A Pharmacological Analysis of the Neuroprotective Efficacy of the Brain- and Cell-Permeable Calpain Inhibitor MDL-28170 in the Mouse Controlled Cortical Impact Traumatic Brain Injury Model

Stephanie N. Thompson,¹ Kimberly M. Carrico,¹ Ayman G. Mustafa,^{1,2} Mona Bains,¹ and Edward D. Hall¹

Abstract

The cytoskeletal and neuronal protective effects of early treatment with the blood–brain barrier- and cell-permeable calpain inhibitor MDL-28170 was examined in the controlled cortical impact (CCI) traumatic brain injury (TBI) model in male CF-1 mice. This was preceded by a dose-response and pharmacodynamic evaluation of IV or IP doses of MDL-28170 with regard to *ex vivo* inhibition of calpain 2 activity in harvested brain homogenates. From these data, we tested the effects of an optimized MDL-28170 dosing regimen on calpain-mediated degradation of the neuronal cytoskeletal protein α -spectrin in cortical or hippocampal tissue of mice 24 h after CCI-TBI (1.0 mm depth, 3.5 m/sec velocity). With treatment initiated at 15 min post-TBI, α -spectrin degradation was significantly reduced by 40% in hippocampus and 44% in cortex. This effect was still observed with a 1-h but not a 3-h post-TBI delay. The cytoskeletal protection is most likely taking place in neurons surrounding the area of mainly necrotic degeneration, since MDL-28170 did not reduce hemispheric lesion volume as measured by the aminocupric silver staining method. This lack of effect on lesion volume has been seen with other calpain inhibitors, which suggests that pharmacological calpain inhibition by itself, while able to reduce axonal injury, may not be able to produce a measurable reduction in lesion volume. This is in contrast to certain other neuroprotective mechanistic approaches such as the mitochondrial protectant cyclosporine A, which produces at least a partial decrease in lesion volume in the same model. Accordingly, the combination of a calpain inhibitor with a compound such as cyclosporine A may be needed to achieve the optimal degree of post-TBI neuroprotection.

Key words: calpain; controlled cortical impact; cytoskeleton; neurodegeneration; traumatic brain injury

Introduction

FOLLOWING THE INITIAL MECHANICAL INJURY to the central nervous system (CNS), additional tissue damage results from delayed secondary processes, including ischemia, loss of ion homeostasis, excitotoxicity, and free-radical production. Among these mechanisms, excessive intracellular calcium (Ca^{2+}) accumulation plays a key role in initiating and mediating numerous events in this secondary damage cascade, including the activation of the neutral proteases, the calpains (Bartus, 1997; Kampfl et al., 1997; McCracken et al., 1999; McIntosh, 1997; Narayan et al., 2002).

Calpains are present in the majority of mammalian cells, with over a dozen different isoforms identified. The two main isoforms implicated in CNS injury appear to be calpain-1

(μ -calpain) and calpain-2 (m-calpain), which require low micromolar and near-millimolar concentrations of Ca^{++} , respectively, for activation *in vitro* (Kampfl et al., 1997; Yuen and Wang, 1996). During normal physiological periods, calpains possess low levels of activity and are believed to aid in cytoskeletal turnover and the regulation of kinases, transcription factors, and receptors (Kampfl et al., 1997). However, excessively activated calpain-1 and calpain-2 results in the proteolysis of numerous proteins, including receptor proteins, calmodulin-binding proteins, signal transduction enzymes, transcription factors, and cytoskeletal proteins (Kampfl et al., 1997). This cleavage of cytoskeletal proteins leads to axonal transport disruption and structural collapse, culminating in secondary axonal injury and possibly cell death. One prototypical calpain substrate is the cytoskeletal protein α -spectrin,

¹University of Kentucky Spinal Cord & Brain Injury Research Center, University of Kentucky College of Medicine, Lexington, Kentucky.

²Department of Anatomy & Cell Biology, Jordan University of Science and Technology College of Medicine, Irbid, Jordan.

with calpain-mediated degradation of this protein producing two stable breakdown products of 150 kDa and 145 kDa weight (SBDP150 and SBDP145) (Roberts-Lewis and Siman, 1993; Roberts-Lewis et al., 1994; Siman et al., 1984). Additionally, the apoptosis-associated protease caspase-3 can cleave spectrin to produce a unique 150-kDa degradation fragment that is further cleaved to a 120-kDa product (Wang, 2000). α -Spectrin cleavage has been used extensively as a quantified measure of calpain activity in both animal models of traumatic brain injury (TBI; Buki et al., 1999; Kampfl et al., 1996; Kupina et al., 2003a, 2001, 2002; Mbye et al., 2009; Saatman et al., 1996), and more recently in TBI patients (Brophy et al., 2009). Based on these published reports, the neuroprotective potential of the calpain inhibitors may be predicted by their ability to attenuate post-traumatic spectrin degradation.

