

An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective

(calpain/protease inhibitor/calcium-binding protein/protease/excitotoxicity)

KEVIN K. W. WANG*, RATHNA NATH*, AVIGAIL POSNER*, KADEE J. RASER*, MICHELLE BUROKER-KILGORE*, IRADJ HAJIMOHAMMADREZA*†, ALBERT W. PROBERT, JR.*‡, FRANK W. MARCOUX*, QIZHUANG YE‡, EMIKO TAKANO§, MASAKAZU HATANAKA§, MASATOSHI MAKI§¶, HAKAN CANER||, JENNIFER L. COLLINS||, ANDREA FERGUS||, KEVIN S. LEE||, ELIZABETH A. LUNNEY**‡, SHERYL J. HAYS††, AND PO-WAI YUEN††

Departments of *Neuroscience Therapeutics, ‡Biochemistry, **Biological and Structural Drug Design and ††Neuroscience Chemistry, Parke–Davis Pharmaceutical Research, Division of Warner–Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105; §The Institute for Virus Research, Kyoto University, Sakyo-ku 606-01, Japan; and ||Department of Neurological Surgery, University of Virginia, Charlottesville, VA 22908

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ABSTRACT Overactivation of calcium-activated neutral protease (calpain) has been implicated in the pathophysiology of several degenerative conditions, including stroke, myocardial ischemia, neuromuscular degeneration, and cataract formation. Alpha-mercaptoacrylate derivatives (exemplified by PD150606), with potent and selective inhibitory actions against calpain, have been identified. PD150606 exhibits the following characteristics: (i) K_i values for μ - and m-calpains of 0.21 μ M and 0.37 μ M, respectively, (ii) high specificity for calpains relative to other proteases, (iii) uncompetitive inhibition with respect to substrate, and (iv) it does not shield calpain against inactivation by the active-site inhibitor *trans*-(epoxysuccinyl)-L-leucyl-amido-3-methylbutane, suggesting a nonactive site action for PD150606. The recombinant calcium-binding domain from each of the large or small subunits of μ -calpain was found to interact with PD150606. In low micromolar range, PD150606 inhibited calpain activity in two intact cell systems. The neuroprotective effects of this class of compound were also demonstrated by the ability of PD150606 to attenuate hypoxic/hypoglycemic injury to cerebrocortical neurons in culture and excitotoxic injury to Purkinje cells in cerebellar slices.

Calpain (EC 3.4.22.17) is a class of cytosolic cysteine protease that is activated by elevated intracellular calcium (1–3). Interest in this class of enzymes has grown substantially in recent years because it has been implicated in the pathophysiology of several degenerative conditions; including cerebral ischemia, myocardial ischemia, and cataract (4–8). The unifying features of these pathological conditions are that calcium serves as a trigger for cellular injury and that calpain may represent a crucial mediator of the degenerative response. Uncontrolled activation of calpain leads to cytoskeletal protein (e.g., spectrin) breakdown, degradation of many receptor proteins (e.g., epidermal growth factor receptor), and enzyme systems (e.g., protein kinase C and calmodulin-dependent kinases) and consequently cell death (1, 8). Several studies have shown that peptidic inhibitors of calpain protect cells in models of ischemic, hypoxic and/or excitotoxic neuronal injury (6, 9–13). However, a clear interpretation of the role of calpain in these studies has been hampered by the lack of selectivity of these peptidic inhibitors (8).

The predominant forms of calpain in mammalian tissues are μ -calpain and m-calpain, requiring low and high micromolar calcium, respectively, for *in vitro* activation. Both of these isoforms are heterodimers in which the large subunit (80 kDa) contains a distinct cysteine protease domain and a calcium-binding domain

with four helix–loop–helix (EF-hand) structures (14). The small subunit (29 kDa) is made up of a glycine-rich region responsible for membrane-interactions and another set of four EF-hand structures (14). To date, most calpain inhibitors are modified peptides that compete for the active site of the protease (8). In this study, we describe the discovery of a class of nonactive site-binding calpain inhibitors. Their selectivity comes from their unique interaction with the calcium-binding domains of calpain, a feature not found in other protease inhibitors.

MATERIALS AND METHODS

Protease Assays and Kinetic Studies. The calpain microplate assay was carried out as described (15). Briefly, a mixture containing 0.5 mg/ml casein, 20 mM DTT, 50 mM Tris-HCl (pH 7.4), 0.01 unit purified μ -calpain (human erythrocytes) or m-calpain (rabbit skeletal muscle) (about 0.08–0.12 μ g) in the presence of various concentrations of an inhibitor (16). CaCl_2 (4 mM) was added to a microtiter plate (250 μ l) and incubated for 60 min at 25°C. The samples were then processed for colorimetric development and read on a Molecular Devices Thermomax microplate reader at an absorbance of 595 nm. Based on a plot percentage inhibition of calpain against log [inhibitor], an IC_{50} was generated using a sigma plot (Jandel, San Rafael, CA). Apparent K_i values were then calculated according to Cheng and Prusoff (17).

