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Allosteric inhibitors of calpains: Reevaluating inhibition by PD150606 and LSEAL

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ABSTRACT

Background: The mercaptoacrylate calpain inhibitor, PD150606, has been shown by X-ray crystallography to bind to a hydrophobic groove in the enzyme's penta-EF-hand domains far away from the catalytic cleft and has been previously described as an uncompetitive inhibitor of calpains. The penta-peptide LSEAL has been reported to be an inhibitor of calpain and was predicted to bind in the same hydrophobic groove. The X-ray crystal structure of calpain-2 bound to its endogenous calpain inhibitor, calpastatin, shows that calpastatin also binds to the hydrophobic grooves in the two penta-EF-hand domains, but its inhibitory domain binds to the protease core domains and blocks the active site cleft directly.
Methods: The mechanisms of inhibition by PD150606 and LSEAL were investigated using steady-state kinetics of cleavage of a fluorogenic substrate by calpain-2 and the protease core of calpain1, as well as by examining the inhibition of casein hydrolysis by calpain and the autoproteolysis of calpain.
Results: PD150606 inhibits both full-length calpain-2 and the protease core of calpain-1 with an apparent non-competitive kinetic model. The penta-peptide LSEAL failed to inhibit either whole calpain or its protease core in vitro.
Conclusions: PD150606 cannot inhibit cleavage by calpain-2 of small substrates via binding to the penta-EF-hand domain.
General significance: PD150606 is often described as a calpain-specific inhibitor due to its ability to target the penta-EF-hand domains of calpain, but we show that it must be acting at a site on the protease core domain instead.

1. Introduction

Calpains are a family of intracellular cysteine proteases that are co-operatively activated by Ca^{2+}. They are responsible for converting localized Ca^{2+} signals into a cellular response through specific and limited cleavage of proteolytic targets. The cellular roles of calpains during signal transduction include reorganization of the cytoskeleton, participation in cell cycle regulation, and apoptosis [1,2]. Over-activation of calpains contributes to pathological effects in conditions such as: heart attack, stroke, neurodegeneration, cancer, Alzheimer's disease, and muscular dystrophy [2–5]. Selective inhibitors of calpains could, therefore, have investigative and therapeutic applications in several diseases.

Calpains-1 and -2, the most extensively studied isoforms, are heterodimers of a distinct catalytic 80-kDa subunit and a common regulatory 28-kDa subunit [1,6–9]. During activation they bind up to ten Ca^{2+}, four to each of the two C-terminal penta-EF-hand (PEF) domains [10,11] and one to each of the two protease core domains [12]. Binding by these ions causes a realignment of the domains to form the active site cleft and a more compact enzyme.

It is the calcium-bound form of the enzyme that is recognized by the endogenous calpain-specific inhibitor, calpastatin. This intrinsically unstructured 70-kDa protein has a domain L of unknown function, followed by four independently active inhibitory domains 1 through 4, each containing subdomains A, B, and C [13]. Recent crystal structures of calcium-bound calpain inhibited by calpastatin have revealed the mode of inhibition [10,11]. Terminal subdomains A and C of the inhibitor form amphipathic α-helices on binding to hydrophobic clefts in the two PEF domains. Subdomain B is responsible for the inhibition of calpain and binds across and occludes the protease core active site.

The ongoing development of calpain inhibitors has focused mainly on peptidomimetic compounds containing electrophilic warheads targeted to the active site cleft. However, the cleft is quite similar in cathepsins and other cysteine proteases, making it difficult to achieve calpain specificity. Allosteric inhibitors of calpains have the potential advantage of binding regions of the enzyme removed from the active site that are unique to calpain. Two types of allosteric calpain inhibitors have

Abbreviations: PEF, penta-EF-hand; FRET, fluorescence resonance energy transfer

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been reported: mercaptoacrylates, such as PD150606 [14] and other variants [15], and peptidic inhibitors, such as the penta-peptide LSEAL [16].

