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Six-Hour Window of Opportunity for Calpain Inhibition in Focal Cerebral Ischemia in Rats

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Background and Purpose—Stroke patients often experience a significant temporal delay between the onset of ischemia and the time to initiation of therapy. Thus, there is a need for neuroprotectants with a long therapeutic window of opportunity. The efficacy of a potent, central nervous system–penetrating calpain inhibitor (MDL 28,170) was evaluated in a temporary model of focal cerebral ischemia to determine the window of opportunity for intracellular protease inhibition.

Methods—An ex vivo brain protease inhibition assay established pharmacodynamic dosing parameters for MDL 28,170. Middle cerebral artery (MCA) occlusion was accomplished by advancing a monofilament through the internal carotid artery to the origin of the MCA. Postmortem infarct volumes were determined by quantitative image analysis of triphenyltetrazolium-stained brain sections.

Results—Maximal inhibition of brain protease activity was observed 30 minutes after injection of MDL 28,170 with an estimated pharmacodynamic half-life of 2 hours. MDL 28,170 caused a dose-dependent reduction in infarct volume when administered 30 minutes after MCA occlusion. A window of opportunity study was conducted to determine the maximal delay between the onset of ischemia and the initiation of efficacious therapy. MDL 28,170 reduced infarct volume when therapy was delayed for 0.5, 3, 4, and 6 hours after the initiation of ischemia. The protective effect of MDL 28,170 was lost after an 8-hour delay.

Conclusions—These data indicate that the therapeutic window of opportunity for calpain inhibition is at least 6 hours in a reversible focal cerebral ischemia model. This protection is observed despite the lethal hypoxic and excitotoxic challenge, suggesting that calpain activation may be an obligatory, downstream event in the ischemic cell death cascade. (Stroke. 1998;29:152-158.)

Key Words: calpain ■ calpain inhibitor ■ cerebral ischemia ■ focal neuroprotection

The clinically demonstrated efficacy of rTPA for thrombolytic stroke is an important breakthrough in the treatment of acute neurological disorders. However, approximately 40% of stroke patients currently do not reach medical personnel within the required 3-hour time window for TA, and patients with hemorrhage must be excluded before initiation of TPA therapy. Therefore, the time between the onset of the ischemic insult and the initiation of therapy is a critical success factor in treating ischemic stroke. It also follows that the longer a neuroprotective treatment can be delayed after ischemia and retain efficacy, the greater the number of patients that might benefit from treatment. These observations suggest a strategy for developing effective pharmacological therapies with broad spectrum clinical utility, which are based on the temporal profile of biochemical events that occur in the brain after an ischemic event.

In addition to hypoxic cell death, a massive release of glutamate occurs in the brain soon after ischemia causing additional excitotoxic cell death. Elevated glutamate triggers a large influx of calcium into neurons, which precipitates an intracellular cascade of events including the activation of calcium-dependent proteases such as calpain. Once activated, calpain cleaves structural and regulatory proteins in the cell, leading to neuronal death.

Pharmacologically targeting a “downstream” event in the cascade, like activated calpain, could theoretically prolong the time for initiation of therapy while retaining efficacy. Due to the technical difficulty of accurately measuring the activation state of calpain in vivo, experiments designed to examine the time course of calpain activity often rely on measurements of the BDPs of one of calpain’s preferred substrates—spectrin. In an animal model of focal cerebral ischemia, spectrin BDPs were detected 1 hour after MCAo in both the striatum and neocortex of the ischemic hemisphere, increasing dramatically until 12 hours posts ischemia.

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spectrin BDPs were detected in the core of the ischemic area at 2 hours postocclusion; and at 3.5 hours post-MCAo, they were present in both the core and penumbral regions. Previous work has indicated that administration of a calpain inhibitor in models of focal and global ischemia decreases ischemic damage, suggesting that calpain inhibition is a viable approach for neuroprotective therapy. However, the lack of a potent, selective calpain inhibitor that rapidly penetrates the CNS has limited the pharmacological studies. For example, administration of AK275 by supracortical perfusion produced significant neuroprotection at 1-hour and 3-hour time points postocclusion, but not at 4 hours. These authors speculated that the therapeutic window for calpain inhibition may be longer than 3 hours due to the drug’s slow rate of perfusion to the ischemic areas of the brain.