Papain (papaya latex), thermolysin (*Bacillus thermoproteolyticus*), and trypsin (bovine pancreas) were assayed similarly to calpain except that the reaction mixture contained 7–10 ng of papain, or 5 μ g of thermolysin, or 1.25 μ g of trypsin instead of calpain (15). Cathepsin B (0.002 unit, bovine spleen) was assayed with 50 mM Mes (pH 5.5), 100 μ M carbobenzoxy-Arg-Arg-4-methoxy- β -naphthylamide (Cbz-Arg-Arg-MNA), an inhibitor, and 2 mM DTT in 200 μ l of the same buffer for 60 min at room temperature. Aliquots of 50 μ l were transferred to a fluorescence-compatible plate and then read with a Perkin–Elmer LS-50B fluorometer (excitation 340 \pm 15 nm and emission 425 \pm 20 nm). Calcineurin (bovine brain, Sigma) (5 μ g, 0.8 unit) was assayed with 10 mM *p*-nitrophenyl

Abbreviations: TNS, 2-(*p*-toluidino)naphthalene-6-sulfonate; E64c, *trans*-(epoxysuccinyl)-L-leucyl-amido-3-methylbutane; AMC, 4-methylcoumarinamine; SLLVY-AMC, succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; PD150606, 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid; PD151746, 3-(5-fluoro-3-indolyl)-2-mercapto-(Z)-2-propenoic acid; μ CBD, calcium-binding domain of μ -calpain; sCBD, calcium-binding domain of s-calpain; CalpInh-I, calpain inhibitor I or acetyl-Leu-Leu-Nle-H; SY5Y, neuroblastoma SH-SY5Y; AMPA, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

†Present address: Pfizer Central Research, Discovery Biology, Sandwich, Kent, CT13 9NJ, United Kingdom.

¶Department of Applied Biological Sciences, School of Agriculture, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-01, Japan.

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phosphate in 1 mM DTT, 1 mM CaCl₂, 1 mM MnCl₂, 200 μ M calmodulin (if added), and 50 mM Tris·HCl (pH 7.4) at 25°C for 60 min or more (18). Production of *p*-nitrophenol was monitored at 405 nm. Basal phosphatase activity and the calmodulin-activated component were calculated separately.

Kinetics studies of calpain hydrolysis of succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (SLLVY-AMC) were performed by incubating 1.2 units of μ -calpain (0.17 μ g) and 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid (PD150606), (10 or 20 μ M) in 20 mM DTT, 1 mM CaCl₂, 50 mM Tris·HCl, and SLLVY-AMC (2, 5, 10, 20, 30, and 50 μ M) (pH 7.4 at 4°C) (200 μ l) in a fluorescence-compatible microtiter plate (19). Fluorescence of the liberated AMC was monitored with a Perkin-Elmer LS-50B fluorometer (excitation 380 \pm 15 nm; emission 460 \pm 20 nm).

Casein Zymography. Casein gels were poured as a 12% (wt/vol) acrylamide solution and 0.2% (wt/vol) sodium casein into mini-gel casts and allowed to polymerize, as described before (20). The casein gels were pre-run with Tris-glycine buffer containing 1 mM EDTA and 1 mM DTT for 15 min (4°C). μ -Calpain (4 μ g) was incubated in 50 mM Tris·HCl (pH 7.4), 3 mM DTT, and 1 mM CaCl₂ in the presence of an inhibitor for 5 min on ice (30 μ l). When indicated, a second inhibitor was introduced and further incubated for 5 min. EGTA (10 mM) in 5 μ l of non-SDS sample buffer was added to the mixture (final volume 38 μ l). The samples were then loaded onto a casein gel and electrophoresed at 125 V for 3 h. The gel was washed twice with 20 mM Tris·HCl (pH 7.4), 10 mM DTT, and 1 mM CaCl₂ for 30 min and incubated overnight at ambient temperature in the same buffer with shaking. Finally, the gel was stained with Coomassie blue G-250.

2-(*p*-Toluidino)naphthalene-6-Sulfonate (TNS) Fluorescence Enhancement by Recombinant Calcium-Binding Domains of Calpain. Recombinant calcium-binding domains from the human calcium-binding domain of μ -calpain (μ CBD, residues 516–714) and porcine small subunit calcium-binding domain of small calpain (sCBD, residues 84–266) (14, 21) were expressed with leader sequences ASMTGGQQMGRIP and ASMTGGQQMGR, respectively, as soluble proteins in *Escherichia coli* using the pET-3d vector with T7 promoter according to Yang *et al.* (22) and Takano *et al.* (23). The proteins were purified by sequential fractionation on a DEAE-Sephacel column (2.5 \times 40 cm) and a Sephacryl S-200 gel-filtration column (1.5 \times 60 cm). Fluorescence emission spectra were obtained by preparation of samples (1 ml) containing 20 μ M TNS in 20 mM Tris·HCl (pH 7.4 at room temperature), 0.1 mM EDTA, 10 μ M of μ CBD, sCBD, or calmodulin (Calbiochem). Samples were incubated at room temperature for 5 min, and the fluorescence spectra were obtained with excitation at 340 nm with a Perkin-Elmer LS-50B luminescence spectrometer (21) with emission at 440 nm, 445 nm, and 447 nm for μ CBD, sCBD, and calmodulin, respectively. Slit widths were set at 5 nm.