The crystal structure of the inhibitor PD150606 bound to the calpain small subunit PEF domain implied an allosteric mechanism of inhibition through binding to a hydrophobic pocket distant from the enzyme's active site [17,18]. Ca^{2+}-binding to the calpain small subunit PEF domain [19] results in the opening of a small hydrophobic pocket near EF-hand 1 into which PD150606 was observed to bind. Binding of PD150606 resulted in the movement of a single residue (Gln173) [17,18]. The calpastatin subdomain C binds to the same region, but primarily in the cleft between helices E1 and F1, and results in a more significant shift of the E1 helix [10,18]. The penta-peptide LSEAL was characterized as a calpain inhibitor and was noted to contain a conserved motif found in the PEF-binding regions of calpastatin [16]. NMR-guided docking studies have suggested that the LSEAL peptide binds hydrophobic clefts in a manner similar to calpastatin subdomains A and C [20].

The recently solved mechanism of action of the calpastatin inhibitor raises questions about how small molecules binding to pre-formed hydrophobic clefts in the PEF domains confer inhibition of the enzyme [10,11]. Calpastatin uses these clefts solely as anchor points to help direct the inhibitory region to the active site cleft. Here we have investigated the inhibition of calpain by PD150606 and LSEAL and show that they are unlikely to work by an allosteric mechanism that involves binding to the PEF domains.

2. Materials and methods

2.1. Materials

Active and inactive (C105S) rat recombinant calpain-2 [21] and calpain-1 protease core [12] were expressed and purified as previously described. Porcine erythrocyte calpain-1 was purchased from Calbiochem. The calpain substrate (EDANS)-EPLFAERK-(DABCYL) and the penta-peptide N-acetyl-LSEAL-amide were synthesized by Biomer Technology. Inhibitors were purchased from Calbiochem (E64, leupeptin, and calpeptin) or Sigma-Aldrich (PD150606), with the exception of SNJ-1945, which was graciously donated by Senju Pharmaceutical Co. Ltd. (Kobe, Japan).

2.2. (EDANS)-EPLFAERK-(DABCYL) hydrolysis assay

The rate of cleavage of the (EDANS)-EPLFAERK-(DABCYL) fluorescent substrate was monitored in a 0.5 mL cuvette using a Perkin Elmer LS55 fluorescence spectrometer with $\lambda_{ex} = 335$ nm, $\lambda_{em} = 500$ nm and slit widths of 10 nm. Triplicate readings of reaction profiles were obtained at a sampling interval of 0.1 s at a temperature of 25 °C. The cuvette contained 100 nM calpain-1 protease core or 10 nM calpain-2, 5 μM substrate, and either 50 μM PD150606 or 100 μM LSEAL peptide (each dissolved in DMSO at stock concentrations of 50 mM) in 10 mM HEPES, pH 7.4; 10 mM DTT and sufficient DMSO to bring the total concentration of DMSO to 0.4% for all assays. After incubating the above compounds for 5 min, the reaction was initiated with the addition of $CaCl_2$ to 4 mM in a final volume of 0.5 mL. For enzyme kinetic analysis with PD150606, the hydrolysis assays were performed under the same conditions but with substrate concentrations in the range of 1–100 μM and PD150606 concentrations in the range of 0–50 μM. The initial reaction rates were determined by fitting a straight line to the linear portion of the progress curves (first 10 s after calcium addition). The resulting rate data were examined with Michaelis–Menten plots for the analysis of enzyme inhibition. K_m and V_{max} values for the reactions were obtained from direct fits of the Michaelis–Menten equation to the data using the non-linear least squares fitting function of gnuplot (version 4.6) [22]. All reactions were corrected for the inner filter effect as previously described by measuring the fluorescence of increasing amounts of substrate with a fixed concentration of free EDANS fluor and thereby calculating a correction factor [23].

2.3. Calpain autolysis/proteolysis assays

Autolysis of 0.5 mg/mL (5 μM) calpain-2 was performed at a temperature of 25 °C in 10 mM HEPES, pH 7.4; 10 mM DTT; and initiated with the addition of $CaCl_2$ to 100 mM in a final volume of 400 μL. Autolysis was performed in the absence and presence of 0.1 mM, 1 mM, and 3 mM PD150606. Immediately, and 20 min after calcium addition, aliquots (40 μL) were removed and mixed with 20 μL of SDS sample buffer and 0.5 M EDTA to stop the reaction. Samples were then analyzed by SDS-PAGE in 10% polyacrylamide gels. Proteolysis of 0.5 mg/mL C105S calpain-2 by 0.5 mg/mL (13 μM) calpain-1 protease core was performed and analyzed in the same manner.

