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A novel calpastatin-based inhibitor improves postischemic neurological recovery

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ABSTRACT

Calpastatin, a naturally occurring protein, is the only inhibitor that is specific for calpain. A novel blood-brain barrier (BBB)-permeant calpastatin-based calpain inhibitor, named B27-HYD, was developed and used to assess calpain's contribution to neurological dysfunction after stroke in rats. Postischemic administration of B27-HYD reduced infarct volume and neurological deficits by 35% and 44%, respectively, compared to untreated animals. We also show that the pharmacologic intervention has engaged the intended biologic target. Our data further demonstrates the potential utility of SBDP145, a signature biomarker of acute brain injury, in evaluating possible mechanisms of calpain in the pathogenesis of stroke and as an adjunct in guiding therapeutic decision making.

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Introduction

Stroke is a leading cause of death and the principal cause of adult disability throughout the world, affecting 15 million people each year. Of these, 5.5 million die and 5 million are left permanently disabled [1,2]. Despite its public health significance, treatment of ischemic stroke is limited to thrombolysis by tissue plasminogen activator administered intravenously within 3 hours of symptom onset [2]. Only a small percentage (<5%) of stroke patients ultimately receive this treatment. Clearly a more efficacious therapeutic strategy with a wider treatment window is needed to augment the effects of reestablishing blood flow with thrombolysis. Calpain is a prominent calcium-activated cysteine protease recognized to play an important role in signal transduction, cell motility, synaptic function, gene expression, and regulation of apoptosis [3–7]. Dysregulated calpain has been implicated in a variety of central nervous system (CNS) disorders, including cerebral ischemia, traumatic brain and spine injury as well as multiple sclerosis, and Alzheimer's, Huntington's and Parkinson's disease [8–13]. These disorders are characterized by intracellular calcium overload leading to excessive activation of calpains [14–16]. *In vivo* inhibition of calpains using small molecule cysteine protease inhibitors (e.g. peptide aldehydes, α-diketones, and α-keto esters and amides) diminishes the extent of neuronal damage following ischemia; however, none of these inhibitors is specific for calpain, since they also effectively inhibit other cysteine proteases as well [17–21]. Therefore, the beneficial effects of the so called calpain inhibitors could be due in part to inhibition of other proteases, including cathepsins B and L and the proteasome. Calpastatin, a naturally occurring protein, is the only inhibitor that is specific for calpain, and it is generally accepted that the interaction of calpastatin with calpain is the most relevant mechanism responsible for the regulation of Ca^{2+}-induced proteolysis [3,22–26]. Previously, we identified two "hot spots" in a 27-mer human calpastatin subdomain 1B peptide (referred to as B27) that contain the residues critical for the potent and specific inhibition of calpain [22,23]. Further, the results suggested a model in which the two hot spots are situated at the interface(s) of the calpain–calpastatin complex and act in a concerted fashion to inhibit calpain. These findings have been confirmed by two crystal structures of the calpain–calpastatin complex that were published recently [27,28]. To the best of our knowledge, none of the studies reported to date on the pharmacologic inhibition of calpain has benefited from the absolute specificity of calpastatin to delineate calpain's contribution to cell death and neurological dysfunction following ischemic stroke. Thus, the present study was undertaken to evaluate B27-HYD, a novel BBB-permeant analogue of B27, for its effect on

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cerebral infarction and neurological functional recovery after focal cerebral ischemia in rats.

Materials and methods

Animal model. The study was approved by the Institutional Animal Care and Use Committee at Henry Ford Hospital. Male Wistar rats (270–290 g) were obtained from Charles River Breeding Co. (Wilmington, MA, USA). Two-hour middle cerebral artery occlusion (MCAO) by the intravascular suture method, drug infusion, measurement of infarct volume and neurological deficits were performed as detailed previously [29–32]. B27-HYD ($D^1PMSSTYIEE^{10}LGKREVTIPP^{20}KY$***VALLP^{27}AVLLALLAP***) and its less active Leu^{11} → βAla^{11} mutant B27βAla^{11}-HYD were produced by FMOC-chemistry solid phase peptide synthesis and characterized for calpain inhibitory activity as described previously [22,23]. B27-HYD (50 μM solution, i.v. infusion at 15 μl/min for 4 h; 3 mg/kg), B27βAla^{11}-HYD or vehicle (1% DMSO in saline) was administered to rats (n = 6–9) immediately after reperfusion following 2 h of MCAO. Sham-operated animals had surgery but no MCA occlusion. Animals to be studied for neurological score and infarct volume survived 7 days.