Calpain Autolysis. μ -Calpain (5 μ g) was incubated with various concentrations of calpain inhibitor I (CalpInh-I) or PD150606 in 50 mM Tris·HCl (pH 7.4), 10 mM DTT, 10 mM CaCl₂ or 10 mM EGTA at ambient temperature for 2 h (500 μ l). The reaction was stopped with 500 μ l of 5% (wt/vol) trichloroacetic acid. After being chilled on ice for 5 min, the calpain protein was pelleted by centrifugation and resolubilized with 20 μ l of 3 M Tris base aided by mechanical force and sonication. Protein samples were then analyzed with SDS/PAGE.

Calpain Activation in Molt-4 and SH-SY5Y Cells. Molt-4 cells were washed three times with serum-free RPMI 1640 medium and resuspended to 2 million/ml and transferred to a 12-well plate (0.5 ml/well). Cells were preincubated for 1 h with inhibitor. To activate endogenous calpain, 15 μ M of A23187 (as 5 mM stock in *N,N*-dimethylformamide) was added and the cells were further incubated for 90 min at 37°C (24). Total cellular protein was extracted as described (25). Protein samples (50 μ g) were run on SDS/PAGE with the Tris-glycine running buffer system and

transferred onto a poly(vinylidene-difluoride) (PVDF) membrane. The blots were probed with an anti- α -spectrin (nonerythroid) antibody (Chemicon) and a second antibody with alkaline phosphatase conjugate. The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Human neuroblastoma SH-SY5Y (SY5Y) cells were grown on 12-well plates to confluency (about 2 million per well) with DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 2.5 μ g/ml of fungizone (amphotericin B). Prior to the experiment, confluent cultures were washed three times with serum-free DMEM. Calpain inhibitors were added at this point for 1 h of preincubation. Medium containing SLLVY-AMC (0.5 ml) was then added to achieve a final concentration of 80 μ M (26). Maitotoxin (0.1 nM) was added if desired at this point (27). The plates were incubated at room temperature. Fluorescence (excitation 380 nm and emission 460 nm with slit widths set at 15 nm and 20 nm, respectively) was measured every 15–30 min up to 120 min with a Millipore Cytoflor 2300 fluorescence plate reader.

Hypoxia/Hypoglycemia in Cerebrocortical Cultures. Cerebrocortical cells were harvested from fetal rats (Sprague-Dawley) on their 18th day of gestation and cultured with DMEM/F12 medium containing 10% horse and 6% fetal bovine serum (heat inactivated) in 96-well poly-L-lysine-coated plates as described (28). Nonneuronal cell division was halted 3 days into culture by adding 25 μ g/ml uridine and 10 μ g/ml 5-fluoro-2'-deoxyuridine. On the 17th day post-plating, the cultures were washed three times with serum-free medium. PD150606 or PD145305 was added at this point for 1 h preincubation. The cultures were then challenged with hypoxia/hypoglycemia for 195 min (exposure atmosphere in gas incubator: 1% O₂, 8% CO₂, 91% N₂; exposure medium: 1.8 mM Ca²⁺, 0.8 mM Mg²⁺, 0.2 g/liter D-glucose) in the presence of PD150606 or PD145305 (29, 30). The plates were then returned to normal serum-free medium (with calpain inhibitor) in an oxygenated incubator (21% O₂, 8% CO₂, 71% N₂) until 24 h after the initiation of the experiment. Normoxic/normoglycemic cultures with the same number of medium changes were used as controls. Neuronal death was then assessed by measuring the cytosolic enzyme, lactic dehydrogenase, released into the medium (25 μ l samples) as described (31). Protein extracts were analysed on Western blots as described above.

Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) Toxicity in Cerebellar Slices. Cerebellar slices were acutely isolated from 8–10-day-old Sprague-Dawley rats and maintained in an oxygenated bath for the duration of the experiment as described (32). In the treated groups, PD150606 or PD145305, 100 μ M was added to a buffer (aCSF) containing 124 mM NaCl/3.3 mM KCl/2 mM CaCl₂/25.7 mM NaHCO₃/2.4 mM MgSO₄/1.25 mM KH₂PO₄/10 mM glucose 60 min before the addition of AMPA (30 μ M for 30 min). The slices were then allowed to recover in normal aCSF for an additional 90 min in the presence of the inhibitor. Damaged cells are identified morphologically upon hematoxylin/eosin staining, using these criteria: cytoplasmic darkening, microvacuolation, and nuclear darkening or chromatin aggregation (32).