2.4. Casein hydrolysis assay

Hydrolysis of 5 mg/mL casein by 0.5 mg/mL (5 μM) calpain-2 and 0.4 mg/mL (10 μM) calpain-1 protease core was performed as previously described [24] for 30 min at 25 °C in the presence of 10 μM leupeptin, calpeptin, SNJ-1945, PD150606, or LSEAL peptide. The reactions were performed with 5 mM $CaCl_2$ or 5 mM EDTA in 50 mM Tris–HCl, pH 8.0 and initiated by the addition of enzyme. Inhibited enzyme activities were calculated relative to control reactions that contained the same final DMSO concentrations (2%) as the inhibitor assays.

2.5. Thermal shift in a differential light scattering assay

Temperature-induced aggregation was measured using differential static light scattering in a 384-well format (StarGazer, Harbinger Biotechnology) as previously described [25]. A solution containing 0.4 mg/mL of either C105S calpain-2 or calpain-1 protease core was prepared in 100 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM DTT; and 1 mM $CaCl_2$. Individual wells contained DMSO as a vehicle control, the compound PD150606, or the penta-peptide LSEAL. Inhibitors were tested at concentrations ranging from 50 μM to 1 mM. Each measurement was done in triplicate and averaged to give the temperature of aggregation (T_{agg}) and the change in temperature of aggregation compared to the control values (ΔT_{agg}).

3. Results

3.1. PD150606 inhibits the protease core of calpain-1

The compound PD150606 was analyzed for inhibitory activity against both full-length calpain-2 and the calpain-1 protease core domains using the peptide FRET substrate (EDANS)-EPLFAERK-(DABCYL). The rate of cleavage of the fluorogenic substrate by calpain-2 in the absence of inhibitor decreased as a function of time (Fig. 1A) mainly due to calpain autoproteolysis. A similar trajectory was seen in the presence of 50 μM PD150606, but the activity level was reduced to about 30% of the uninhibited reaction. Similar inhibition by PD150606 was also observed with full-length calpain-1 (not shown), as previous studies have observed [14]. The rate of cleavage of the fluorogenic substrate by calpain-1 protease core, which was linear with time because the core is resistant to autoproteolysis, was reduced to 50% of the uninhibited reaction by the presence of 50 μM PD150606 (Fig. 1B). In comparison to PD150606, leupeptin at 10 μM showed a greater degree of inhibition of both the full-length calpain-2 and the calpain-1 protease core.

3.2. LSEAL inhibits neither the protease core of calpain-1 nor full-length calpain-2

When the LSEAL penta-peptide was tested for the inhibition of both full-length calpain-2 and the calpain-1 protease core, no significant

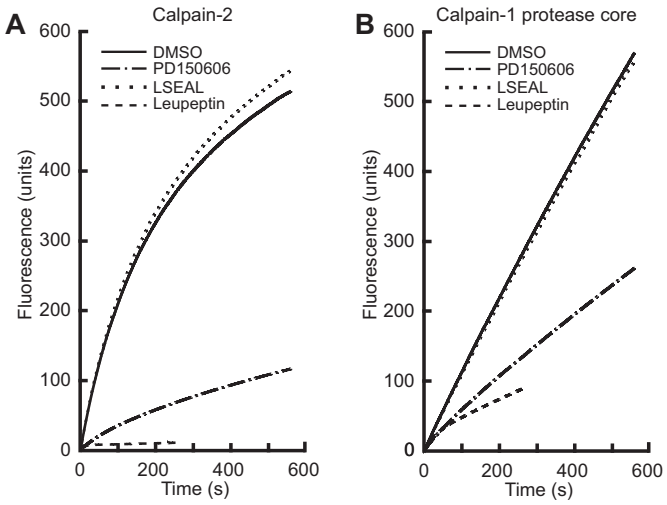

Fig. 1. Inhibition of calpain-2 and calpain-1 protease core. The lines represent the average of the triplicates of fluorescence-based substrate cleavage assays that were performed with (EDANS)-EPLFAERK-(DABCYL), and either calpain-2 (A) or calpain-1 protease core (B) in the absence of inhibitors (solid line) and in the presence of 50 μM PD150606 (dash–dot line), 100 μM LSEAL peptide (dotted line) or 10 μM leupeptin (dashed line).

difference in the rate of fluorogenic substrate cleavage was observed between untreated and treated reactions (Fig. 1A and B).