Brain protein extraction and Western blot analysis. Following MCAO and drug infusion, the animals were sacrificed after 24 or 48 h of survival. At that time, the brains were extracted and separated into right and left, cortical and subcortical regions, snap-frozen in liquid nitrogen and stored at −80 °C until used. For Western blot analysis, the brain samples were pulverized to a fine powder with a small mortar and pestle set over dry ice. The pulverized brain tissue powder was homogenized in a solution of 0.25 M sucrose, 25 mM 2-(*N*-morpholino)-ethanesulfonic acid, 1 mM EDTA, pH 6.5, containing 0.025 mM E-64, 2.0 mM AEBSF, 0.5 mM PMSF, 0.02 mM leupeptin, 0.05 mM pepstatin, and 0.001 mM aprotinin (homogenization buffer) at 4 °C in a Potter–Elvehjem homogenizer. The homogenates were then centrifuged at 4 °C for 20 min at 16,000*g*. Aliquots of the supernatant were stored at −80 °C until used for experiments. Protein concentrations were determined using micro BCA Protein Assay kit (Pierce, Rockford, Ill). SDS–PAGE was carried out according to Laemmli [33] and immunoblots were performed according to the technique of Towbin et al. [34]. Mouse monoclonal anti-αII-spectrin antibody (MAB 1622, 1:1,000) and horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (AP124P, 1:10,000) were from Chemicon (Temecula, CA). Labeled proteins were detected with the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Biosciences, Piscataway, NJ, USA). Bands were quantified by densitometry using Gel-Doc 2000 (Bio-Rad, Richmond, VA) and the free open source software ImageJ (http://rsb.info.nih.gov/ij/).

Results

Infarct volume

From the H&E sections, the ischemic core area with diffuse pallor of the eosinophilic background and the border area with vacuolation or sponginess and neutrophils can be identified under the microscope. As can be seen from the representative H&E stained serial coronal sections A through G in Fig. 1A, the brain region mostly affected by the ischemia is the cortex in the MCA distribution (black arrows). The subcortical region in the core slices of C and D is also affected (white arrows). The ischemic damage (i.e. cell death, loss of tissue, or encephalomalacia) is less in the B27-HYD-treated animals compared to the vehicle-treated controls. By comparison, the contralateral side appeared undamaged by both gross inspection and in histological sections. As shown in Table 1 and Fig. 1B, B27-HYD significantly reduced infarct volume compared to B27βAla^{11}-HYD-treated (24.9 ± 3.8% vs. 33.8 ± 4.2%, percent hemispheric infarct volume ± SE; $^{\#}P < 0.05$, independent t-test; n = 9 per group) and vehicle-treated (24.9 ± 3.8% vs. 38.4 ± 2.1%, percent hemispheric infarct volume ± SE; $^{*}P < 0.05$, independent t-test; n = 9 per group) controls, respectively.