Multiple calpain inhibitors have been developed (Yuen and Wang, 1996), of which several have demonstrated beneficial effects in TBI models, including E64D (Posmantur et al., 1997), AK295 (Saatman et al., 1996, 2000), SJA-6017 (Kupina et al., 2001), and MDL-28170 (Ai et al., 2007; Ayala-Grosso et al., 2004; Buki et al., 2003; Czeiter et al., 2009; Kawamura et al., 2005; Markgraf et al., 1998; Yu and Geddes, 2007). Of these, MDL-28170 (carbenzoxymethyl-phenylalanine, calpain inhibitor III) is among the best at crossing cell membranes and the blood-brain barrier (BBB), as well as possessing a high selectivity for calpain relative to the other proteases trypsin, plasmin, caspase-1, and cathepsin D (Mehdi et al., 1988; Yuen and Wang, 1996). Consistent with that profile, MDL-28170 has been reported to have neuroprotective effects in numerous rodent neurotrauma models, including spinal cord injury (Arataki et al., 2005; Yu and Geddes, 2007; Yu et al., 2008), neonatal hypoxia-ischemia (Kawamura et al., 2005), and focal cerebral ischemia (Markgraf et al., 1998). Additionally, MDL-28170 has been shown to attenuate post-traumatic axonal injury in rat diffuse TBI models (Ai et al., 2007; Buki et al., 2003; Czeiter et al., 2009). However, the effect of MDL-28170 has not been examined in a controlled cortical impact (CCI) model of TBI, in which axonal injury and focal cortical contusion are both observed (Hall et al., 2005, 2008).

Therefore, in this study using this focal TBI model in male mice, we tested the neuroprotective effects of MDL-28170 in terms of a reduction of calpain-mediated cytoskeletal degradation and hemispheric lesion volume at the times at which each has been shown to peak (Deng et al., 2007; Hall et al., 2005, 2008). Prior to our TBI experiments, we established the dose-response and pharmacodynamic profile of MDL-28170 using an *ex vivo* assay of calpain-2 activity. We then examined if we could reduce post-injury calpain-mediated spectrin degradation in mouse CCI-TBI using dosing protocols derived from the drug's pharmacodynamic analysis. We also evaluated the therapeutic time window in terms of its ability to attenuate cytoskeletal proteolysis. Lastly, we determined if MDL-28170 could affect the extent of neurodegeneration occurring at 48 h using the de Olmos silver staining histochemical method, which selectively stains degenerating neurons, and their axons in particular (de Olmos et al., 1994; Switzer, 2000).

Methods

All procedures followed the guidelines set by the U.S. Public Health Service Policy on Humane Care and Use of

Laboratory Animals, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Ex vivo calpain activity assay

Calcium-dependent proteolytic activity was measured in cortical tissue using a 4,4-difluoro-5,7-dimethyl-4-boro-3a, 4a-diaz-s-indacene-3-propionic acid label casein (BODIPY-FL; Molecular Probes, Eugene, OR) microplate assay (Thompson et al., 2000). Non-injured male CF-1 mice (28–30 g) received a single injection (IV or IP) of either MDL-28170 (Calbiochem, San Diego, CA) or vehicle (9:1 PEG 300:EtOH). The animals were sacrificed at various time points following injection (15 min to 4 h), and the cortices from both hemispheres were immediately removed, snap frozen using dry ice, and stored at -80°C until analysis. The cortices were crushed, weighed, and homogenized in dilution buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM KCl, and 0.1% 2-mercaptoethanol) at a concentration of 1 mg/10 μL of buffer. Homogenates were centrifuged at 15,000 rpm for 20 min at 4°C . The supernatant (25 μL) was added to a black microtiter plate well containing 75 μL of dilution buffer. Due to low basal levels of endogenous calpain in naïve mice, 0.6 U of calpain-2 (purified from porcine kidney; Calbiochem) was added to a subgroup of wells containing sample immediately prior to assay initiation. The addition of 100 μL of BODIPY-FL-casein (10 $\mu\text{g}/\text{mL}$) in assay buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM KCl, 10 mM CaCl_2 , and 0.1% 2-mercaptoethanol) initiated the assay. Each plate contained an EDTA blank (25 μL of 100 mM EDTA, 50 μL of dilution buffer, 25 μL of calpain-containing sample, and 100 μL of BODIPY-FL-casein), and a Ca^{2+} blank (100 μL of dilution buffer and 100 μL of BODIPY-FL casein). A standard curve was established with 0.1–112.5 U of calpain-2 in 100 μL dilution buffer and 100 μL assay buffer. All samples, standards, and blanks were assayed in duplicate. The covered microtiter plate was incubated for 30 min at room temperature on a shaking rocker. The reaction was terminated by adding 25 μL of 100 mM EDTA. Following incubation the plate was read on a spectrofluorometer (BioTek Synergy HT; BioTek, Winooski, VT) at 485-nm excitation and 528-nm emission. The average values of Ca^{2+} and EDTA blanks were subtracted from the fluorescent unit readings for the samples, and calpain activity was calculated using the standard curve. The obtained fluorescent unit readings were normalized to milligrams of soluble protein using a modified Lowry assay (Bio-Rad DC Assay with 0.1 mM iodoacetamide; Bio-Rad Laboratories, Inc., Hercules, CA). All data were expressed as units of calpain per milligram of total protein, because the number units per milligram of calpain varied with the individual lots of porcine calpain-2 used to prepare the standard curve (Calbiochem). One unit of calpain is defined as the amount of enzyme that hydrolyzed 1.0 pmol Suc-LLVY-AMC in 1 min, at 25°C using the Calpain Activity Assay Kit, Fluorogenic (Calbiochem).

