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# Calpastatin Exon 1B-Derived Peptide, a Selective Inhibitor of Calpain: Enhancing Cell Permeability by Conjugation with Penetratin

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**The ubiquitous calpains, μ- and m-calpain, have been implicated in essential physiological processes and various pathologies. Cell-permeable specific inhibitors are important tools to elucidate the roles of calpains in cultivated cells and animal models. The synthetic N-acetylated 27-mer peptide derived from exon B of the inhibitory domain 1 of human calpastatin (CP1B) is unique as a potent and highly selective reversible calpain inhibitor, but is poorly cell-permeant. By addition of N-terminal cysteine residues we have generated a disulfide-conjugated CP1B with the cell-penetrating 16-mer peptide penetratin derived from the third helix of the Antennapedia homeodomain protein. The inhibitory potency and selectivity of CP1B for calpain *versus* cathepsin B and L, caspase 3 and the proteasome was not affected by the conjugation with penetratin. The conjugate was shown to efficiently penetrate into living LCLC 103H cells, since it prevents ionomycin-induced calpain activation at 200-fold lower concentration than the non-conjugated inhibitor and is able to reduce calpain-triggered apoptosis of these cells. Penetratin-conjugated CP1B seems to be a promising alternative to the widely used cell-permeable peptide aldehydes (*e.g.* calpain inhibitor I) which inhibit the lysosomal cathepsins and partially the proteasome as well or even better than the calpains.**

*Key words:* Apoptosis / Calpain / Calpain inhibitors / Calpastatin / Ionomycin / Penetratin.

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## Introduction

The ubiquitous calpains, μ- and m-calpain (μ- and mCP), are cytosolic calcium-dependent cysteine proteases that are present in almost all mammalian cells (see Sorimachi and Suzuki, 2001, for a recent review). Usually the calpains coexist with their specific endogenous protein inhibitor, calpastatin, which binds reversibly to the $Ca^{2+}$-activated enzymes and inhibits them selectively and efficiently (Takano *et al.*, 1995).

Although results with transgenic (knockout) mice deficient in calpain small subunit gene (Arthur *et al.*, 2000) point to essential cellular functions of the calpains, and a great number of putative calpain substrates have been demonstrated *in vitro*, not yet many calpain-dependent processes have been definitely identified. The ubiquitous calpains seem to be involved in signaling pathways (Sato and Kawashima, 2001), controlling cell proliferation (Carragher *et al.*, 2002), differentiation (Yajima and Kawashima, 2002) and motility (Glading *et al.*, 2002) as well as cell death (Squier *et al.*, 1999; Wang, 2000; Lu *et al.*, 2002; Gil-Parrado *et al.*, 2002). Proteolysis by undue activity of ubiquitous calpains has been implicated in various pathologies, such as neuronal and myocardial ischemia/reperfusion, inflammation or neurodegenerative disorders (reviewed in Vanderklish and Bahr, 2000; Huang and Wang, 2001). Therefore, calpains constitute potential therapeutic targets in diseases.

A great number of conclusions concerning the role of calpains in physiological or pathological processes is based on the effects of calpain inhibitors in cultured cells or animal models. Manipulation of the cellular level of calpastatin by overexpression or antisense techniques have been used in cell culture studies (*e.g.* Hiwasa *et al.*, 2002), but the large majority of experiments have been performed with exogenous synthetic calpain inhibitors that are sufficiently cell-permeable to reach their intracellular targets.

The search for cell-permeable calpain inhibitors has been an ongoing process following the discovery of the enzyme over 30 years ago (Donkor, 2000). Nonselective calpain inhibitors such as EDTA and EGTA ($Ca^{2+}$-chela-

tors) were followed by more potent and/or more selective calpain inhibitors isolated from natural sources or synthesized (reviewed in Wang and Yuen, 1997; Donkor, 2000). Most of them react covalently with the catalytic cysteine residue of calpain, but, with few exceptions, their selectivity for the calpains is low. For instance, various peptide aldehydes that have been extensively used for *in vivo* calpain inhibition (*e.g*. Ac-Leu-Leu-Nle-CHO, here abbreviated as AcLLNal, 'calpain inhibitor I'), are potent inhibitors also of the lysosomal cathepsins L and B (Sasaki *et al.*, 1990) and of the proteasome (Stein, *et al.*, 1996). Moreover, the calpain inhibitor calpeptin (Z-Leu-Nle-CHO) was shown to inhibit protein tyrosine phosphatases as well (Schoenwaelder and Burridge, 1999). During the last decade much effort has been spent in the development of peptidic, peptidomimetic or non-peptide calpain inhibitors of improved selectivity and good bioavailability, but few of these inhibitors have been completely characterized in terms of selectivity or have been used extensively as tools in basic research (Donkor, 2000; Hernandez and Roush, 2002).