3.3. PD150606 inhibits calpain autoproteolysis by a direct effect on the protease core

The natural substrates of calpain are proteins rather than small peptides, and the autoproteolysis of the enzyme is an effective way to test this activity. In addition, the autoproteolysis assay is not subject to the fluorescence quenching seen with the FRET substrate and this allowed us to test much higher concentrations of PD150606. As previously observed [12], when calpain-2 is activated by Ca^{2+}, the large 80 kDa subunit is autoproteolyzed to produce the 38 kDa protease core and the large subunit PEF domain, which co-migrated with the small subunit PEF domain (see the 20-min time point in Fig. 2A). The addition of 3 mM PD150606 completely inhibited autoproteolysis (Fig. 2B). In the presence of 1 mM PD150606 partial autoproteolysis was evident with the appearance of a 60-kDa band that represents incomplete release of the protease core from the neighboring C2L domain. At 0.1 mM PD150606 the level of autoproteolysis after 20 min was only marginally less than in the complete absence of inhibitor. To test if PD150606 was inhibiting autoproteolysis allosterically by binding to a site on the PEF domains or by direct action on the protease core we used a variation of the autoproteolysis assay where the substrate was the active site-inactivated calpain-2 (C105S mutant) and the enzyme was the calpain-1 protease core. In the control reaction the calpain-1 protease core cleaved the inactive C105S mutant of calpain-2 to produce fragments similar to those produced by calpain-2 autoproteolysis, including the 38 kDa protease core fragment, the 60 kDa product, and a slightly larger form of the large subunit PEF domain (Fig. 2C). Both 1 mM and 3 mM PD150606 produced complete inhibition of calpain-1 protease core activity as judged by the absence of the 60-kDa and PEF large subunit products, while inhibition by 0.1 mM PD150606 was incomplete (Fig. 2D).

3.4. PD150606 is a relatively weak calpain inhibitor as shown by the fixed-point casein hydrolysis assay

Using the traditional casein hydrolysis assay for calpain, we compared the effectiveness of PD150606 on both full-length calpain-2 as well as the protease core of calpain-1 in relation to a number of other established calpain inhibitors (Fig. 3). For full-length calpain-2,

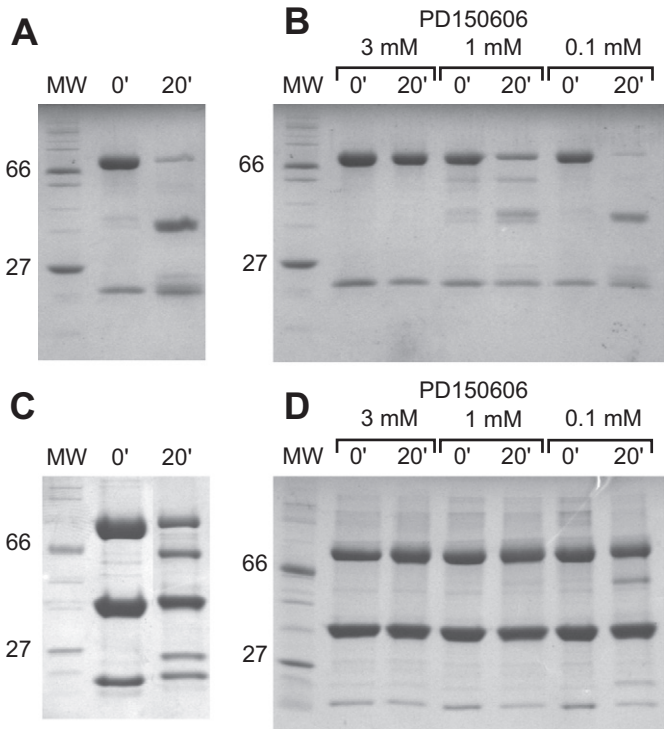

Fig. 2. The effect of PD150606 inhibition on calpain-2 autoproteolysis and calpain-1 protease core cleavage of inactive C105S calpain-2. Calpain-2 autoproteolysis was done in the absence (A) or presence (B) of PD150606. Calpain-1 protease core cleavage of inactive C105S calpain-2 was carried out in the absence (C) or presence (D) of PD150606. Samples were taken initially and after 20 min, as indicated. All reactions were initiated with $CaCl_2$. Reactions were sampled and terminated by the addition of EDTA and 3× SDS sample buffer and analyzed by SDS-PAGE. The two most intense bands of the molecular weight marker have masses of 66 kDa and 27 kDa, as indicated.