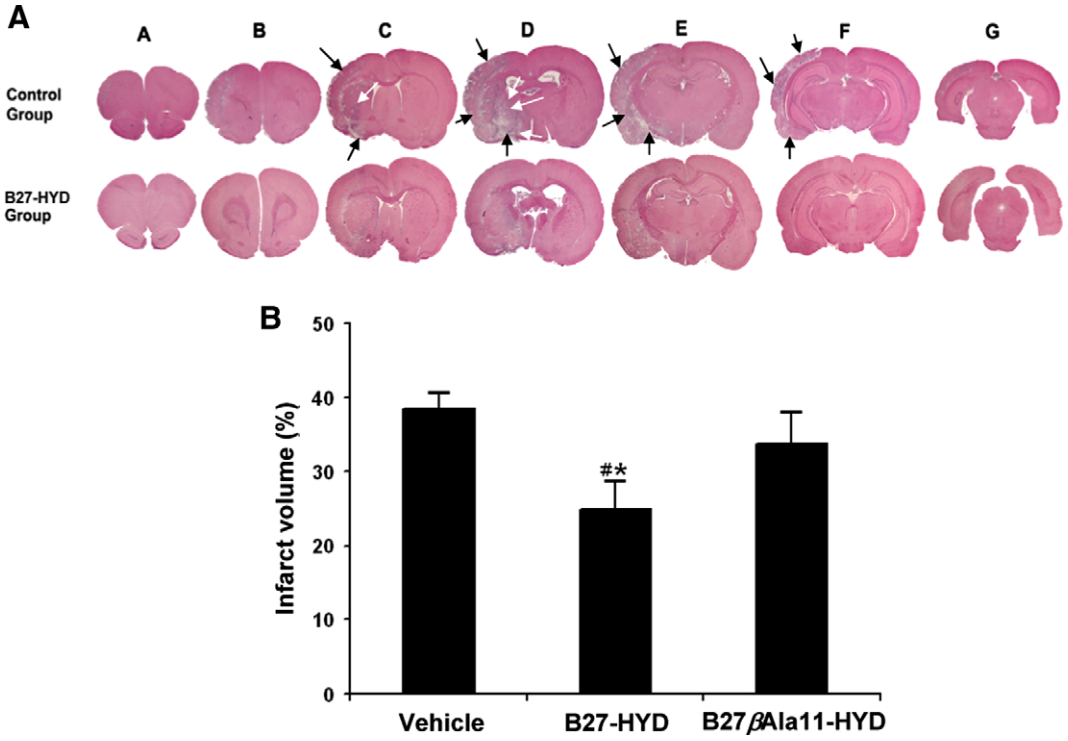

Fig. 1. (A) Histology of the ischemic brain. Representative serial coronal H&E stained sections from the vehicle control and B27-HYD-treated groups are shown. (B) B27-HYD reduces cerebral infarction after MCAO in rats.

Table 1
Summary of data for infarct volume and neurological functional outcome.

Group	Lesion volume % of hemisphere	Neurological score			
		0 Day	1 Day	4 Days	7 Days
Saline ($n = 9$)	38.4 ± 2.1	2.89 ± 0.11	2.33 ± 0.24	1.89 ± 0.24	1.78 ± 0.22
B27-HYD ($n = 9$)	24.9 ± 3.8$^{*,\#}$	2.89 ± 0.11	2.33 ± 0.24	1.44 ± 0.18	1.00 ± 0.17$^{*,\#}$
B27βAla^{11}-HYD ($n = 9$)	33.8 ± 4.2	2.89 ± 0.11	2.11 ± 0.20	1.56 ± 0.18	1.44 ± 0.18

Values are mean ± SE.
* $P < 0.05$ B27-HYD vs. vehicle.
$^{\#}$ $P < 0.05$ B27-HYD vs. B27βAla^{11}-HYD.

Neurological score

Neurological scores have been previously defined as follows: no observable deficit = 0, forelimb flexion = 1, decreased resistance to lateral push with forelimb flexion = 2, same behavior as 2 plus circling = 3 [30,32]. The behavioral data indicate that the neurological scores slightly improved in all the experimental groups during the first 4 days post-injury (Table 1 and Fig. 2). However, the difference in performance between the B27-HYD-treated animals and the vehicle controls (1.00 ± 0.17 vs. 1.78 ± 0.22, $^{*}P < 0.05$, $n = 9$ per group; independent t-test) and the B27βAla^{11}-HYD-treated group (1.00 ± 0.17 vs. 1.44 ± 0.18, $^{\#}P < 0.05$, $n = 9$ per group; independent t-test) was statistically significant at day 7 post-injury.