The α-mercaptoacrylic acid derivative PD150606, a non-peptide cell-permeable calpain inhibitor that binds to the calmodulin-like domains of the large and the small subunit and inhibits calpain uncompetitively, has been considered to be selective for calpain (Wang *et al.*, 1996), but has recently been shown to interfere with excitoxicity-dependent motor neuron death purely mediated through $Ca^{2+}$-permeable AMPA receptors despite other calmodulin and calpain inhibitors were not effective (Van Den Bosch *et al.*, 2002).

The most selective synthetic inhibitor of calpains that is presently available is a N-acetylated and C-amidated 27-mer peptide derived from exon B of the inhibitory domain 1 of human calpastatin (CP1B; Maki *et al.*, 1989) which binds reversibly to the catalytic subunit of μ- and m-calpain (Maki *et al.*, 1988). This peptide, also called 'acetyl calpastatin 27-peptide' (AC27P; Gil-Parrado *et al.*, 2002), does not inhibit papain or trypsin (Maki *et al.*, 1989) and is considered to be highly selective for the ubiquitous calpains, although so far quantitative data on its inhibitory specificity have not been reported. CP1B, which is commercially available, has successfully been used by several investigators for inhibition of calpain activity in living cells (Eto *et al.*, 1995; Yamazaki *et al.*, 1997a,b; Kusakawa *et al.*, 2000; Blomgren *et al.*, 2001; Shiraishi *et al.*, 2001; Gil-Parrado *et al.*, 2002). Using a rhodamine-labeled peptide, uptake of CP1B into cells was confirmed (Eto *et al.*, 1995). However, high concentrations were needed by all investigators to achieve intracellular effects, indicating that the 27-mer is poorly cell-permeable.

Here we report quantitative *in vitro* inhibition data confirming the presumed high selectivity of CP1B for μ-calpain over other typical intracellular proteases, such as cathepsin L and B, caspase 3 and the proteasome. Cell permeability of this highly selective calpain inhibitor was substantially increased by conjugation with the cell-penetrating peptide penetratin, a 16-mer peptide derived from the third helix of the homeodomain of the Antennapedia protein (Derossi *et al.*, 1998) which has been widely used for intracellular delivery of peptides and oligonucleotides (Fischer *et al.*, 2001). The resulting new calpain inhibitor, penetratin-calpastatin peptide 1B (PCP1B), was

