction was monitored spectroscopically at 430 nm and after 4–5 h stirring at room temperature the mixture was washed with AcOEt to remove the 5-nitro-2-thiopyridine. The aqueous layer was then lyophilized and the product was purified by RP-HPLC: 22% yield; homogeneous on HPLC (t_R 6.3 min); ESI-MS: m/z = 1485.8 $[M + 3H]^{3+}$, 1114.6 $[M + 4H]^{4+}$, 892.0 $[M + 5H]^{5+}$, 743.4 $[M + 6H]^{6+}$, 637.4 $[M + 7H]^{7+}$; calcd. 4454.35.

Inhibition Assays and Determination of K_i-Values

Enzyme assays with μ-calpain and cathepsin L and the peptidic inhibitors were performed using the fluorogenic substrate Suc-Leu-Tyr-AMC as described previously [14].

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Cell Culture and Protease Activity Assay in Living Cells

LCLC 103H cells were cultured and the ionomycin-induced peptidolytic activity in living LCLC 103H cells was measured with the fluorogenic substrate Suc-Leu-leu-Val-Tyr-AMC in the absence and presence of compounds **1**–**3** following the protocoll reported previously [14]. The basal activity in the absence of ionomycin was subtracted for each individual concentration of the inhibitor and the resulting relative residual activity was expressed in % of the ionomycin-induced activity in the absence of inhibitor. LCLC 103H cells were incubated for 30 min with the fluorogenic substrate Suc-LLVY-AMC in the presence of 2 μM ionomycin at 37 °C without and with compounds **1**–**3** at varying concentrations.

REFERENCES

1. Vanderklish PW, Bahr BA. The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. *Int. J. Exp. Pathol.* 2000; **81**: 323–339.
2. Huang Y, Wang KK. The calpain family and human diseases. *Trends Mol. Med.* 2001; **7**: 355–362.
3. Sorimachi H, Ishiura S, Suzuki K. Structure and physiological function of calpains. *Biochem. J.* 1997; **328**: 721–732.
4. Wang KK, Yuen PW. Development and therapeutic potential of calpain inhibitors. *Adv. Pharmacol.* 1997; **37**: 117–152.
5. Maki M, Takano E, Osawa T, Ooi T, Murachi T, Hatanaka M. Analysis of structure-function relationship of pig calpastatin by expression of mutated cDNAs in *Escherichia coli*. *J. Biol. Chem.* 1988; **263**: 10254–10261.
6. Maki M, Bagci H, Hamaguchi K, Ueda M, Murachi T, Hatanaka M. Inhibition of calpain by a synthetic oligopeptide corresponding to an exon of the human calpastatin gene. *J. Biol. Chem.* 1989; **264**: 18866–18869.
7. Eto A, Akita Y, Saiso TC, Suzuki K, Kawashima S. The role of the calpain-calpastin system in tyrotropin-releasing hormone-induced selective down-regulation of a protein kinase C isoenzyme, nPKCε, in rat pituitary GH4C1 cells. *J. Biol. Chem.* 1995; **270**: 25115–25120.
8. Gonscherowski V, Becker BF, Moroder L, Motrescu E, Gil-Parrado S, Gloe T, Keller M, Zahler S. Calpains: a physiological regulator of the endothelial barrier? *Am. J. Physiol. Heart Circ. Physiol.* 2006; **290**: H2035–H2042.
9. Derossi D, Joliot AH, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* 1994; **269**: 10444–10450.
10. Derossi D, Chassaing G, Prochiantz A. Trojan peptides: the penetratin system for intracellular delivery. *Trends Cell Biol.* 1998; **8**: 84–87.
11. Fischer PM, Krausz E, Lane DP. Cellular delivery of impermeable effector molecules in the form of conjugates with peptides capable of mediating membrane translocation. *Bioconjug. Chem.* 2001; **12**: 825–841.
12. Snyder EL, Dowdy SF. Cell-penetrating peptides in drug delivery. *Pharm. Res.* 2004; **21**: 389–393.
13. Temsamani J, Vidal P. The use of cell-penetrating peptides for drug delivery. *Drug Discov. Today* 2004; **9**: 1012–1019.
14. Gil-Parrado S, Assfalg-Machleidt I, Fiorino F, Deluca D, Pfeiler D, Schaschke N, Moroder L, Machleidt W. Calpastatin exon 1B-derived peptide, a selective inhibitor of calpain: enhancing cell permeability by conjugation with penetratin. *Biol. Chem.* 2003; **384**: 395–402.
15. Fischer PM, Zhelev ZN, Wang S, Melville JE, Fåhraeus R, Lane DP. Structure-activity relationship of truncated and substituted analogues of the intracellular delivery vector penetratin. *J. Pept. Res.* 2000; **55**: 163–172.
16. Schaschke N, Deluca D, Assfalg-Machleidt I, Höhneke C, Sommerhoff CP, Machleidt W. Epoxysuccinyl peptide-derived cathepsin B inhibitors. Modulating membrane permeability by conjugation with the C-terminal heptapeptide segment of penetratin. *Biol. Chem.* 2002; **383**: 849–852.
17. Bepler G, Koehler A, Kiefer P, Havemann K, Beisenherz K, Jaques G, Gropp C, Haeder M. Characterization of the state of differentiation of six newly established human non-small-cell lung cancer cell lines. *Differentiation* 1988; **37**: 158–171.
18. Gabrijelcic-Geiger D, Mentele R, Meisel B, Hinz H, Assfalg-Machleidt I, Machleidt W, Möller A, Auerswald EA. Human μ-calpain: simple isolation from erythrocytes and characterization of autolysis fragments. *Biol. Chem.* 2001; **382**: 1733–1737.