αII-Spectrin breakdown

Pathologic activity of calpain and *in vivo* efficacy of B27-HYD were assessed by Western blot analysis of αII-spectrin breakdown products (SBDPs) generated in the rat brain following MCAO. As can be seen in Fig. 3, brain samples from sham-operated animals present mainly the intact αII-spectrin (280-kDa) while the stroke-injured vehicle-treated animals (vehicle controls) had elevated levels of 150- and 145-kDa SBDPs (SBDP150 and SBDP145) in the cortical and subcortical regions of the ipsilateral hemisphere in the first 24 h post-injury (panels A and B). B27-HYD treatment blocked SBDP150 and SBDP145 formation to basal levels at 24 h post-injury (panel A, B, and E). In the vehicle control animals, SBDP145 levels continued to increase in the brain, becoming the predominant αII-spectrin cleavage product at 48 h post-injury (see panels C and D). It is interesting to note that additional SBDPs of 120 (SBDP120) and 110 kDa (SBDP110), which were not present in the first 24 h post-injury, were generated by 48 h post-injury. B27-HYD treatment immediately after MCAO/reperfusion significantly reduced SBDP145 levels in the cortical and subcortical regions of the brain at 48 h post-injury ($^{*}P < 0.05$ vs. vehicle; $n = 6$; panel C, D, and E).

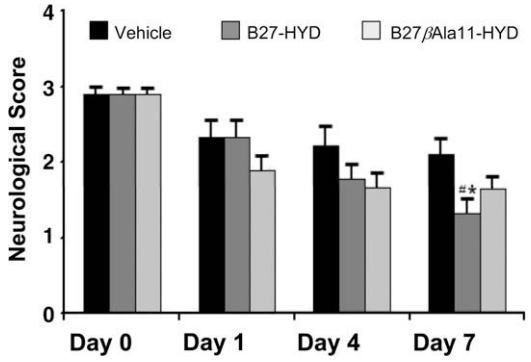

Fig. 2. B27-HYD reduces neurological deficits after MCAO in rats.

Discussion

In this study, we have used a pharmacological approach that exploits calpastatin's absolute specificity for calpain and a BBB-permeable drug delivery strategy to demonstrate that postischemic inhibition of calpain significantly reduces infarct volume and improves neurological functional recovery after focal cerebral ischemia. The novel calpain inhibitor B27-HYD is composed of a signal sequence-derived membrane translocation peptide motif (***VALLPAVLLALLAP***) [35] conjugated to the C-terminus of human calpastatin subdomain B [22,23,27,28]. B27-HYD is a potent, water-soluble, and specific inhibitor of calpain that is non-toxic to cultured cells. Since B27-HYD is calpastatin-based, evidence is provided to support a causal role for calpain in neurological dysfunction after stroke.

Investigation of the pathobiology of cerebral ischemia indicates that the initial ischemic insult induces massive release of glutamate from damaged synapses which leads to activation of glutamate receptor-associated and voltage-dependent calcium channels [8–12,14–16]. Such activation induces influx of calcium ions into the neuron and release of calcium ions from intracellular stores. Loss of intracellular calcium homeostasis contributes to cell death by activating various enzymes, including proteases, kinases, phosphatases, and phospholipases [12,14]. Integral to the mechanism of calcium-mediated brain injury is the pathologic activation of calpains [3,8–12]. Under normal physiological conditions, calpain exists at very low activity in cells and is proposed to participate in the turnover of cytoskeletal proteins and the regulation of kinases, transcription factors, and receptors [3–7,12]. However, pathologic calpain activation results in proteolytic destruction of many cellular proteins including receptor proteins, calmodulin binding proteins, signal transduction enzymes, transcription factors, and cytoskeletal proteins [8–12]. Furthermore, uncontrolled calpain activity prevents increased expression of several key proteins, including growth-associated protein-43 (GAP-43), synaptophysin, and collapsin receptor mediator proteins (CRMPs), that play a major role in regeneration and neuroplasticity after ischemic and traumatic brain injury [36–42]. Thus, calpains can contribute to the pathogenesis of ischemic brain injury via multiple molecular and cellular pathways. These previous observations, together with the data from the present study, suggest that selective inhibition of calpains may lead to both cerebroprotective effects and an enhancement of neuronal plasticity/repair mechanisms.