Thus, pharmacological evidence supports the utility of a therapeutic approach targeting calpain inhibition in the injured brain. Furthermore, biochemical evidence suggests that calpain activity is a downstream event in the lethal cascade following stroke. These observations, taken together with the data showing that the therapeutic window for calpain inhibition may exceed 3 hours, prompted a systematic investigation of the therapeutic window of opportunity for a rapidly CNS-penetrating calpain inhibitor, MDL 28,170 (Fig 1). First, a pharmacodynamic profile of the blood-brain barrier penetration of MDL 28,170 was established in naive animals. Second, the optimally efficacious dose for MDL 28,170 was determined in a rodent model of ischemia-reperfusion. This information was used to evaluate the maximal window of therapeutic efficacy for calpain inhibition by delaying the onset of pharmacotherapy with MDL 28,170 for up to 8 hours after the initiation of the infarction.

Materials and Methods

Study Design

Experiment 1: Time Course of Proteinase Inhibition

Naive rats were used to determine a pharmacodynamic time course for blood-brain barrier penetration of MDL 28,170 after a single 30 mg/kg intravenous bolus injection. MDL 28,170’s ability to inhibit proteinases in the brain was examined at four time points: 0.5, 1, 2, and 4 hours postinjection (n=6 rats per time point), and compared with vehicle-injected group (n=6).

Experiment 2: Optimal Dose of MDL 28,170 for Neuroprotection

The optimal neuroprotective dose for MDL 28,170 was determined in a temporary focal cerebral ischemia model. MDL 28,170 was administered 30 minutes after initiation of ischemia by intravenous administration. Infarct volume was determined 24 hours after the occlusion. For each dose group, a vehicle control group was run (n=10 to 12 per group).

Experiment 3: Therapeutic Window of Efficacy Using MDL 28,170

Using the optimal dose determined in Experiments 1 and 2, the delay between initiation of ischemia and administration of MDL 28,170 was systematically increased to 0.5, 3, 4, 6, and 8 hours posts ischemia. Infarct volume was determined 24 hours after the occlusion. Each time point group was compared with a matched vehicle-treated control group (n=10 to 15 per group) as in the previous experiment.

Subjects

For the ischemia studies, male Wistar rats were obtained from Charles River Laboratories (Wilmington, Mass), and the weight range was regulated between 270 and 300 g. Rats were acclimated to the vivarium for 1 week before surgical preparation. They were maintained on a 12-hours light/dark schedule with free access to food and water. All procedures followed in the present studies were in accordance with institutional guidelines.

Proteinase Inhibition Assay

Awake male Fischer rats (Charles River Laboratories, Wilmington, Mass) from 75 to 125 g were given a single intravenous bolus injection of MDL 28,170 or vehicle into the tail vein and killed by decapitation at indicated times after the injection. The brain was immediately removed and the supratentorial region rapidly frozen in liquid nitrogen and stored at -70°C until analysis. Frozen brains were weighed, thawed, and homogenized on ice at 0.25 g tissue/mL buffer (50 mmol/L Tris, pH 8.0, containing 2.0 mmol/L EDTA and 1 mmol/L phenylmethylsulfonyl fluoride). Homogenates were centrifuged at 100 000 × g for 60 minutes at 4°C, and supernatants were collected and stored on ice for enzyme assay. Supernatants were assayed for protease activity at 25°C in 3.0 mL of 100 mmol/L MOPS (pH 7.5) containing 100 mmol/L KCl, 5 mmol/L CaCl2, 5 mmol/L dithiothreitol, 50 mmol/L substrate (N-t-boc-val-leu-lys-7-amido-4-methyl coumarin) using 30 μL of brain supernatant from each sample. The increase in fluorescence was monitored continuously using a SLM SPF 500 spectrofluorometer (excitation 381 nm, emission 460 nm). The initial reaction rates were determined within the first 120 seconds, and these data were analyzed by least-squares fit and reported as relative fluorescence units per second. Reaction rates were normalized to milligrams of soluble protein using the Bradford protein assay.

Focal Cerebral Ischemia

Focal ischemia-reperfusion was produced by a modification of the monofilament method described by Zee Longa et al.11 Rats were anesthetized with halothane in N2O/O2 and were allowed to breathe spontaneously during the brief procedure. A ventral neck incision was made and an indwelling intravenous catheter of PE 50 tubing (Clay Adams) was placed in the left jugular vein and exteriorized through the back of the neck. A short length of the catheter (30 cm) protruded and was capped. The left ECA and ICA were exposed and carefully isolated. After placement of two retaining sutures around the ECA, a permanent ligature was placed distally on the ECA and ICA, and was capped. The left ECA and ICA were exposed and carefully isolated. After placement of two retaining sutures around the ECA, a permanent ligature was placed distally on the ECA, and a small arteriotomy was made between the retaining sutures. A 40-mm length of 3–0 nylon monofilament (Look Inc), its tip rounded by flame-heating, was inserted into the ECA and advanced into the ICA with minimal stretching and pulling of the vessels. The monofilament was inserted 30 minutes after initiation of ischemia by intravenous administration. Infarct volume was determined 24 hours after the occlusion. For each dose group, a vehicle control group was run (n=10 to 12 per group).