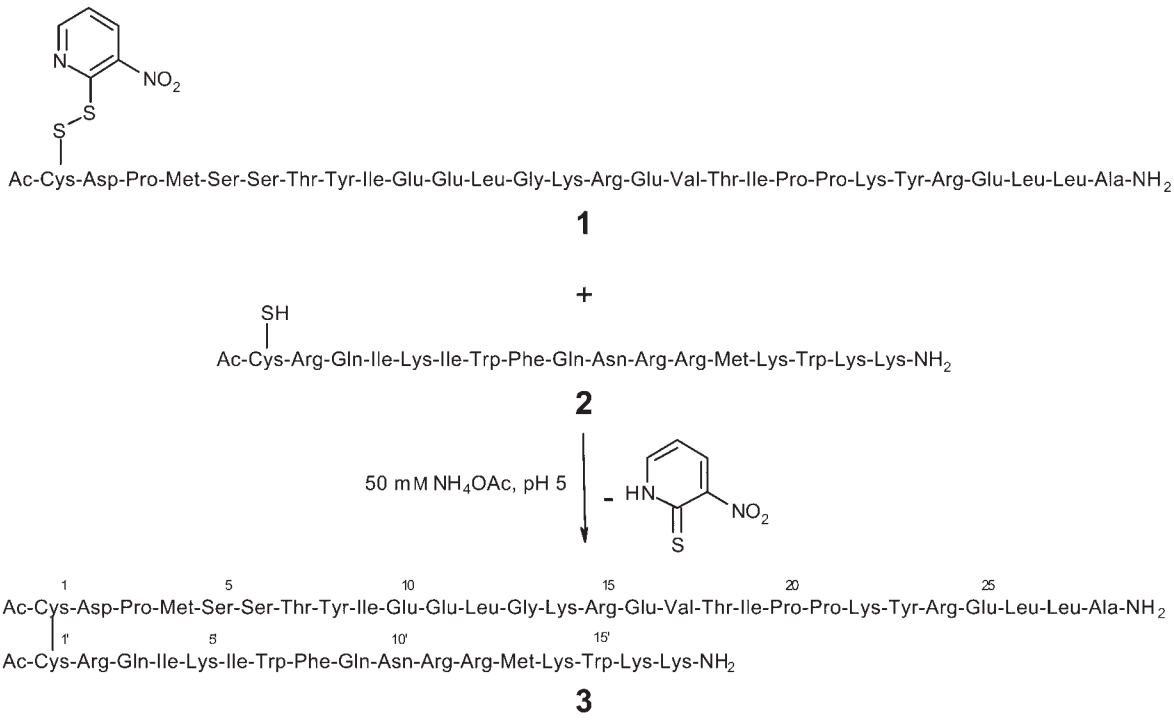


**Fig. 1** Structure and Synthesis of the Calpastatin Exon 1B-Derived Peptide-Penetratin Conjugate (PCP1B).

able to inhibit ionomycin-induced calpain activation in living cells at submicromolar concentrations and to prevent calpain-triggered apoptosis. These features constitute a promising substitute for widely used cell-permeable calpain inhibitors of low selectivity.

## Results

### Synthesis of Penetratin-Conjugated Calpastatin Exon 1B-Derived Peptide (PCP1B)

The CP1B-penetratin conjugate **3** was synthesized as shown in Figure 1. To crossbridge the calpastatin-derived peptide with penetratin *via* a disulfide bridge, both peptides were appropriately modified by N-terminal elongation with N-acetyl cysteine. Selective formation of the disulfide bond was achieved by reacting S-Npys-activated peptide **1** with the free thiol of modified penetratin **2** (Bernatowicz *et al.*, 1986). The resulting conjugate **3,** penetratin-calpastin peptide 1B (PCP1B), was fully characterized by RP-HPLC, quantitative amino acid analysis, and ESI-MS (data not shown). The solubility of CP1B was substantially increased by the conjugation with the basic penetratin peptide.

### CP1B and PCP1B Are Potent and Selective Calpain Inhibitors

The inhibitory profile of CP1B against four typical cellular cysteine proteases and the human 20S proteasome demonstrates that the calpastatin-derived peptide is a very potent ($K_i$ = 0.2 nM) and highly selective inhibitor of calpain (Table 1). Whereas the activity of cathepsin B, caspase 3 and the chymotryptic activity of the 20S proteasome are virtually not affected, the selectivity for μ-calpain over cathepsin L is still 30 000-fold. Inhibition of cathepsin L by CP1B is comparable to that obtained with a 'scrambled' peptide of the same amino acid composition (Eto *et al.*, 1995). In contrast, the cell-permeable peptidyl aldehyde Ac-LLNal (calpain inhibitor I) has about the same affinity for cathepsin B and even 160-fold higher affinity for cathepsin L when compared with μ-calpain (Table 1).

The high inhibitory potency of CP1B for calpain remains essentially unchanged after conjugation with penetratin, whereas the N-acetylated Cys-penetratin vector (AcP) alone does not inhibit μ-calpain. $K_i$ values of PCP1B for the two cathepsins are still in the micromolar range, and a similar micromolar $K_i$ was determined with AcP itself. Inhibition of cathepsin L and B by AcP and PCP1B was temporary, indicating that the free and the conjugated penetratin peptide is slowly degraded as a substrate. This was confirmed by the detection of cleavage products after incubation with higher concentrations of cathepsin L (≥0.25 nM). PCP1B was cleaved by cathepsin L in the penetratin portion between Gln9' and Asn10', and AcP additionally between Arg11' and Arg12' (see sequences in Figure 1). In contrast, in PCP1B no cleavages were observed with μ-calpain, even with 100-fold higher enzyme concentrations than used in the inhibition assays. From this we expect that the penetratin moiety may be proteolytically degraded by intracellular proteases after cellular uptake, as has been suggested (Waizenegger *et al.*, 2002). Penetratin contains several basic residues which are preferred cleavage sites of proteases, and a half life of 1 h has been estimated in mammalian reticulocytes (Lindgren *et al.*, 2000a).