The primary endpoint of this study was functional outcome assessed by the method of Bederson et al. [32]. Treatment with B27-HYD immediately after 2-h MCAO reduced neurological deficits by 24% and 44% on day 4 and day 7, respectively, compared to vehicle-treated stroke controls. Infarct volume was a secondary outcome and was measured at day 7 after stroke. The extent of cerebral infarction was reduced by 35% in the B27-HYD-treated animals compared to vehicle-treated animals. Because of the predictable location of areas of infarction and consistent production of neurological deficits in the MCAO model used in this study [29–30,32],

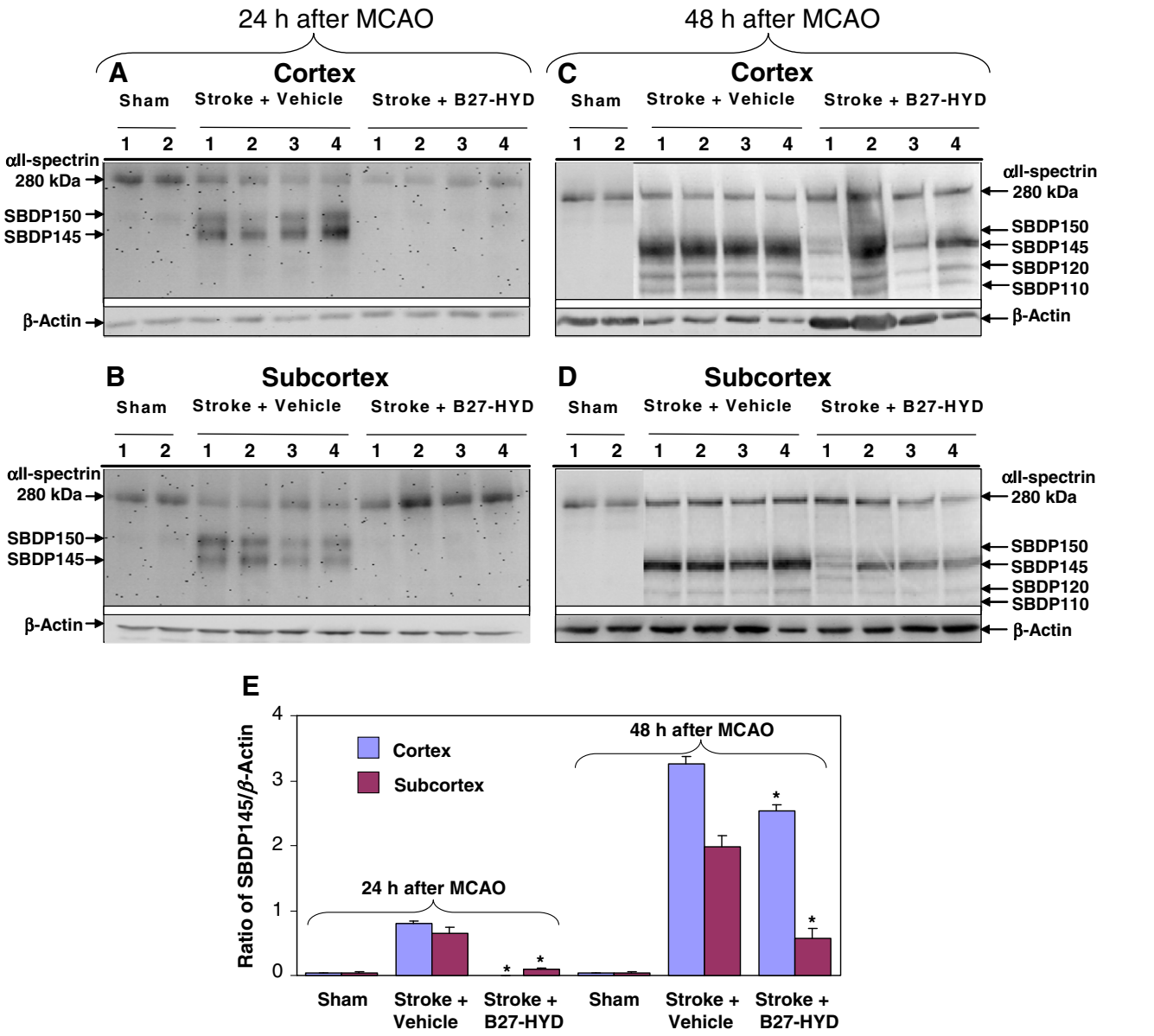

Fig. 3. B27-HYD blocks calpain-mediated cleavage of αII-spectrin in rat brain following MCAO. (A–D) Representative immunoblots of αII-spectrin in ipsilateral cortical (A,C) and subcortical (B,D) regions of sham-operated, ischemic vehicle-treated, and ischemic B27-HYD-treated animals at 24 h (A,B) and 48 h (C,D) following MCAO. Intact αII-spectrin (280 kDa), SBDP150, SBDP145, SBDP120 and SBDP110 are demonstrated. (E) Densitometric analysis of SBDP150/SBDP145 levels in ipsilateral cortex and subcortex at 24 and 48 h following MCAO.