MDL-28170 preparation

MDL-28170 was purchased from Calbiochem and dissolved in a 9:1 ratio of polyethylene glycol 300 to ethanol on the day of injection. This vehicle was chosen based on its previous use by other investigators (Markgraf et al., 1998).

Controlled cortical impact injury

Young adult CF-1 male mice weighing 29–31 g (Charles River, Portage, MI) were used in all experiments. The mice were anesthetized using isoflurane (2.5%), shaved, and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), with the head positioned in the horizontal plane and the nose bar set at zero. Following a midline incision to expose the skull, a 4-mm craniotomy was made lateral to the sagittal suture, and centered between the lambda and the bregma. The skull at the craniotomy site was removed without disrupting the underlying dura. A pneumatically-driven piston (Precision Systems and Instrumentation, LLC, Fairfax, VA) containing a 3-mm-diameter flat tip depressed the cerebral tissue 1.0 mm (3.5 m/sec velocity) for 500 msec. Following the injury, a cranial cap constructed from denture material was placed over the craniotomy and adhered to the skull using cyanoacrylate. The injured region was sutured and the anesthetized mice were then placed in an incubator (37°C) until they regained consciousness. All animals had *ad libitum* access to food and water. Further details of the procedure are available in our previous publications (Deng-Bryant et al., 2008; Deng et al., 2007; Hall et al., 2005; Mbye et al., 2009).

Tissue processing and immunoblotting

To measure calpain-specific α -spectrin degradation, the mice were euthanized at 24 h post-impact, the initial time point when α -spectrin degradation is maximally elevated in both the ipsilateral cortex and hippocampus post-CCI-TBI (Deng et al., 2007; Thompson et al., 2006). Another group has also shown that neurofilament proteolytic damage after CCI-TBI in rats peaks at 24 h (Posmantur et al., 1994). The mice received an overdose of sodium pentobarbital (200 mg/kg IP). The ipsilateral cortex and hippocampus were rapidly dissected on an ice-chilled stage and immediately transferred to Triton lysis buffer (1% Triton, 20 mM Tris HCL, 150 mM NaCl, 5 mM EGTA, 10 mM EDTA, and 10% glycerol) containing protease inhibitors (Complete MiniTM Protease Inhibitor Cocktail; Roche Diagnostics Corp., Indianapolis, IN). The samples were sonicated and then centrifuged for a period of 30 min (15,000 rpm at 4°C). The supernatants were collected and the pellet was discarded. Protein concentrations were determined using the BioRad DC Protein Assay, and samples were diluted to 1 mg/mL of protein.

Calpain-mediated spectrin degradation following severe CCI was determined using Western immunoblot analysis. Protein samples (5 μ g) were run on 3–8% Tris-Acetate CriterionTM XT Precast gels (Bio-Rad), and then transferred to a nitrocellulose membrane via a semidry electro-transferring unit. The membranes were incubated in a blocking solution of TBS plus 5% milk for 1 h. This was followed by an incubation in TBST blocking solution containing mouse monoclonal anti- α -spectrin antibody (1:5000, Affiniti FG6090; Affiniti, Mamhead Castle, U.K.) overnight at 4°C. The next incubation (1 h in darkness at room temperature) consisted of TBST plus a goat anti-mouse secondary antibody conjugated to an infrared dye (1:5000, IRDye800CW; Rockland Immunochemicals, Inc., Gilbertsville, PA). The membranes were imaged and quantified using the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). In the current experiments, the band analyzed for α -spectrin breakdown was the calpain-specific 145-kDa band. Included on each blot was a

loading control that was used to normalize band densities between blots.

Silver staining

We examined post-TBI neurodegeneration at its 48-h peak using the de Olmos aminocupric silver staining method (de Olmos et al., 1994; Switzer, 2000) as previously described (Deng-Bryant et al., 2008; Deng et al., 2007; Hall et al., 2005). The mice were overdosed with sodium pentobarbital (200 mg/kg IP), and transcardially perfused with a wash solution containing 0.8% sodium chloride, 0.4% dextrose, 0.8% sucrose, and 0.023% CaCl₂, and then fixed using 4% paraformaldehyde, 4% sucrose, and 1.4% sodium cacodylate. After decapitation, the heads were stored in the fixative solution for 24 h. The brains were then removed and shipped for histological processing to Neuroscience Associates Inc. (Knoxville, TN). The brains used for this study were embedded in single gelatin blocks (Multiblock Technology; Neuroscience Associates), except for 4 historical shams from a previous experiment. Thirteen 35- μ m coronal sections obtained 420 μ m apart between 1.0 mm anterior and 4.4 mm posterior to the bregma were silver stained and counterstained with nuclear fast red. The sections were photographed with an Olympus Provis A70 microscope at 1.25 $\times$ magnification using an Olympus Magnafire digital camera. The images were analyzed by Image-Pro Plus (v 4.0; Media Cybernetics Inc., Silver Spring, MD) software by a blinded observer. Using densitometric thresholding, the area of silver staining and the overall area in the ipsilateral or the contralateral hemisphere was measured for each brain section. After setting the threshold for the sections on one slide, it was not changed for analysis of the subsequent slides. The lesion area of each section was combined with silver staining measurements to yield measurements of the total volume of neurodegeneration in the injured (ipsilateral) hemisphere. Each area was multiplied by the distance between sections (420 μ m) to calculate a subvolume, with all subvolumes then summed to yield the total volume. The volume of the contralateral hemisphere was also determined in an identical fashion, and silver staining + lesion in the injured hemisphere was expressed as a percentage of contralateral hemisphere volume.