### PCP1B Is a Cell-Permeable Calpain Inhibitor

LCLC 103H cells, a cell line derived from a human large cell lung carcinoma (Bepler *et al.*, 1988), were used for cell permeation studies, because we had shown previously that ionomycin-induced calpain activity in these cells is effectively inhibited by preincubation with AC27P (CP1B) (Gil-Parrado *et al.*, 2002). Here we compared the inhibitory effect of PCP1B and CP1B on the intracellular calpains. Ionomycin-induced hydrolysis of the cell-permeable fluorogenic substrate Suc-LLVY-amc was meas-

**Table 1** Selectivity of Calpain Inhibition by Calpastatin-Derived Peptide 1B (CP1B) and Its Penetratin Conjugate (PCP1B).

| Inhibitor | $K_i$ (μM)[a] | | | | |
|---|---|---|---|---|---|
| | μ-Calpain | Cathepsin L | Cathepsin B | Proteasome[b] | Caspase 3 |
| CP1B | 0.0002 | 6 | ≥500 | ≥200 | ≥500 |
| CP1B scrambled[c] | 6 | 6 | ≥500 | ≥200 | ≥200 |
| PCP1B | 0.0005 | 0.8[e] | 12[e] | ≥200 | ≥200 |
| AcP[c] | ≥50 | 1.0[e] | ≥130[e] | ≥200 | ≥200 |
| AcLLNal[c] | 0.003[d] | 0.00003 | 0.002[d] | 0.5[e] | ≥9000 |

[a]Mean of 5–10 experiments with individual inhibitor concentrations (S.D.<10%). ≥ values are 10-fold the highest used inhibitor concentration that resulted in less than 10% inhibition.
[b]Chymotryptic activity of the human 20S proteasome.
[c]Data obtained with 'scrambled' CP1B (see 'Results') and AcP (N-acetyl-Cys-penetratin) are included as controls, data with AcLLNal ('calpain inhibitor I') for comparison.
[d]Data taken from Schaschke *et al.* (1996).
[e]Temporary inhibition (see text for explanation).

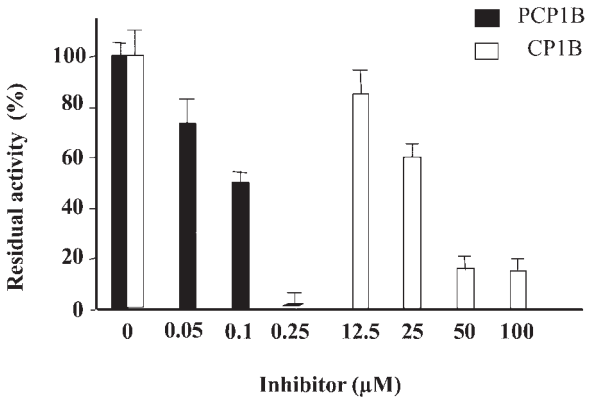


**Fig. 2** Inhibition of Calpain Activity and Calpain-Mediated Apoptosis in Living LCLC 103H Cells by Penetratin-Linked Calpastatin Exon 1B-Derived Peptide (PCP1B).
Inhibition of ionomycin-induced Suc-LLVY-amc hydrolyzing activity. Residual ionomycin-induced Suc-LLVY-amc hydrolyzing activity (%) measured in cells after 30 min of incubation with the fluorogenic substrate (37 °C) following pre-incubation with CP1B or PCP1B at the indicated concentrations. 100% represents the Suc-LLVY-amc hydrolyzing activity induced by addition of 2 μM ionomycin in the absence of inhibitors. All experiments were performed in triplicate; SD are indicated by bars; all depicted data are significantly different ($p<0.01$) from the controls (0, ionomycin-treated cells in the absence of inhibitors).