RESULTS

Mercaptoacrylic Acid Derivatives Are Calpain Inhibitors. Initially more than 150,000 compounds were randomly screened for inhibition of human μ -calpain with a proteolysis assay in which a preferred calpain substrate, casein, was utilized (15). From this assay, we identified a series of mercaptoacrylic acid derivatives exhibiting appropriate features for a selective calpain inhibitor. Subsequent directed synthesis resulted in compounds with improved potency.## Two representative compounds, PD150606 and 3-(5-fluoro-3-indolyl)-2-mercapto-(Z)-2-propenoic acid (PD151746), are shown in Fig. 1). PD150606 exhibited apparent inhibition constants (*K_i*) of 0.21 \pm 0.01 μ M and 0.37 \pm 0.03 μ M against μ -calpain and m-calpain, respectively (Table 1). Interestingly, PD151746

showed a 20-fold selectivity for μ -calpain (K_i $0.26 \pm 0.03 \mu\text{M}$) over m-calpain (K_i $5.33 \pm 0.77 \mu\text{M}$). In fact, a complete inhibition of m-calpain was not achieved even with a high concentration (200 μM) of PD151746. The double bond in both of these compounds appeared to be critical for their inhibitory actions because a saturated analog (PD145305) was inactive when tested at concentrations up to 500 μM ($n = 4$) (Fig. 1). In addition, unmodified sulfhydryl and carboxylic acid groups were also necessary for the inhibition of calpain. Inasmuch as these ionized groups could serve to chelate calcium ions associated with calpain, it was postulated that these groups may perturb the normal conformation of the calcium-binding site(s) and thus modulate the activity of the enzyme (Fig. 1). It is important to note, however, that PD150606 was not a simple calcium ion chelator, as it did not have high affinity for free calcium ions in solution (data not shown).

The selectivity of PD150606 and PD151746 was evaluated by testing their inhibitory activities against a panel of six proteases (Table 1). The peptidic inhibitor CalpInh-I (acetyl-Leu-Leu-Nle-H) was also tested in parallel for the purpose of comparison (33). The inhibitory effects of PD150606 and PD151746 were highly selective for calpains (Table 1). PD150606 appeared to inhibit cathepsin B only at higher concentrations (K_i $127.8 \pm 9.2 \mu\text{M}$). This result could be partly attributed to fluorescence quenching at high concentrations of PD150606, since a fluorogenic substrate (Cbz-Arg-Arg-MNA) was used. In contrast, CalpInh-I was more selective for cathepsin B than for calpains. The mercaptoacrylates and CalpInh-I were also tested for their inhibitory actions on another EF-hand calcium-binding protein, calmodulin. We measured the effects of these calpain inhibitors on calmodulin-stimulated phosphatase activity of calcineurin. In these experiments, neither PD150606 nor PD151746 inhibited the basal activity of calcineurin. However, both compounds did inhibit calmodulin stimulation of calcineurin when applied at higher concentrations. As expected, CalpInh-I did not inhibit the basal activity or the calmodulin-stimulated activity of calcineurin.

The inhibitory properties of PD150606 were further investigated by performing kinetic analyses with a synthetic substrate for calpain (SLLVY-AMC). An Eadie-Hofstee plot of the kinetic data demonstrated that the uninhibited curve and the inhibited curve converged as substrate concentration decreased (Fig. 2A). Although the convergence point of the two lines was slightly below the x axis, the curves clearly did not converge on the y axis and were not parallel, thus ruling out competitive and noncompetitive inhibition. Taken together, these findings indicate that the inhibitory action of PD150606 was uncompetitive with respect to substrate. This conclusion was confirmed by additional kinetics studies using a protein substrate, casein. In these experiments, uncompetitive kinetics were again observed with PD150606 (data not shown).

A characteristic feature of calpain is its ability to autolytically degrade to small inactive fragments in the absence of added substrate (2). Under our assay conditions, the large subunit of μ -calpain (80 kDa) underwent rapid fragmentation to its 76-kDa form, after which additional fragments of 55 kDa and 43 kDa were formed. The 29-kDa subunit also rapidly degrades to a 18-kDa fragment (Fig. 2B). CalpInh-I, which blocks the active site of the calpain molecule, virtually eliminated the autolytic degradation of μ -calpain, at 1–10 μM (Fig. 2B). In contrast, PD150606 only partially inhibited autolysis at concentrations up to 100 μM .

The mode of action of PD150606 was further examined using casein zymography (20). Under our conditions, calpain treated with a reversible inhibitor, such as CalpInh-I, would show no significant reduction of calpain activity on the casein gel (Fig. 2C). However, the use of an irreversible active site inactivator [e.g., *trans*-(epoxysuccinyl)-L-leucyl-amido-3-methylbutane (E64c)] re-

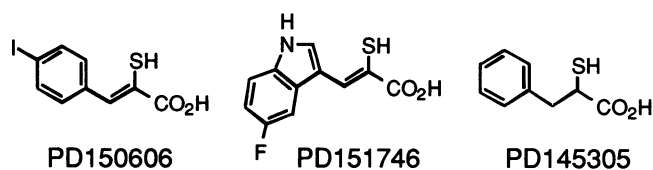


FIG. 1. Chemical structure of calpain inhibitors PD150606 and PD151746 and inactive analog PD145305.

sulted in sustained inhibition (34). PD150606 did not inhibit calpain upon reactivation, indicating its reversibility (Fig. 2C). In addition, we observed that incubation of calpain with CalpInh-I prior to the addition of E64c prevented the irreversible inhibition of calpain in the zymogram because both compounds act on the same site. On the other hand, pretreatment of calpain with PD150606 did not protect against the inactivation by E64c (Fig. 2C). Taken together, our results (Fig. 2) suggest that mercaptoacrylate derivatives operate by inhibiting a region of the calpain molecule other than the active site.