Statistical analysis

All data were analyzed using a one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls (SNK) *post-hoc* analysis (Statview 5.0; SAS Institute, Cary, NC). In all analyses, a *p* value <0.05 was considered significant.

Results

Pharmacodynamic and dose-response analysis for calpain inhibition by MDL-28170 in uninjured cortical tissue

MDL-28170 underwent a pharmacodynamic time course evaluation to determine its dose-related ability to suppress calpain activity, and the duration of its effect following a single administration (either tail vein IV or IP injection). The information obtained was needed to design the optimal dosing regimens for the TBI experiments. The pharmacodynamic evaluation of MDL-28170 was performed in uninjured mice, because they provide a greater challenge for the compound,

FIG. 1. *Ex vivo* pharmacodynamic analysis of the presence of calpain inhibitory concentrations of MDL-28170 in cerebral cortical tissue following either intravenous (IV) or intraperitoneal (IP) injections. (A) A single IV injection of 20 mg/kg MDL-28170 suppressed calpain-2 activity in cortical homogenates spiked with 0.6 U of calpain-2 that had been harvested at 30 min post-injection, but not at later time points. (B) IV dose-response graph showing that a 20-mg/kg IV dose was required to produce the *ex vivo* calpain-2 inhibitory effect shown in A. (C) A single IP injection of 40 mg/kg suppressed calpain-2 activity in cortical homogenates spiked with 0.6 U of calpain-2 that had been harvested as late as 2 h after injection, as well as at 30 min and 1 h. (D) IP dose-response graph showing that either a 20- or a 40-mg/kg IP dose was able to produce a significant *ex vivo* calpain-2 inhibitory effect. Values are mean $\pm$ standard error for 5–6 animals per group (* p < 0.05 versus vehicle by Fisher's *post-hoc* test following significant one-way analysis of variance).

since it must penetrate the brain in the absence of any post-traumatic BBB disruption. Following injection, calpain-2 activity was measured in cortical tissue using a BODIPY-FL-labeled casein microplate assay. Preliminary assays demonstrated that basal levels of endogenous calpain-2 in uninjured mice were too low to distinguish between MDL-28170 and vehicle-treated animals (as shown in Fig. 1). However, we found that adding a small amount of exogenous calpain-2 (0.6 U) to the samples immediately prior to assaying calpain activity allowed for the differentiation of MDL-28170-treated and vehicle-treated mice.

We first determined the pharmacodynamic time course of MDL-28170 following IV injection. When the animals were sacrificed 30 min post-injection, those receiving MDL-28170 (20 mg/kg IV) demonstrated significantly less cortical calpain activity (44%) than those receiving vehicle in the presence of exogenous calpain (p < 0.001; Fig. 1A). However, at the later time points of 1, 2, and 4 h post-IV-injection, MDL-28170's calpain inhibitory effect had abated. Thus the effects of MDL-28170 administered via tail vein IV injection are short-lived, and after 30 min post-IV-injection, additional MDL-28170 dosing would be required to maintain brain tissue calpain

FIG. 2. Comparison of the effects of a short two-dose MDL-28170 dosing regimen (20 mg/kg intravenous [IV] at 15 min post-injury plus a second intraperitoneal [IP] dose given at 45 min.) versus a more extended four-dose regimen (20 mg/kg IV at 15 min post-injury plus a second IP dose at 45 min, a third dose at 2 h 45 min, and a fourth dose at 4 h 45 min), on α -spectrin degradation in ipsilateral cortical and hippocampal samples harvested at 24 h post CCI-TBI, which is the peak time for post-traumatic calpain-mediated cytoskeletal proteolysis in the model. (A) The two dose regimen, which based on the pharmacodynamic analysis shown in Figure 1, would have maintained effective calpain-2 inhibitory brain levels of MDL-28170 only out to 2 h 45 min post-injury, failed to produce a reduction in either hippocampal or cortical levels of the calpain-specific 145-kDa α -spectrin fragment at 24 h. (B) In contrast, the four-dose regimen, which would have maintained effective concentrations of MDL-28170 out to 6 h 45 min post-injury, was able to significantly reduce SBDP145 levels in both brain regions at the 24-h peak time point. Values and mean $\pm$ standard error for 8 mice/group ($\#p < 0.05$ compared to sham animals; $*p < 0.05$ versus vehicle-treated animals by Student-Newman-Keuls *post-hoc* analysis following significant one-way analysis of variance). (C) Sample Western immunoblots from the cortical samples from animals treated with the longer dosing regimen shown in B, demonstrating the degree of proteolysis of α -spectrin into the breakdown products SBDP150 and SBDP145 in sham- or traumatic brain-injured (TBI)-mice treated with and without MDL-28170 (CCI-TBI, controlled cortical impact traumatic brain injury).