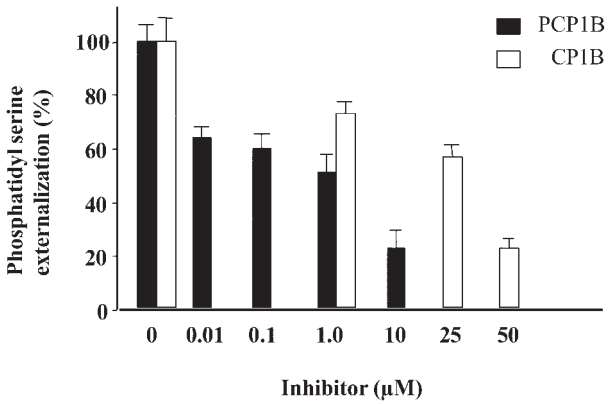


**Fig. 3** Inhibition of Ionomycin-Induced Apoptosis by Free and Penetratin-Linked CP1B.
To cells treated with the indicated inhibitors, annexin V-fluorescein and propidium iodide were added before counting and sorting by FACS as described in Materials and Methods. Phosphatidylserine externalization (%) represents the percentage of cells positive for phosphatidylserine externalization and negative for propidium iodide uptake after addition of 2 μM ionomycin for 3 h at 37 °C, related to the total amount of cells. All experiments were performed in triplicate; SD are indicated by bars; all depicted data are significantly different ($p<0.01$) from the controls (0, ionomycin-treated cells in the absence of inhibitors).

ured in non-treated control cells (100%) and after preincubation with increasing concentrations of CP1B and PCP1B, respectively (Figure 2). Whereas ionomycin-induced activity was reduced to 15% by 50 μM CP1B (a concentration widely used in the cell culture models cited in the Introduction section), a concentration of PCP1B as low as 0.25 μM yielded almost complete inhibition. Preincubation of the cells with the penetratin vector (AcP) itself had no significant effect (data not shown). These data clearly demonstrate a substantial increase of cell-permeability due to the conjugation of CP1B with penetratin. The penetratin-conjugated peptide is able to penetrate into LCLC 103H cells where it prevents ionomycin-induced calpain activation at roughly 200-fold lower concentrations (in the surrounding medium) than the non-conjugated inhibitor.

### PCP1B Protects LCLC 103H Cells against Ionomycin-Induced Apoptosis

We have recently shown that ionomycin-activated calpain triggers apoptosis in LCLC 103H cells and that apoptosis was prevented by preincubation of these cells with 50 μM CP1B (AC27P; Gil-Parrado *et al.*, 2002). Here we used this model to compare the effects of CP1B and PCP1B on the percentage of apoptotic cells, estimated by FACS analysis for phosphatidyl serine externalization (annexin V binding) and propidium iodide uptake 3 h after incubation with 2 μM ionomycin (Figure 3). Whereas apoptotic cells were reduced to roughly 25% by 50 μM CP1B, the same reduction was achieved by only 10 μM PCP1B. The penetratin vector itself had no significant effect on the number of apoptotic cells (data not shown).

## Discussion

As long as the three-dimensional structure of a calcium-actived calpain or a substrate/inhibitor complex of calpain is not available, structure-based modeling of synthetic calpain inhibitors will be not feasible. It remains to be established whether the recently published structure of a $Ca^{2+}$-activated 'minicalpain' comprising the catalytic domain II plus domain I (Moldoveanu *et al.*, 2002) will enable the design of more selective inhibitors, since this very papain-like fragment displays less than 5% of the specific enzymatic activity of complete calpain. It seems probable that other domains of the calpain molecule participate in substrate recognition and are responsible for the highly selective binding of protein substrates and of the endogenous protein inhibitor calpastatin. This is underscored by the results of structure-activity relationship (SAR) studies (Donkor, 2000) indicating that affinity and selectivity of calpain for small peptide substrates and inhibitors are low when compared to other cysteine proteases.

The shortest peptide binding to calpain with outstanding affinity and selectivity is the 27-mer derived from exon 1B of calpastatin domain 1 (CP1B). The mechanism of inhibition of this peptide and its binding site on calpain are unknown, but it has been shown that N-terminal or C-terminal truncation by a few residues reduces its inhibitory

potency drastically (Uemori *et al.*, 1990). A similar synthetic 24-mer peptide, derived from a consensus sequence of all four inhibitory domains of porcine calpastatin has been described (Croall and McGrody, 1994). Maki *et al.*, who introduced CP1B (Maki *et al.*, 1989), reported that it does not inhibit either papain or trypsin. Here we confirmed and extended these findings by quantitative data on the inhibition of selected intracellular proteases. Although only few typical cellular proteases were considered, our data suggest that CP1B is a potent and highly selective calpain inhibitor.