the inhibitors leupeptin (10 μM), calpeptin, (10 μM) and SNJ1945 (10 μM) reduced activity to 10% or less of the control values. In the presence of PD150606 (10 μM) activity was reduced to ~65%. When using the protease core of calpain-1 in the fixed-point casein hydrolysis assay, these inhibitors produced similar levels of inhibition to those seen with the full-length enzyme. With both forms of the enzyme PD150606 inhibition is much less effective than leupeptin, calpeptin and SNJ1945. When the LSEAL penta-peptide was tested for the

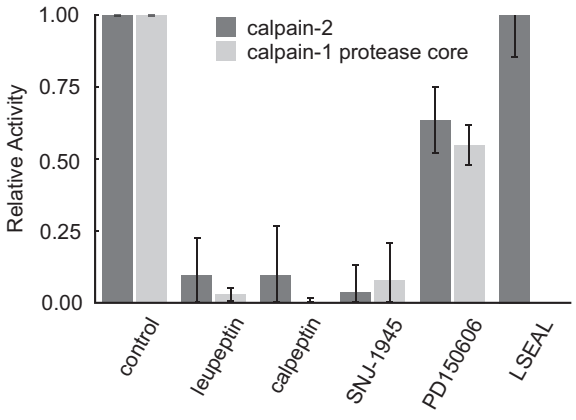

Fig. 3. The inhibition of calpain-2 and calpain-1 protease core in a fixed-point casein hydrolysis assay. The hydrolysis of casein by calpain-2 (dark gray) or calpain-1 protease core (light gray) was assayed in the absence or presence of inhibitors. Data from triplicate assays were corrected for controls containing EDTA and normalized to the activity of the sample containing DMSO and lacking inhibitor. Error bars represent the standard deviation.

Table 1
The effect of PD150606 and LSEAL on calpain-2 and calpain-1 protease core aggregation temperatures.

Enzyme	Compound	T_{agg} (°C) enzyme alone	T_{agg} (°C) enzyme plus compound	ΔT_{agg} (°C)
Calpain-2	PD150606 (500 μM)	54.3 ± 0.2	75.1 ± 0.3	20.8
	LSEAL (1 mM)	54.5 ± 0.2	53.5 ± 0.2	−1
Calpain-1 protease core	PD150606 (500 μM)	60.8 ± 0.3	60.4 ± 0.2	−0.4
	LSEAL (1 mM)	60.4 ± 0.2	60.0 ± 0.2	−0.4
	SNJ-1945 (1 mM)	60.4 ± 0.2	64.1 ± 0.1	−3.7

inhibition of full-length calpain-2 in the casein hydrolysis assay, no significant inhibition was observed (Fig. 3).

3.5. PD150606 binding stabilizes full-length calpain-2 but not the calpain-1 protease core

PD150606 and LSEAL were analyzed for binding to full-length C105S calpain-2 and calpain-1 protease core using a thermal denaturation assay. C105S calpain-2 had a T_{agg} of 54.3 ± 0.2 °C (Table 1). This increased by over 20 °C (ΔT_{agg} = 20.8 °C) when incubated with 500 μM PD150606. No such stabilization was seen with the calpain-1 protease core (T_{agg} = 60.8 ± 0.2 °C), which in the same 500 μM PD150606 had a ΔT_{agg} of −0.4 °C. There was no significant stabilization of either form of calpain by the penta-peptide LSEAL even at concentrations as high as 1 mM.

3.6. PD150606 does not follow traditional Michaelis–Menten kinetics with full-length calpain-2

Using the fluorogenic substrate hydrolysis assay, the mode of inhibition of PD150606 was investigated with respect to full-length calpain-2. PD150606 binds to calpain-2 slowly in the presence of calcium but inhibition is stronger when PD150606 is pre-incubated with the calpain enzyme without calcium (Fig. 4). This suggests that PD150606 does not follow the traditional Michaelis–Menten kinetics in enzyme inhibition, but rather exhibits slow-binding inhibition [26].