inhibition. When the MDL-28170 IV dose-response was examined, it was found (Goss et al., 2003) that while 20 mg/kg of MDL-28170 significantly suppressed calpain-2 activity ($p < 0.01$), lower doses (5 and 10 mg/kg) failed to alter calpain activity at 30 min post-injection (Fig. 1B). Evidence of a dose-related effect is derived from the fact that there was statistically significant difference between the 10 mg/kg and 20 mg/kg IV doses ($p < 0.01$).

A similar pharmacodynamic analysis determined the time course and optimal dose of MDL-28170 when administered via the IP route (Fig. 1C). MDL-28170 inhibited calpain-2 activity by 30 min post-IP-injection ($p < 0.01$), and continued to inhibit it at 1 and 2 h post-injection ($p < 0.02$ and 0.01, respectively). By 4 h post-injection, this effect had ended, suggesting a half-life of appropriately 2 h. Both 20 mg/kg and 40 mg/kg of MDL-28170 significantly reduced calpain activity at 30 min post-injection ($p < 0.02$ and 0.004, respectively), while 10 mg/kg of MDL-28170 did not (Fig. 1D). Additionally, there was no statistically significant difference between the 20- and 40-mg/kg IP doses, although there was between the 10- and 40-mg/kg IP doses ($p < 0.02$), showing that the effect was indeed dose-related. These studies demonstrated that MDL-28170 can cross the BBB and penetrate cortical tissue in concentrations adequate to produce *ex vivo* suppression of calpain activity after spiking the samples with exogenous calpain-2. This suggested that in experiments examining the neuroprotective effects of MDL-28170 in mouse TBI, a repeated dosing regimen involving an initial IV dose plus repeated IP doses would be required to maintain effective calpain inhibition.

Effect of MDL-28170 administration on calpain-mediated cytoskeletal degradation after CCI-TBI

Using dosing protocols based on the MDL-28170 pharmacodynamic analysis obtained with the *ex vivo* calpain activity assay, we next examined the ability of MDL-28170 to reduce

post-injury calpain-mediated cytoskeletal (i.e., α -spectrin) degradation in injured cortical and hippocampal tissue at 24 h post-TBI, when it is known to reach its peak in this mouse CCI-TBI model (Deng et al., 2007; Thompson et al., 2006), as well as in the rat lateral fluid percussion TBI model (McGinn et al., 2009). At this time point, injury caused pronounced cytoskeletal degradation, as seen in Figure 2, with the calpain-specific degradation of the 280-kDa α -spectrin to SBDP145, being highly significant in both the ipsilateral hippocampus and cortex of vehicle-treated animals compared to non-injured sham animals (Fig. 2A and B). Post-injury administration of MDL-28170 in an abbreviated two-dose regimen confined to the first hour post-TBI (20 mg/kg IV 15 min post-TBI + 40 mg/kg IP at 45 min post-TBI) failed to affect the 24 h post-injury SBDP145 levels in either the ipsilateral hippocampus or cortex (Fig. 2A). In contrast, a more extended MDL-28170 dosing regimen (20 mg/kg IV 15 min post-TBI + 40 mg/kg IP at 45 min, 2 h 45 min, and 4 h 45 min post-TBI) reduced post-traumatic spectrin degradation by 40% in the ipsilateral hippocampus and cortex by 44% (Fig. 2B). Figure 2C displays an example Western blot from some of the cortical samples from animals that had been treated with the more prolonged MDL-28170 dosing regimen shown in Figure 2B.

Therapeutic window analysis of the effect of MDL-28170 on calpain-mediated cytoskeletal degradation after CCI-TBI

A therapeutic window study was then conducted to determine if the more prolonged MDL-28170 dosing regimen (20 mg/kg IV 15 min post-TBI + 40 mg/kg IP at 45 min, 2 h 45 min, and 4 h 45 min post-TBI) maintained its effectiveness when the initial injection was delayed beyond 15 min post-injury, to the more clinically relevant time points of 1 and 3 h post-injury. Accordingly, we administered MDL-28170 starting at either 1 or 3 h after injury, with additional IP booster injections at 30 min, 2 h 30 min, and 4 h 30 min following the