PD150606 Interacts with Both Calcium-Binding Domains of Calpain. Several lines of evidence were consistent with the hypothesis that the mercaptoacrylate derivatives interact with the calcium-binding site(s) of calpain. This evidence included: (i) the uncompetitive nature of inhibition, (ii) the lack of cross-reactivity with other cysteine proteases, (iii) the apparent low-affinity antagonism for calmodulin (Table 1), and (iv) that the unsubstituted sulfhydryl and carboxylic acid groups could serve to chelate calcium ions associated with the calcium-binding domain(s) of calpain. To test this hypothesis, we studied the possible interaction between PD150606 and the recombinant calcium-binding domain from either the large (μ CBD) or the small subunit of μ -calpain (sCBD) (21, 22). We took advantage of a previous observation that binding of TNS to an EF-hand calcium-binding protein resulted in an enhanced fluorescence signal (22). In fact there are two components of fluorescence enhancement: calcium-dependent and calcium-independent (Fig. 3). The presence of PD150606 reduced the calcium-dependent TNS-fluorescence enhancement by μ CBD or sCBD by 58–61%, whereas the calcium-independent fluorescence was only reduced by 14–17%. These data show a direct calcium-dependent interaction between PD150606 and both calcium-binding domains of calpain. In contrast, the calcium-dependent TNS-fluorescence enhancement by calmodulin was only reduced by 21%, consistent with the selectivity of PD150606 for calpain over calmodulin (Fig. 3).

Inhibition of Cellular Calpain Activity. It has been reported that μ -calpain is autolyzed in human leukemic Molt-4 cells with treatment of calcium ionophore A23187 (24). It is also well established that α -spectrin, which is one of its preferred cytoskeletal substrates, was degraded into two proteolytic fragments (150 kDa and 145 kDa) (5, 24, 35, 36). This distinct pattern of fragmentation of α -spectrin has been used as an assay for calpain activity in intact cells (28, 37, 38). Under our conditions, the 145-kDa fragment was more prominent with higher concentrations of A23187, suggesting that the two fragments were formed sequentially (Fig. 4A). Using this model, we examined the ability of PD150606 to cross the cell membrane and to inhibit cellular calpain activity. PD150606 inhibited α -spectrin proteolysis in a dose-dependent manner, virtually eliminating the formation of the 145-kDa fragment at a concentration of 10 μM (Fig. 4B). However, the formation of the 150-kDa fragment was only partially blocked by PD150606 at concentrations up to 10 μM . Even at higher concentrations, PD150606 did not completely block the formation of the 150-kDa fragment (data not shown). This result could be explained by the uncompetitive mode of inhibition for PD150606 (see *Discussion*). Lastly, the inactive PD145305 did not attenuate α -spectrin breakdown product formation.

††A detailed account of the synthetic scheme and structure-activity relationship of the mercaptoacrylates as calpain inhibitors will be submitted elsewhere.

Table 1. Selectivity of PD150606, PD151746, and CalpInh-I as calpain inhibitors

	K_i , $\mu\text{M} \pm \text{SEM}$		
	PD150606	PD151746	CalpInh-I
μ -Calpain	0.21 ± 0.01 (6)	0.26 ± 0.03 (4)	0.086 ± 0.023 (4)
m-Calpain	0.37 ± 0.03 (4)	5.33 ± 0.77 (4)	0.192 ± 0.023 (4)
Cathepsin B	127.8 ± 9.2 (4)	>200 (2)	0.022 ± 0.009 (3)
Papain	>500 (2)	>500 (2)	2.24 ± 0.22 (3)
Trypsin	>500 (2)	>500 (2)	>500 (2)
Thermolysin	204.1 ± 23.3 (2)	>500 (2)	>500 (2)
Calcineurin-basal	>200 (2)	>200 (2)	>200 (2)
Calcineurin-CaM	12.98 ± 0.47 (4)	84.54 ± 3.81 (4)	>200 (2)

Proteases are assayed as described in *Materials and Methods*. Apparent K_i are means \pm SEM (n in parentheses). Calcineurin-basal and Calcineurin-CaM represent the basal and calmodulin-stimulated component of calcineurin activity.

A second cell culture model was used to verify the efficacy of mercaptoacrylates in intact cells. The human neuroblastoma cell line SY5Y was treated with a nonselective calcium channel activator (maitotoxin) (27). A fluorogenic substrate, SLLVY-AMC, was used because it can cross intact cell membranes and is subjected to cellular calpain hydrolysis (19, 25, 26) (Fig. 5). Pretreatment of SY5Y with either PD150606, PD151746, or CalpInh-I (all at 10 μM) effectively attenuated the SLLVY-AMC hydrolysis induced by maitotoxin (Fig. 5). In contrast, the inactive compound PD145305 provided no inhibition at concentrations up to 50 μM .