Extensive analysis was done to ascertain the mode of inhibition of PD150606 after pre-incubation with calpain, again using the fluorogenic substrate hydrolysis assay. The Michaelis–Menten plots (v vs [S]) are shown in Fig. 5A. There appears to be a reduction in the V_{max} suggesting that the mode of inhibition is not competitive. The reaction data were fit using the simultaneous non-linear regression method [27] to determine the most likely kinetic model for the inhibition (Table 2). The model with the lowest error estimates was the noncompetitive inhibition mechanism. It was not possible to further analyze the data in terms of the slow-binding inhibition by the method of Morrison [26] due to the fact that autoproteolysis results in no steady-state velocity (see the DMSO control curve in Fig. 4).

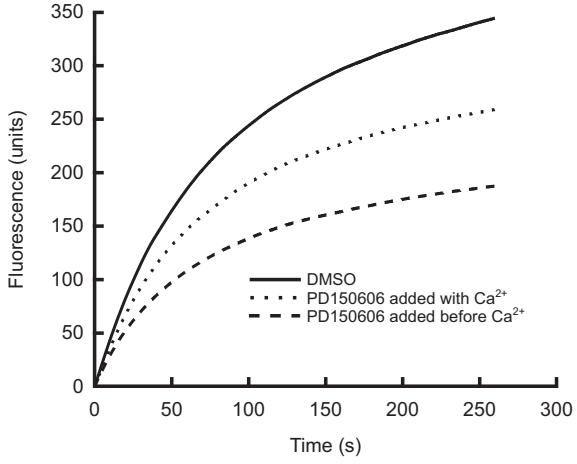

Fig. 4. Differential binding of PD150606 to apo- and Ca^{2+}-bound calpain-2. Triplicate fluorescence-based substrate cleavage assays were performed with (EDANS)-EPLFAERK-(DABCYL) and calpain-2. DMSO was used as a control lacking inhibitor (solid line). The reactions were initiated by the addition of $CaCl_2$. PD150606 inhibitor was added 5 min before (dashed) or with (dotted) calpain activation by Ca^{2+}. The averaged plots are shown.

3.7. PD150606 appears to inhibit the protease core of calpain-1 in a non-competitive manner

As PD150606 inhibits the protease core of calpain-1 alone, the hydrolysis of the substrate (EDANS)-EPLFAERK-(DABCYL) was again employed for kinetic analyses with the calpain-1 protease core. The initial rate of substrate cleavage by the calpain-1 protease core was illustrated by Michaelis–Menten plots (Fig. 5B). Again, simultaneous non-linear regression using all of the reaction data suggests a noncompetitive model of inhibition of the calpain-1 protease core by PD150606. Attempts were made to confirm the mode of inhibition by using higher concentrations of inhibitor. However, these reactions were problematic because PD150606 absorbs at the excitation wavelength of the (EDANS)-EPLFAERK-(DABCYL) substrate.

4. Discussion

4.1. Calpastatin binds to PEF domains only to anchor its inhibitory B peptide

We opened this investigation because the mechanism of inhibition of calpain by calpastatin showed that binding of the A and C peptides to the PEF domains was only responsible for the anchoring and orientation of the inhibitor. The inhibitory region of calpastatin is the B peptide, which binds to the active site cleft and is far removed from the A and C peptides. It is important to note that peptides corresponding to the A and C subdomains of calpastatin do not inhibit calpain activity in direct proteolysis assays under calcium-saturating conditions (not shown), and truncations of calpastatin are not inhibitory without the B subdomain [28]. On the contrary, the A and C subdomains of calpastatin have been shown to be activators of calpain [29] although this is a result we have not been able to reproduce.

4.2. PD150606 does not induce changes in structure or dynamics of calpain

From the various calpain crystal structures, we now know that the hydrophobic clefts on the PEF domains targeted by the A and C peptides become more exposed upon calcium-activation of calpains [10,11,30]. The driving force behind calpain-activation conformational changes comes from calcium binding to the PEF and protease core domains, resulting in an overall compaction of the enzyme and aligning the active site residues. As calpastatin is an intrinsically disordered protein with some mild propensity for α-helical and β-turn formation [31], ordered structure requires binding to calcium-activated calpain and the preformed hydrophobic clefts. Those hydrophobic clefts only shift slightly upon binding the calpastatin A or C peptide [10,11,18]. From this perspective, it seems unlikely that a small molecule or peptide with no secondary structure would, by binding to the exposed clefts in the