FIG. 3. Therapeutic window analysis of MDL-28170 as measured by calpain-mediated α -spectrin degradation 24 h post CCI-TBI. Injury produced an increase in SBDP145 levels in both the hippocampus and cortex at 24 h post-TBI. Delayed treatment of MDL-28170 initially administered at 1 h post-injury (20 mg/kg intravenous [IV] 15 min + 40 mg/kg intraperitoneal [IP] 45 min, 2 h 45 min, and 4 h 45 min) attenuated α -spectrin degradation in the hippocampus and cortex. However, if MDL-28170 administration was delayed for 3 h following TBI, this effect lost significance. Values are mean $\pm$ standard error for 8 mice/group ($\#p < 0.05$ compared to sham animals; $*p < 0.05$ versus vehicle-treated animals by Student-Newman-Keuls *post-hoc* analysis following significant one-way analysis of variance; CCI-TBI, controlled cortical impact traumatic brain injury).

initial IV injection. The animals were sacrificed at 24 h following CCI-TBI and spectrin degradation was analyzed. MDL-28170 maintained its effectiveness when delayed for 1 h post-injury in both the hippocampus and cortex (Fig. 3). However, when treatment administration was delayed for 3 h, MDL-28170's protective effect against α -spectrin proteolysis lost statistical significance in both brain regions.

Effect of MDL-28170 on post-traumatic neurodegeneration

Although suppression of calpain-mediated cytoskeletal proteolysis can be argued to constitute a neuroprotective effect that is particularly relevant to an attenuation of axonal degeneration, the ultimate test of the neuroprotective efficacy of MDL-28170 depends on its ability to reduce histologically-demonstrated neurodegeneration. Thus, we next conducted an examination of the extent of post-traumatic neurodegeneration as measured at its previously demonstrated peak at 48 h post-injury (Deng et al., 2007; Hall et al., 2005, 2008), using the de Olmos aminocupric silver staining method. This staining technique specifically labels degenerating neuronal axons and dendrites (de Olmos et al., 1994; Switzer, 2000), and CCI-TBI animals demonstrate widespread silver staining in the injured cortex and subcortical tissues, including the hippocampus and thalamus (Hall et al., 2005, 2008). Additionally, by 48 h post-injury, cortical tissue loss is also apparent in the form of a lesion cavity at the CCI epicenter. The volume of this contusion lesion was added to the volume of the silver-stained tissue in the current analysis. As shown in Figure 4, CCI-TBI resulted in a total volume of neurodegeneration of approximately 15% of the ipsilateral hemisphere by 48 h post-injury in vehicle-treated animals. This was unaffected by an MDL-28170 dosing regimen that was able to reduce calpain-mediated α -spectrin degradation 24 h earlier (Fig. 2).

Discussion

As noted earlier, elevated intracellular free Ca^{2+} is hypothesized to play a central role in the initiation of the pathophysiological cascade leading to neurodegeneration after neurotrauma, and a major part of the damaging effects of calcium overload is related to the activation of the cysteine protease calpain (Bartus, 1997; McIntosh et al., 1997). In multiple TBI models, calpain-mediated cytoskeletal damage, as measured via neurochemical (Buki et al., 1999; Hall et al., 2005; Kupina et al., 2003; McGinn et al., 2009; Posmantur et al., 1994, 1996) or immunohistochemical (Buki et al., 1999; McGinn et al., 2009) methods, has been shown to occur and to be associated with histological damage, particularly to axons (Buki et al., 1999; McGinn et al., 2009). Thus calpain represents a rational and feasible target for neuroprotective therapeutic interventions.

Supporting this viewpoint is the demonstrated efficacy of multiple small-molecule calpain inhibitors to either lessen cytoskeletal degradation (Posmantur et al., 1997), to protect axonal structure (Ai et al., 2007; Buki et al., 2003) and axonal function (Ai et al., 2007), or to improve cognitive and motor recovery (Kupina et al., 2001; Saatman et al., 1996) following experimental TBI. However, making an association between cytoskeletal protection and improvements in post-traumatic

FIG. 4. Lack of an effect of MDL-28170 (20 mg/kg intravenous [IV] 15 min +40 mg/kg intraperitoneal [IP] 45 min, 2 h 45 min, and 4 h 45 min) on neurodegeneration as measured by de Olmos aminocupric silver staining in the ipsilateral hemisphere at 48 h post CCI-TBI. Scatterplots show individual volumes of neurodegeneration (percentage of silver staining + cortical contusion lesion) in each animal, as well as the mean value for each group of mice ($n = 8$ for the injured vehicle and MDL-28170 groups, and $n = 6$ for the sham group; $\#p < 0.05$ compared to sham animals by Student-Newman-Keuls *post-hoc* analysis following significant one-way analysis of variance; CCI-TBI, controlled cortical impact traumatic brain injury).

behavioral recovery has been elusive. Specifically, the calpain inhibitors AK295 (Saatman et al., 1996) and SJA6017 (Kupina et al., 2001), both of which improve motor recovery, do not show any correlating decrease in calpain-mediated cytoskeletal degradation. Further, the calpain inhibitor AK295, when administered in doses that improve motor and cognitive recovery after TBI, does not simultaneously reduce cortical lesion volume or the numbers of apoptotic neurons in the cortex or hippocampus (Saatman et al., 2000). However, we conceived this study with the idea that the incongruities seen in previous efforts to clarify the neuroprotective pharmacology of calpain inhibition in TBI models stem from the fact that none of the calpain inhibitors have been carefully studied using a systematic pharmacological approach. Specifically, none of the above-cited studies of any of the calpain inhibitors carefully explored the dose-response characteristics, optimum treatment duration, and therapeutic time window of their protective effects on axonal cytoskeletal proteins and neurodegeneration (e.g., lesion volume), when given at doses that achieve calpain-inhibitory concentrations in brain tissue for a prolonged period.