As shown previously (Kawasaki *et al.*, 1989), inhibition of calpain by CP1B is reversible and competitive with substrates. Considering a $K_i$ value of 0.2 nM as determined in this work, an intracellular concentration of about 20 nM CP1B would be required for 99% inhibition of μ-calpain. However, even 100 μM CP1B in the surrounding medium was not sufficient to completely inhibit calpain activity in living cells. This discrepancy is obviously due to the poor cell permeability of CP1B that has been observed in previous cell culture studies (see references cited in 'Introduction') and apparently precludes this selective calpain inhibitor from use in isolated organ perfusion and animal models as well as from potential therapeutic application.

The most promising approach to increase cell permeability of a large hydrophilic peptide like CP1B seemed to conjugate it with a cell-penetrating peptide vector (reviewed in Lindgren *et al.*, 2000b; Fischer *et al.*, 2001). We decided in favor of penetratin, a 16-mer peptide derived from the third helix of the homeodomain of the Antennapedia protein (Derossi *et al.*, 1998). This peptide penetrates membranes through an energy-independent mechanism that is not precisely known and translocates conjugated cargo peptides or even proteins into the cytoplasm and the cell nucleus. Recently, we have used the C-terminal heptapeptide segment of penetratin as a vector for intracellular delivery of an epoxysuccinyl peptide-derived selective cathepsin B inhibitor (Schaschke *et al.*, 2002). Here, as in most previously reported applications of penetratin, we disulfide-linked full-length Cys-penetratin to Cys-CP1B. The disulfide-linked cargo is supposed to be rapidly released in the reductive intracellular milieu (Hällbrink *et al.*, 2001).

It has been shown recently by fluorescence correlation spectroscopy that penetratin-conjugated fluorophores are imported into living cells even at nanomolar concentrations (Waizenegger *et al.*, 2002). By fluorescence microscopy we detected uptake of biotinylated penetratin into LCLC 103H cells at concentrations above 100 nM (data not shown). Accordingly, our penetratin-CP1B conjugate effected complete inhibition of ionomycin-induced intracellular calpain activity at concentrations in the nanomolar range (see Figure 2), close to the concentration required for complete inhibition of isolated μ-calpain *in vitro* (see above). The high selectivity for calpain over the cathepsins L and B, caspase 3 and the 20S proteasome remained essentially unchanged after conjugation with penetratin (see Table 1). Therefore, PCP1B should allow effective and selective calpain inhibition in cell culture models, isolated organs or whole animals at concentrations in the submicromolar or low micromolar range.

Surprisingly, the protective effect of PCP1B on calpain-induced apoptosis of LCLC 103H cells (Figure 3) was much less pronounced than was expected from inhibition of intracellular calpain activity (Figure 2). On the basis of available data we cannot satisfactorily explain this discrepance. However, phosphatidyl serine (PS) externalization, the mechanism of which is not fully understood (Naito *et al.*, 1997), is a complex process at the end of the intrinsic apoptotic pathway triggered by calpain (Gil-Parrado *et al.*, 2002) and may not strictly correlate with intracellular calpain activity determined by cleavage of a cell-permeable peptide substrate. More than 25% of ionomycin-induced PS externalization were inhibited neither by 10 μM PCB1B nor by 50 μM CP1B and seem to be not calpain-dependent (Figure 3). This portion should include cells that become apoptotic in the absence of ionomycin (up to 35% in previous studies; see Figure 3A in Gil-Parrado *et al.*, 2002).

A related approach using a different cell-penetrating peptide, the signal sequence of Kaposi's fibroblast growth factor (residues 7–22), peptide bond-linked to the consensus sequence of the four inhibitory domains of porcine calpastatin, has previously been reported for calpain inhibition in platelets (Croce *et al.*, 1999). This conjugate, called 'calpastat', displayed an $IC_{50}$ of 70 nM for inhibition of isolated μ-calpain, and $IC_{50}$ values between 20 and 50 μM for biological effects in platelets. The use of a consensus sequence (Croall and McGrody, 1994) instead of CP1B seems to be responsible for the 350-fold lower inhibitory potency of 'calpastat' when compared to PCP1B, but biological effects have been reported with similar concentrations (Croce *et al.*, 1999).