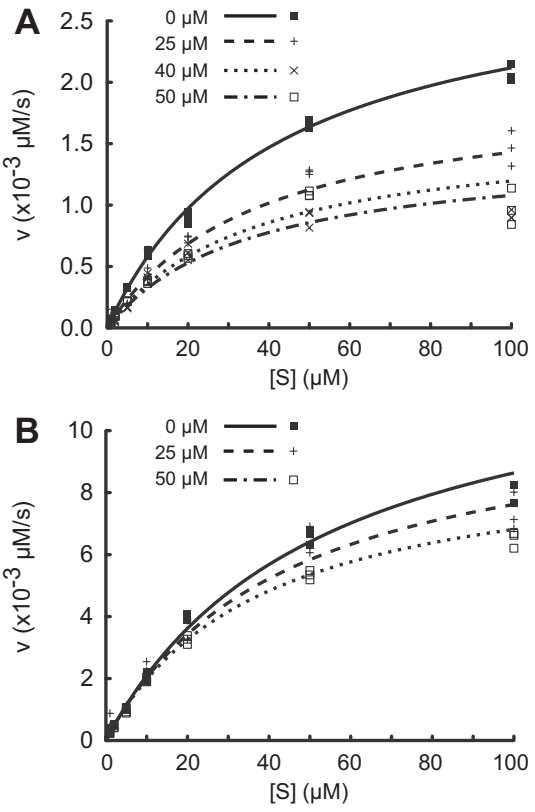

Fig. 5. Enzyme kinetics curves for calpain-2 and calpain-1 protease core inhibited by PD150606. Triplicate fluorescence-based substrate cleavage assays were performed with increasing concentrations of (EDANS)-EPLFAERK-(DABCYL) and of PD150606 (0, 25, 40 and 50 μM as indicated on the graph). Progress curves for calpain-2 were analyzed using the initial linear portion to determine a reaction velocity, and this data were fit to the Michaelis–Menten (A) equation. Progress curves for calpain-1 protease core were analyzed and data were fit in a similar manner (B). K_m and V_{max} values for both enzymes were obtained from the fit to the Michaelis–Menten equations.

presence of Ca^{2+}, induce changes in the calpain conformation leading to inhibition. In the case of PD150606, the overall structure of the calpain small subunit is unchanged when comparing PD150606-bound to PD150606-free structures except for the movement of a single glutamine sidechain [18].

Recently, the concept of allostery has expanded to include the idea that binding of a ligand at a distant site may cause alterations in the dynamic properties of proteins rather than simply changes in their structure [32,33]. At this point, based on the crystal structures of the small subunit PEF domains, there is no evidence to suggest that binding of PD150606 is able to alter the dynamics or the structure of the PEF domain and hence transmit those changes to the protease core to reduce calpain activity.

4.3. Thermal shift assays are neutral with the protease core

Thermal shift assays showed no effect for binding of PD150606 to the protease core of calpain, whereas the signal was strong for its binding to full-length calpain. This is not surprising because most inhibitors of the core do not stabilize its structure, even if covalently bound, probably because of the flexibility of the two core subdomains (PC1 and PC2) about a central pivot region [12]. For example, the alpha-ketoamide inhibitor SNJ-1945 inhibits the calpain protease core well (Fig. 3) and forms a stable covalent complex in the active site cleft [34] and yet has a negligible stabilizing effect on the protease core in the thermal shift assay (Table 1). Perhaps PD150606 is able to increase the T_{agg} of the full-length calpain by binding to the PEF domain but may inhibit it only by binding to a site on the protease core. Given the lack of stabilization by SNJ-1945, then it would not be surprising that PD150606 also does not increase the T_{agg} of the protease core.