PD150606 Protects Cortical Neurons Against Hypoxia/Hypoglycemia and Cerebellar Purkinje Neurons from AMPA Toxicity. The ability of mercaptoacrylate derivatives to inhibit calpain in intact cell systems raises the possibility that these compounds may be of therapeutic value under conditions in which calpain activation contributes to cellular injury (8, 39–40). The effects of PD150606 were therefore examined in two models of cerebral injury. In our first model, fetal rat cerebrocortical cultures were subjected to a combination of hypoxia and hypoglycemia (30, 37). Pretreating with PD150606

significantly inhibited the release of lactate dehydrogenase after hypoxia/hypoglycemia, whereas PD145305 was ineffective (Fig. 6A). Light microscopic analysis of these cultures confirmed the protective effects of PD150606. In parallel, hypoxia/hypoglycemia-triggered α -spectrin breakdown was attenuated by PD150606 (Fig. 6B). CalpInh-I (1 and 10 μM) also provided neuroprotection in this model (results not shown). The neuroprotective effects of PD150606 were not likely due to direct antagonism on glutamate receptors as it (10 and 50 μM) did not significantly alter the binding of [^3H]glutamate, [^3H]N-methyl-D-aspartate ([^3H]NMDA), [^3H]AMPA, or [^3H]kainate to rat brain membrane preparations (data not shown) (28, 41).

The potential neuroprotective effects of PD150606 were further examined using a model of direct neuronal excitotoxicity. *In vitro* slices of rat cerebellum were treated with the glutamate receptor agonist AMPA (30 μM for 30 min) (32). This manipulation resulted in injury to between 80 and 90% of the Purkinje cells in the slices (Fig. 7). Pretreatment of the slices with PD150606 significantly and markedly inhibited AMPA-induced damage. In contrast, the inactive compound, PD145305, did not significantly reduce cell damage (Fig. 7).

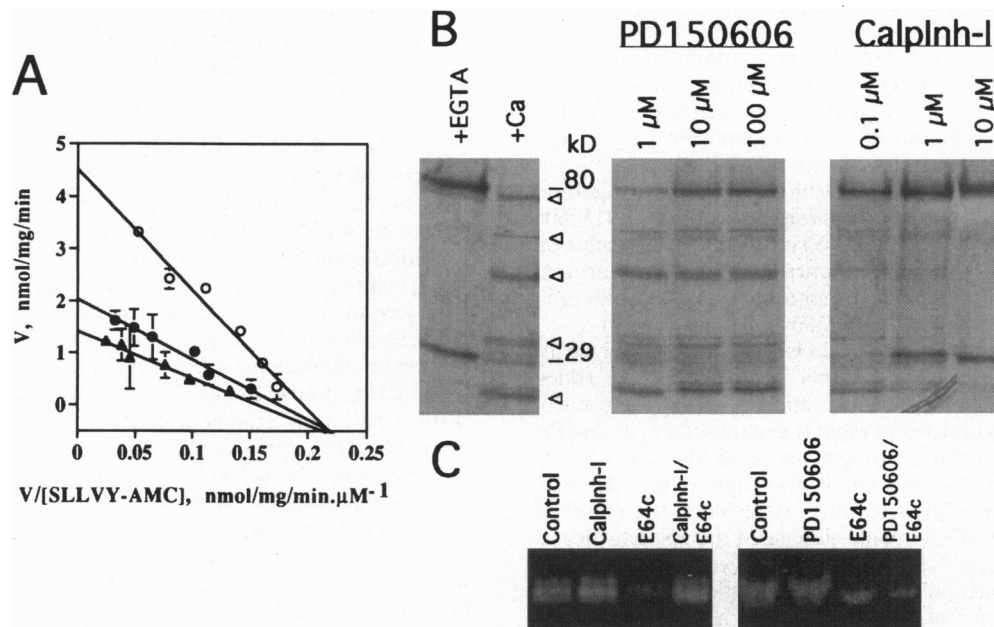


FIG. 2. Inhibition of μ -calpain by PD150606. (A) Calpain hydrolysis of SLLVY-AMC was performed by incubating μ -calpain and PD150606 [none (open circles), 10 μM (solid circles), or 20 μM (solid triangles)] with SLLVY-AMC (2, 5, 10, 20, 30, and 50 μM). Initial rate of hydrolysis (V) versus $V/[\text{SLLVY-AMC}]$ was plotted ($n = 3$; means \pm SEM). (B) μ -Calpain (5 μg) was subjected to autolysis either with EGTA ($-Ca$), calcium ($+Ca$) alone, or together with indicated concentrations of CalpInh-I or PD150606 at ambient temperature for 2 h (500 μl). Proteins samples were then analyzed with SDS/PAGE and Coomassie blue staining. The intact μ -calpain subunits (80 kDa and 29 kDa) are indicated by a solid line, whereas the major autolytic fragments (76 kDa, 55 kDa, 40 kDa, and 18 kDa) are indicated by triangles. (C) Casein gels were loaded with μ -calpain alone (control), μ -calpain treated with CalpInh-I, E64c, or PD150606 or calpain pre-treated with CalpInh-I or PD150606 before addition of E64c (CalpInh-I/E64c; PD150606/E64c). Only the portions of gels that contain the calpain bands are shown. Results shown are representative of three experiments.