Due to the lack of selective calpain inhibitors that are readily cell-permeant, even very recent studies addressing the physio(patho)logical relevance of calpain have been performed using inhibitors of low selectivity or poor cell permeability (*e.g.* Bordone and Campbell, 2002; Rutledge and Whiteheart, 2002; Chen *et al.*, 2002, among many others). The new selective and cell-permeable inhibitor described here may help to confirm or question results obtained with less specific inhibitors and to discriminate between the effects of calpain and other cellular proteases (Yamashima, 2000). The inherent drawback of a rather complicated synthesis of this long peptide should be accepted as long as equally cell-permeable and specific calpain inhibitors of smaller size are not available.

## Materials and Methods

### Materials

μ-Calpain was isolated from human erythrocytes (Gabrijelcic-Geiger *et al.*, 2001). Human 20S proteasome was obtained from Affiniti Research Products (Mamhead, Exeter, UK). Cathepsins L

and B, active recombinant caspase 3, the scrambled calpastatin peptide and calpain inhibitor I (Ac-LLNal) were purchased from Calbiochem (Bad Soden, Germany). Calpastatin peptide (CP1B) was from Sigma or Calbiochem. Substrates Suc-LY-amc, Suc-LLVY-amc, Z-FR-amc and Ac-DEVD-amc were from Bachem (Heidelberg, Germany), ionomycin from Calbiochem, avidin-FITC from Zymed Laboratories (South San Francisco, CA, USA). All other reagents were of the highest purity commercially available. The LCLC 103H cell line (ACC 384) was supplied by DSMZ-GmbH (Braunschweig, Germany).

#### Peptide Synthesis and Purification

Compound **1** (Figure 1), calpastatin exon 1B peptide functionalized at its N-terminus with a S-Npys-activated N-acetylated cysteine residue, was initially custom synthesized by the Microchemical Facility of Emory University, Winship Cancer Center (Atlanta, GA, USA) and later synthesized in our laboratory by the method described below for Compound **2.** Compound **2** (Figure 1), penetratin N-terminally elongated with a N-acetylated cysteine residue, was synthesized on a Applied Biosystems peptide synthesizer (model 431A) 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.). N-terminal acetylation was carried out with acetic anhydride/DIEA (1:1.5; 10 equiv.) and final cleavage/deprotection was performed with 95% aq TFA/triisopropylsilane (98.5:1.5) yielding StBu-protected **2** which was purified by RP-HPLC on Nucleosil C18 using a linear gradient form 18% B to 45% B within 60 min (eluent A: 0.1% aq TFA; eluent B: 0.08% TFA in acetonitrile). The resulting product was deprotected by treatment with tri-n-butylphosphine (5 equiv.) in 95% aq TFE. Conjugate **3** was synthesized by adding under an argon atmosphere peptide **2** (1 equiv.) dissolved in 50 mM $NH_4OAc$-buffer (pH 5, degassed and saturated with argon) slowly to a solution of **1** (1 equiv.) in the same buffer. After purification by gel chromatography on Fractogel HW 40 S using 1% aq AcOH as eluent, **3** was fully characterized by RP-HPLC, quantitative amino acid analysis, and ESI-MS.