the effects of B27-HYD on neurological outcome and size of infarction produced by focal cerebral ischemia could be reliably studied. It must be noted, however, that, infarction volumes could correlate poorly with functional outcome because small lesions in cortical locations can produce major functional deficits. Conversely, large lesions in relatively silent areas cause little detectable function loss. Reduction of neurological deficits after stroke has been attributed to synaptic and functional reorganization in the cerebral cortex and in subcortical structures after ischemic injuries [43–46]. Since pathologic calpain activity can result in disruption of axonal transport and structural collapse as well as a net decrease in the level of proteins involved in the neuronal plasticity that occurs after brain injury, B27-HYD is expected to effectively treat an array of calpain-mediated physiological changes associated with stroke to improve functional outcome.

The most well-studied calpain target is αII-spectrin, a 280-kDa neuronal protein that localizes to axons and functions in cortical cytoskeleton matrix support. αII-spectrin is proteolyzed by calpain to generate 150- and 145-kDa spectrin breakdown products (SBDP150 and SBDP145) or by caspase-3 to generate 150- and 120-kDa fragments (SBDP150i and SBDP120) following ischemic and traumatic brain injury [12,47]. Calpain-generated SBDP145 is a signature biomarker for neuronal necrotic/oncotic cell death after ischemic and traumatic brain injury while caspase-3-generated SBDP120 is a signature biomarker for neuronal apoptosis [12,17,47]. Recently, the clinical significance of calpain and caspase-3 specific SBDPs as biomarkers for acute brain injury was demonstrated in humans [48–49]. Our data confirm that necrotic/oncotic and apoptotic cell death mechanisms overlap but appear to be activated at distinct time patterns after MCAO. As shown in Fig. 3A and B, the extent of SBDP145 formation was similar in both cortical and subcortical regions of the ischemic hemisphere at 24 h post-injury. Interestingly, the SBDP145 levels increased about 3- and 2-fold in the cortex and subcortex, respec-

tively, by 48 h after MCAO, suggesting continued calpain-mediated proteolysis of αII-spectrin in the ischemic hemisphere probably due to a new surge in intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels in this region of the brain that resulted in an additional increase in calpain activation between 24 and 48 h following the initial ischemic injury. While acute postischemic administration of B27-HYD completely blocked SBDP145 accumulation at 24 h post-injury, the treatment significantly reduced SBDP145 levels in the cortex and the subcortex at 48 h. In addition to its use in assessing neuronal structural degradation and evaluating possible mechanisms involved in the evolving brain damage after stroke, we have demonstrated the potential utility of SBDP145 biomarker as an adjunct in guiding therapeutic decision making during the development of calpain-target-based neuroprotective strategies.

To date, preclinical studies reported on anti-calpain treatment strategies for stroke have used inhibitor designs based on di- or tri-peptide and peptidomimetic address labels modified with a chemical war-head [18–21]. Even though these small molecule synthetic inhibitors are effective in inhibiting calpain, they do, to varying degrees, react with other proteases implicated in the pathophysiology of stroke. It could be argued that single-drug therapy with a broad-spectrum inhibitor that blocks several proteases involved in the progression of ischemic brain injury would be more effective than treatment with a highly selective inhibitor that targets one particular protease. However, the lack of specificity of a protease inhibitor often raises other issues related to tissue toxicity. Furthermore, the onset, duration, and extent (amount) of abnormal protease activity generated after the initial ischemic event are likely to be different for each protease target. Previously, we demonstrated the cerebroprotective effects of CP-1, a cysteine protease inhibitor which does not block calpain or caspase but, rather, is selective for cathepsins B and L [30–31]. Our current findings suggest that a protease-targets-based combination therapy aimed at selective inhibition of unique steps of protease-mediated brain injury would result in synergistic brain tissue protection and improvement of functional outcome after stroke.

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