Accordingly, in our study we performed a pharmacological analysis of the brain-penetrable calpain inhibitor MDL-28170, and defined its dose-response and pharmacodynamics with regard to calpain inhibition. Then using the best dose and dosing regimen, we examined its effects on cytoskeletal and neuronal damage in the CCI-TBI paradigm in which the regional and temporal characteristics of calpain-mediated brain damage and neurodegeneration has been previously defined (Deng et al., 2007; Thompson et al., 2006). In other words, after defining the dose-response for MDL-28170 in terms of the ability of IV or IP dosing to generate brain tissue levels capable of showing *ex vivo* calpain inhibition, we used these doses and pharmacodynamic data concerning the duration of calpain inhibitory action to design a combined IV and IP dosing regimen that would maintain calpain inhibitory concentrations in brain tissue for either 3 or 7 h. We then compared the ability of these two different treatment durations to attenuate 24-h post-TBI α -spectrin degradation. The longer of the two treatment durations, but not the shorter one, significantly attenuated the levels of the calpain-specific 145-kDa α -spectrin fragment in both the injured cortex and hippocampus. However, while early initiation (15 min post-injury) of the 7-h-long MDL-28170 dosing regimen was able to decrease spectrin cleavage at its post-traumatic peak at 24 h, the effect was only partial (–44% in cortex and –40% in hippocampus; see Fig. 2B). This is actually consistent with the finding that even when MDL-28170 is administered in maximally effective calpain inhibitory doses in non-injured mice, the maximal *ex vivo* inhibition of calpain activity was also only partial (Fig. 1). The limit on calpain-inhibitory efficacy in non-injured mice, which is similar to that shown in pharmacodynamic studies in non-injured rats (Markgraf et al., 1998), may imply that MDL-28170 is not as brain-penetrable as previously believed. However, the quantitatively similar partial effect in the CCI-TBI model in which BBB disruption is known to occur progressively within the first post-injury hour (Smith et al., 1994), suggests that brain penetrability is unlikely to be the reason for the limited effect of MDL-28170 on post-traumatic calpain-mediated α -spectrin degradation. As an alternative explanation, it may be that the direct inhibition of calpain is simply not adequate to achieve complete suppression of calpain activity. However, neither of these possibilities can be dismissed or verified by studies with only one calpain inhibitor, since future calpain inhibitors with higher affinity, selectivity, and brain and cellular permeability may be found to be capable of a more complete cytoskeletal protective effect.

Targeting a downstream event in the pathological cascade such as calpain proteolytic activity should theoretically be associated with a clinically practical therapeutic window. While calpain-mediated damage to the cytoskeleton has been observed within the first minutes following experimental TBI, maximal proteolytic activity does not peak until 24 h (Deng et al., 2007; Newcomb et al., 1997; Posmantur et al., 1994, 1996, 1997; Thompson et al., 2006). An immunohistochemical time course analysis of α -spectrin degradation has shown that during the first minutes and hours, α -spectrin is selectively seen in the neuronal soma and dendrites. On the other hand, α -spectrin fragments are not seen in the axons until 24 h after injury (Newcomb et al., 1997). In the case of mild TBI, calpain-mediated cytoskeleton degradation can occur as late as several days (Saatman et al., 1996). However, protection of

neuronal cytoskeletal proteins by the longer of the two MDL-28170 dosing regimens was only significantly effective if drug administration was instituted within the first post-injury hour; a delay of 3 h produced an apparent, but not statistically significant, effect. Interpreting this in light of the immunohistochemical studies of Newcomb and colleagues (Newcomb et al., 1997), suggests that the window for reducing calpain-mediated cytoskeletal damage in the dendrites and soma is relatively short, whereas it may be longer for that occurring in the axons. This suggests that the partial efficacy of MDL-28170 treatment seen with initiation as early as 15 min post-injury may be because it is already too late to protect against the early calpain proteolytic damage done to the neuronal soma and dendrites, whereas axonal cytoskeletal protection has a longer window for successful calpain inhibitory intervention. In other words, the MDL-28170-attenuated α -spectrin degradation may be mainly occurring in the axons, whereas the non-attenuated portion may represent that occurring more rapidly in the soma and dendrites. The testing of this hypothesis requires careful immunohistochemical study at the ultrastructural level, with high-affinity antibodies that are selective for the calpain-generated α -spectrin fragments.