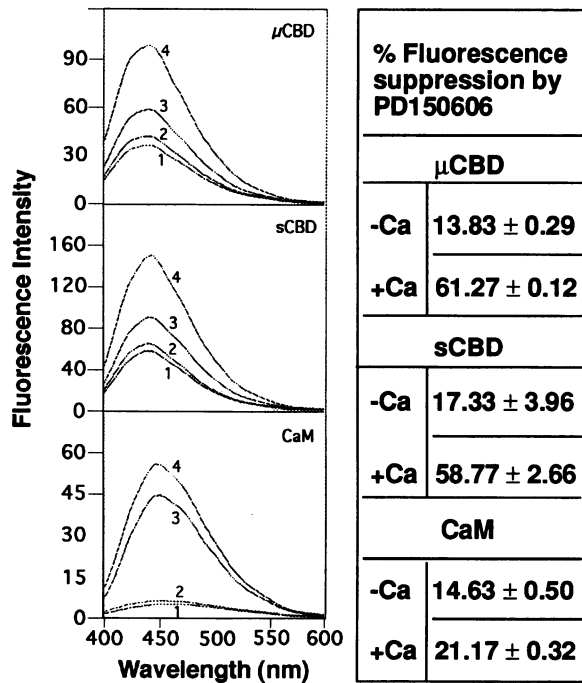


FIG. 3. Interaction between PD150606 and recombinant calcium-binding domain of μ -calpain and s-calpain. The samples contain either no calcium (traces 1 and 2) or 1.1 mM CaCl_2 (1.0 mM of free Ca^{2+}) (traces 3 and 4). Certain samples also contain PD150606 (traces 1 and 3). The suppression of calcium-independent (-Ca) and calcium-dependent (+Ca) fluorescence by PD150606 was expressed as the percentage of respective control in the right panel ($n = 3$; means \pm SEM).

DISCUSSION

In this study, we have described a novel class of selective non-peptide calpain inhibitors, exemplified by PD150606. This lack of cross-reactivity to other proteases (Table 1) is consistent with its uncompetitive mode of action with respect to substrate (Fig. 2A). Unlike CalpInh-I, PD150606 did not prevent μ -calpain inactivation by E64c (Fig. 2C). Together these results suggest that PD150606 interacts with a molecular domain that is distinct from

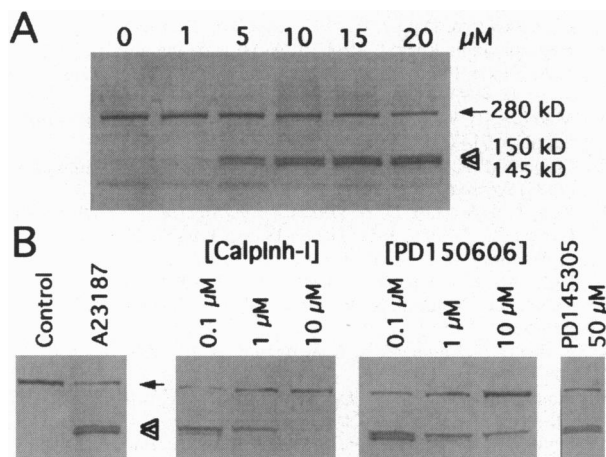


FIG. 4. Effects of calpain inhibitors on calpain-mediated α -spectrin breakdown in A23187-treated Molt-4 cells. (A) Molt-4 cells in suspension were subjected to various concentrations of calcium ionophore A23187 for 60 min. (B) Cells were either untreated (Control) or treated with 15 μM of A23187 in the absence (A23187) or the presence of various concentrations (indicated) of CalpInh-I, PD150606, or PD145305. Total cellular protein (50 μg) was subjected to Western blot analysis with an anti- α -spectrin antibody. The arrow indicates intact α -spectrin (280 kDa) and the arrowheads indicate α -spectrin fragments (150 kDa and 145 kDa). Results are representative of three experiments.

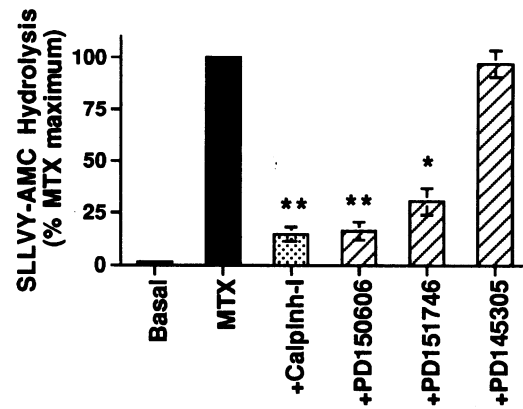


FIG. 5. *In situ* hydrolysis of SLLVY-AMC by calpain in neuroblastoma SY5Y cells and its inhibition by calpain inhibitors. Cells were either untreated (open bar) or treated with maitotoxin in the absence (solid bar) or the presence (dotted bar) of 10 μM of CalpInh-I, PD150606, or PD151746, or 50 μM of PD145305 (hatched bars). The hydrolysis of SLLVY-AMC was detected by fluorescence of liberated AMC at 60 min ($n = 3$; means \pm SEM). Data significantly different from maitotoxin alone are indicated by **, ($P < 0.0001$, Student's *t*-test) and *, ($P < 0.0005$).