#### Inhibition Assays and $K_i$ Determination

Inhibition of μ-calpain (0.5 – 1 nM) was measured at 12 °C (to slow down inactivation by autolysis) with the fluorogenic substrate Suc-LY-amc (250 μM) in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.015% Brij-35 (calpain buffer), 150 – 200 μM $CaCl_2$, 1 mM dithiothreitol (DTT). Cathepsin L (0.25 nM) was assayed at 25 °C, pH 5.5 with 25 μM Suc-LY-amc and cathepsin B (25 pM) at 30 °C with Z-FR-amc (10 μM) in 0.25 mM sodium acetate, pH 5.5, 2 mM EDTA, 0.015% Brij-35. The chymotryptic activity of the human 20S proteasome (2 nM) was measured at 30 °C with Suc-LLVY-amc (10 μM) in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 0.025% SDS and caspase 3 activity at 25 °C with Ac-DEVD-amc (5 μM) in 50 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 10% sucrose, 0.1% CHAPS and 10 mM DTT. Continuous assays were performed recording fluorescence (excitation 380 nm, emission 460 nm) essentially as described in Machleidt *et al.* (1993). After preactivation of the enzymes with DTT (exept the proteasome) in the presence of substrate and of $CaCl_2$ (μ-calpain), about 5 – 10 different inhibitor concentrations, dissolved and prediluted in calpain buffer (or DMSO for AcLLNal), were added (max. 1% of the total test volume), and the reaction was followed until equilibrium. $K_i$ values for the inhibition of μ-calpain by the free and the penetratin-conjugated calpastatin peptide were obtained from presteady-state kinetics, fitting the progress data by nonlinear regression analysis to the integrated equation of Morrison (Morrison, 1982). From the rate constants $k_{on}$ and $k_{off}$ the $K_i$-values were calculated as $K_i = k_{off}/k_{on}$. All other inhibition constants were calculated from the initial and steady state rates using the equation for classical inhibition. $K_i$ values were corrected for competition with the substrates. In cases of less than 10% inhibition, the $K_i$-value was assumed to be at least 10-fold the highest used inhibitor concentration.

#### Cell Culture

For routine cell culture, RPMI 1640 **m**edium (Gibco-BRL) was supplemented with 10% fetal calf sera (Sigma) and 0.6% L-glutamine (Gibco-BRL). Cells were maintained at 37 °C in a humidified 5% $CO_2$ atmosphere. For inhibition experiments, the cells were maintained in serum-free HEPES-RPMI 1640 (Gibco-BRL). When being subcultured, cells were lifted using trypsin-EDTA solution (Gibco-BRL).

#### Protease Activity and Inhibition Assay in Living Cells

Suc-LLVY-amc hydrolyzing activity was determined by measuring hydrolysis of the peptidyl 7-amino bond of this fluorogenic substrate in LCLC 103H cells, plated on 24-well plates ($10^5$ cells/well), in Hepes-buffered serum-free growth medium. Cells were pre-incubated with Suc-LLVY-amc (160 μM) for 1 h at 37 °C in a humidified 5% $CO_2$ incubator. Substrate hydrolysis was monitored using a fluorescence reading system (Fluoroskan ascent) with filter settings of 355 ± 20 nm for excitation and 460 ± 20 nm for emission. Prior to addition of 2 μM ionomycin, the basal fluorescence, $F_0$, was recorded. Fluorescence readings, $F_t$, were collected every 5 min up to 120 min at 37 °C. Subsequently, plates were removed from the scanner, and incubated with lysis buffer (50 mM Hepes/KOH, 150 mM NaCl, 1% NP 40, pH 7.4) for 2 h at room temperature for protein concentration measurements. As there were no statistically significant differences in protein content between the wells, ionomycin-dependent proteolytic activity was expressed in arbitrary units and calculated as the linear slope $\Delta F = (F_t\text{-}F_o)/(t\text{-}t_o)$.

Inhibition of ionomycin-induced Suc-LLVY-amc hydrolyzing activity was measured after preincubation of the cells for 1 h at 37 °C with the inhibitors CP1B and PCP1B dissolved in calpain buffer and diluted in Hepes-buffered serum-free growth medium at the indicated concentrations (see Figure 2) before addition of substrate. Note that these inhibitor concentrations were chosen taking into account prior results (Gil-Parrado *et al.*, 2002) and the maintenance of cell viability. The residual activity (%) was expressed as $[(\Delta F - \Delta F_{inh})/\Delta F] \times 100$, where $\Delta F_{inh}$ is the ionomycin-induced activity in the presence of inhibitor. Triplicate measurements were performed for each sample.

#### FACS Analysis of Apoptotic Cells

Cells were treated with 2 μM ionomycin for 3 h at 37 °C. Before being counted and sorted by FACS, annexin V-fluorescein and propidium iodide were added as described (Gil-Parrado *et al.*, 2002). Phosphatidylserine externalization (%) represents the percentage of cells positive for phosphatidylserine externalization and negative for propidium iodide uptake, related to the total amount of cells. Inhibition of phosphatidylserine externalization was measured after pre-incubation of the cells for 1 h with different concentrations of the inhibitors CP1B and PCP1B, followed by incubation with ionomycin and FACS counting. All experiments were performed in triplicate.

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