While MDL-28170 was able to decrease α -spectrin cleavage at 24 h (albeit only partially), the same dosing regimen failed to have any effect on the overall volume of post-traumatic neurodegeneration as measured by image analysis of the volume of hemispheric silver staining plus the cortical contusion volume at 48 h, the time point at which the histological damage has been shown to peak (Deng et al., 2007). Examination of the cortical contusion volume alone also failed to show an effect of MDL-28170 (data not shown). The simplest explanation may be that the degree of cytoskeletal protection afforded by MDL-28170 is inadequate to produce an observable decrease in lesion volume. Consistent with that possibility, other studies in our laboratory, in which we examined the cytoskeletal and histological protective effects of the antioxidant compound tempol in the same mouse CCI-TBI model, revealed that the partial attenuation of post-traumatic cortical α -spectrin degradation similar to that seen with MDL-28170 was also not associated with a significant reduction in neurodegeneration in the injured hemisphere. However, we did observe a significant 32% attenuation of cortical lesion volume with tempol treatment (Deng-Bryant et al., 2008). The most effective neuroprotective agents we have studied that are able to reduce lesion volume in the mouse CCI-TBI model are the mitochondrial permeability transition pore inhibitors cyclosporine A and NIM811, which significantly attenuated hemispheric silver staining volume by 36% and 38%, respectively (Mbye et al., 2009). However, the magnitude of their 24-h post-injury suppression of α -spectrin degradation was roughly similar to that seen with MDL-28170 or tempol. Thus the idea that the manifestation of a reduction in histological damage requires some threshold level of cytoskeletal protection to be achieved is not consistent with the available data. Indeed, it may be that post-traumatic cytoskeletal protection and attenuation of histological damage, while each representing a neuroprotective effect, may be somewhat independent actions. The fact is that none of the calpain inhibitors thus far studied in rodent TBI models has been convincingly shown to reduce cytoskeletal degradation in parallel with a reduction in

histological lesion volume. The only possible exception to this statement comes from the study of Posmantur and colleagues (Posmantur et al., 1997), of calpain inhibitor II in the rat CCI-TBI paradigm, in which a reduction in cortical lesion volume along with a decrease in α -spectrin degradation was reported. However, careful examination of that article reveals that the effect on cortical lesion volume was only seen in a single set of photomicrographs that showed the cortical contusion lesion in a single vehicle-treated animal versus that seen in a calpain inhibitor II-treated one. No quantitative data were included, implying that the purported effect on lesion size was not systematically investigated. Thus there is no convincing evidence that calpain inhibition by itself is able to decrease post-traumatic lesion volume. On the other hand, it has been shown that a reduction in brain necrotic lesion volume in controlled cortical contusion models is not a prerequisite for improvements in cognitive or motor recovery. The most notable example of this is seen with progesterone, which on the basis of extensive preclinical data obtained in the rat bilateral CCI-TBI model is now the subject of clinical trials in TBI patients. Progesterone has been shown to significantly improve spatial memory function (i.e., Morris water maze performance), without any effect on brain necrotic lesion volume (Goss et al., 2003).

Another quandary in the current overall body of published data concerning the neuroprotective effects of small-molecule calpain inhibitors in TBI models and their functional significance is the fact that two different calpain inhibitors, AK295 (Saatman et al., 2000) and SJA6017 (Kupina et al., 2001), have demonstrated improved post-traumatic motor recovery without any effect on cortical α -spectrin degradation or histological damage. Thus in those investigations, no neuroprotective correlate for the improved motor functional recovery seen was documented. So even though in the current study we took a more systematic pharmacological approach to studying one of the more promising calpain inhibitors, and added important insights into the topic of calpain inhibitor neuroprotection, much remains to be done to provide a clear picture of the translational potential of the calpain inhibitory strategy for acute TBI. Regarding MDL-28170 specifically, despite the lack of any effect on hemispheric or cortical lesion volume, the attenuation of cytoskeletal degradation no doubt represents a neuroprotective effect that is relevant to a reduction in axonal injury. Two other groups of TBI investigators have shown that MDL-28170 administration is able to reduce traumatic structural and/or functional damage to both corpus callosal axons following lateral fluid percussion TBI (Ai et al., 2007), and brainstem axons after diffuse impact-acceleration TBI (Buki et al., 2003). Whether these effects related to selective calpain inhibition are adequate to produce behavioral recovery worthy of clinical application requires further study. The fact that a reduction in histological damage has been demonstrated with other neuroprotective strategies such as mitochondrial protection with cyclosporine A (Mbye et al., 2009; Scheff and Sullivan, 1999; Sullivan et al., 2000a, 2000b), or its non-immunosuppressive analog NIM811 (Mbye et al., 2009), but not via calpain inhibition alone, strongly suggests that a combination of a mitochondrial protectant and a calpain inhibitor should be investigated in TBI models, to see if together they produce more robust neuroprotective and behavioral recovery-promoting effects than either agent used alone.

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Address correspondence to:

Edward D. Hall, Ph.D.

Spinal Cord & Brain Injury Research Center

University of Kentucky College of Medicine

741 S. Limestone Street, BBSRB Room 483

Lexington, KY 40536-0509

E-mail: edhall@uky.edu