4.4. Mode of inhibition by PD150606 is not clear

The mode of inhibition of PD150606 remains unclear. For the full-length calpain-2, it appears that PD150606 has some affinity for the enzyme in the absence of Ca^{2+}. This is inconsistent with our result of a best fit of the kinetic data to noncompetitive inhibition and to previous reports of uncompetitive inhibition. Both of these kinetic models would require binding in the presence of substrate and hence Ca^{2+}. The apparent affinity in the absence of Ca^{2+} is also inconsistent with the structural explanation that PD150606 inhibits by binding to the hydrophobic clefts exposed on the calcium-activated PEF domains. For the calpain-1 protease core, PD150606 also appears to inhibit in a noncompetitive manner. Again this kinetic model implies that PD150606 binds only to the calcium-activated protease core with and without substrate bound to induce an inactivating conformational change. It is difficult to reconcile this kinetic model with the structure of the protease core and the active site cleft. Compared to full-length calpain the protease core is relatively small and has no clefts other than the active site that are common to the full-length protein and that would provide an obvious binding site for PD150606.

4.5. LSEAL does not inhibit calpain in vitro and shows no affinity for PEF domains

LSEAL did not display any inhibition of calpain in the in vitro fluorescence-based substrate cleavage assay or the casein hydrolysis assay. This does not rule out the possibility that LSEAL can act as a calpain inhibitor in vivo. For example, the calcium-dependent clefts on

Table 2
Results of simultaneous non-linear regression to the Michaelis–Menten equations for different kinetic models of inhibition of calpain-2 and calpain-1 protease core by PD150606 with standard error estimates. Error estimates are also given as percentages in parentheses.

Enzyme	Inhibition model	k_{cat} (s^{-1})	K_m (μM)	K_{I1} (μM) [a]	K_{I2} (μM) [b]
Calpain-2	Noncompetitive	2.9 ± 0.1 (5%)	39 ± 3 (7%)	60 ± 5 (8%)	
	Uncompetitive	4.0 ± 0.4 (10%)	75 ± 9 (12%)		20 ± 3 (15%)
	Competitive	2.3 ± 0.1 (6%)	29 ± 3 (10%)	40 ± 5 (12%)	
	Mixed	3.1 ± 0.2 (7%)	44 ± 5 (11%)	70 ± 10 (14%)	44 ± 10 (23%)
Calpain-1 protease core	Noncompetitive	0.121 ± 0.005 (4%)	46 ± 3 (7%)	330 ± 70 (21%)	
	Uncompetitive	0.132 ± 0.007 (5%)	53 ± 4 (8%)		120 ± 30 (25%)
	Competitive	0.114 ± 0.004 (4%)	43 ± 3 (7%)	270 ± 80 (30%)	
	Mixed	0.129 ± 0.007 (5%)	51 ± 4 (8%)	800 ± 800 (100%)	150 ± 50 (33%)

[a] K_{I1} is the dissociation constant for inhibitor binding to free enzyme.
[b] K_{I2} is the dissociation constant for inhibitor binding to the ES complex.

calpain could conceivably govern sub-cellular localization [35–37] after activation by helping the enzyme to interact with hydrophobic residues on ligands and substrates [38–41]. This may explain the ability of the inhibitors discussed here to reduce cleavage of some protein substrates [16,18,42]. Studies investigating the penta-peptide LSEAL used tau and α-synuclein cleavage to assess inhibition [16]. In this context, LSEAL may interfere with hydrophobic interactions between enzyme and substrate. Calpastatin could possibly function in this manner as well. It may block interactions of various protein substrates with the active enzyme in addition to inhibiting proteolysis at the active site. However, we conclude that binding to hydrophobic clefts in the calpain PEF domains does not directly confer proteolytic inhibition.

5. Conclusion

The calpain assays presented here all showed no inhibition by the penta-peptide LSEAL on either full-length calpain-2 or the protease core of calpain-1. The assays also show that PD150606 inhibits both the protease core of calpain-1 and full-length calpain-2. Thus it can inhibit calpain in the absence of the C2L domain and the PEF domains of the large and small subunits. While PD150606 inhibits PEF domain-containing calpains, it was also previously shown to inhibit cathepsin B and thermolysin albeit to a much lesser extent [14]. In more recent literature, PD150606 has been found to inhibit other proteases such as MMP-2 [43], which have no similarity to calpains. PD150606 has also been shown to inhibit kainate-induced Ca^{2+} influx independent of its action on calpain [44]. Our results show that PD150606 has a similar inhibitory effect on both the full-length calpain-2 and the calpain-1 protease core, providing further support for a single mode of inhibition that does not require interaction with the PEF domains. We cannot completely rule out an additional allosteric effect contributing to calpain core inhibition, but it seems unnecessary and unlikely that this small compound would have a second mechanism of inhibition.

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