the active site of the enzyme. The absolute requirement of unmodified sulfhydryl and carboxylic acid groups for calpain inhibitory activity suggests that the mercaptoacrylates are actually involved in the chelation of the bound calcium ion. The presence of aromatic ring structure suggests that there is also hydrophobic interaction. Indeed, we observed that PD150606 interfered with the respective calcium-dependent binding of TNS to the recombinant calcium-binding domain from both the large and small subunits (Fig. 3). It is likely that the binding of PD150606 to the calcium-binding sites disrupts the conformational changes that are necessary to activate the catalytic domain. Inhibition of

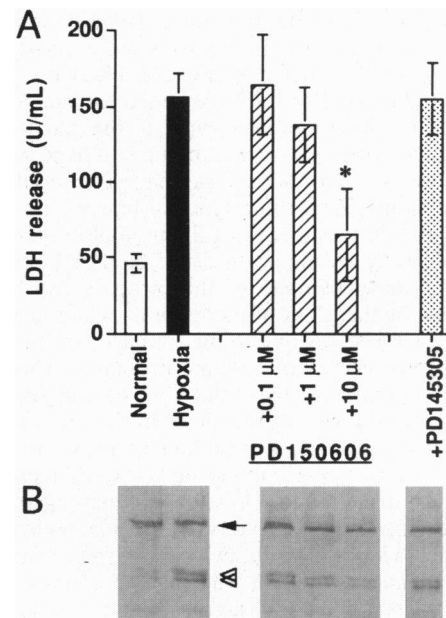


FIG. 6. Protective effects of PD150606 against hypoxia/hypoglycemia-induced neuronal injury. (A) The cultures are either normoxic/normoglycemic (open bar) or challenged with hypoxia/hypoglycemia (Hypoxia) (solid bar) in the absence or the presence of indicated concentration of PD150606 (hatched bars) or 50 μM PD145305 (dotted bar). Data shown are means \pm SEM ($n = 6$). Data significantly different from hypoxia/hypoglycemia alone are indicated by *, ($P < 0.02$, Student's *t*-test). (B) Protein extract from the corresponding condition described in A was analyzed for α -spectrin breakdown (see Fig. 4).

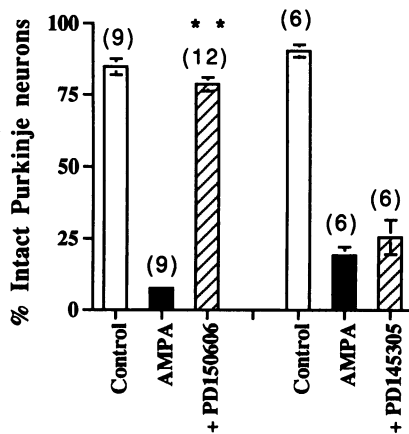


FIG. 7. Neuroprotective effects of PD150606 against AMPA toxicity to Purkinje cells. Cerebellar slices are either untreated (open bars) or treated with 30 μ M of AMPA for 30 min in the absence (solid bars) or the presence (hatched bars) of 100 μ M of PD150606 or PD145305. The data shown are means \pm SEM. The number of experiments performed is in parentheses. Values significantly different from AMPA alone are indicated by **, ($P < 0.0001$, Student's *t*-test).

calpain by PD150606 appears to be partial as evidenced by the lack of a complete blockade of μ -calpain autolysis *in vitro* (Fig. 2B) and the formation of the 150-kDa fragment of α -spectrin in Molt-4 cells (Fig. 4). This may be a result of uncompetitive mode of action of PD150606. An uncompetitive inhibitor can bind to the protease with high affinity only when substrate is bound to the protease. Thus activated calpain might have the opportunity to elicit limited substrate hydrolysis prior to its inhibition by PD150606. It is worth noting that unlike PD150606, PD151746 showed a 20-fold selectivity for μ -calpain over m-calpain (Table 1). This can be attributed to the differences in the calcium binding domain between μ - and m-calpain. It suggests that it is possible to develop isoform-specific calpain inhibitors.

Calpain overactivation is thought to contribute to neurodegeneration (8). PD150606, but not PD145305, was found to make cerebral glutamatergic neurons more resistant to hypoxic/hypoglycemic challenge (Fig. 6). Likewise, PD150606 also protected cerebellar Purkinje neurons from AMPA toxicity (Fig. 7). These findings support the participation of calpain in the expression of excitotoxic and hypoxic neuronal injury and show that the mercaptoacrylate could provide neuroprotection against these types of injury.

To date, the development of calpain inhibitors has focused almost exclusively on the synthesis of modified peptides that compete for the active site of the protease (8). The major disadvantage of this class of compounds is the lack of selectivity (8), which is likely due to the similarity of the active site among different classes of cysteine proteases. The discovery and refinement of selective inhibitors of calpain therefore remain important goals for this field. In the present study, we have identified a novel class of calpain inhibitors. Its selectivity for calpain and cell-permeability make the mercaptoacrylate PD150606 an attractive tool in studying the long sought after physiologic functions of calpains. PD145305, which was ineffective both *in vitro* and *in situ* in blocking calpain activity, can serve as a negative control. The neuroprotective effects of PD150606 shown here also suggest that compounds of this class may be therapeutically useful in treating various forms of disorders in which calpain overactivation is implicated.

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