Selected Abbreviations and Acronyms

BDPs = breakdown products
CCA = common carotid artery
CNS = central nervous system
ECA = external carotid artery
ICA = internal carotid artery
MCAo = middle cerebral artery occlusion
NMDA = N-methyl-D-aspartate
rTPA = recombinant tissue plasminogen activator
TTC = triphenyltetrazolium hydrochloride

![Figure 1. Chemical structure of MDL 28,170 (carbobenzylxoxy-Val-Phe-H).](image)
advanced intracranially to occlude the origin of the MCA and was secured with an additional tie on the ICA. Correct placement of the monofilament was established when resistance was felt after the monofilament had been inserted at least 18 mm from the CCA/ICA bifurcation. The neck wound was then closed and the animal was fitted with a harness, tether, and swivel apparatus (Stoelting Inc) to allow continuous intravenous infusion on awakening. The MCA was occluded for a period of 180 minutes, after which time the rat was reanesthetized and the monofilament was retracted to the bifurcation of the ICA and CCA. The incision was reclosed and the animal was returned to the infusion chamber.

**Histology and Image Analysis**
Any animal that died before the 24-hour time point was excluded from the study. Twenty-four hours after MCA occlusion, each rat was weighed and decapitated and then the brain removed. Using a brain block, 6 sections, each 2 mm thick, were cut and incubated in 2% TTC for 30 minutes. After incubation, slices were transferred to 10% formalin. Image analysis was performed by a single experimenter (P.A.C.), who was blinded to the experimental treatment group. Within 72 hours, each slice was photographed using Polaroid PolaChrome 35-mm instant color slide film (Polaroid Corp). These photographic images were digitized and used to determine the area of infarct and the area of each hemisphere for each slice on a Compix system computer (C Imaging 1280 System; Compix Inc Image Systems). Infarct volume was derived by the integration of the area measurements.

**Drug Administration**
MDL 28,170 was dissolved in PEG 300/EtOH (9:1). For the proteinase inhibition study, a 30 mg/kg bolus was administered via the tail vein at the times indicated above. For the ischemia studies, MDL 28,170 was administered via the previously placed indwelling catheter as a slow bolus injection given by hand, followed by a continuous infusion delivered by an external pump (KDS Scientific) at the times and doses indicated in the Table.

**Statistical Analyses**
Group data are expressed as mean±SEM. For the two focal ischemia studies, infarct volume and weight loss were each compared among groups using one-way ANOVA, followed by orthogonal contrast analysis (ischemia studies) or Bonferroni method (ex vivo studies) for post hoc comparisons, as appropriate.

**Results**

**Time Course of Proteinase Inhibition**
A pharmacodynamic assay was developed to assist in defining dosing parameters for MDL 28,170 in the ischemia models. This assay allowed estimation of the onset and duration of enzyme inhibition in the brain after a single intravenous bolus injection of MDL 28,170. It also required the compound to penetrate barriers associated with the blood–brain barrier and the cell wall, as well as to inhibit the proteases responsible for substrate degradation.

As shown in Fig 2, intravenous administration of MDL 28,170 produced an inhibition of protease activity in the brain at the earliest time point measured (30 minutes). Activity declined over 4 hours, with an estimated $t_{1/2}$ duration of effect in the brain of 2 hours. No deaths were seen in any of the MDL 28,170– or vehicle-treated groups. Enzyme inhibition did not result from residual MDL 28,170 in the circulation, because a preliminary experiment revealed no differences between acutely excised brains and brains from animals that had been transcardially perfused with saline before excision (data not shown).

**Optimal Dose of MDL 28,170 for Neuroprotection**
To determine the optimally efficacious dose for MDL 28,170 in a temporary ischemia model, experiments were conducted with the compound administration beginning 30 minutes after the initiation of ischemia. Each group of MDL 28,170–treated animals was matched to a corresponding vehicle control group that was run in parallel. To analyze the results of these experiments, the average infarct volume of the vehicle control group was determined, and the infarcts from the drug-treated animals are expressed as a percentage of the average infarct volume for its matched control.

The dose-response experiments were designed to evaluate the bolus dose and infusion dose separately. To optimize the
bolus dose, the infusion was held constant at 3.33 mg/kg per hour×6 hours as determined from the above experiments, and the bolus dose was varied from 3 to 20 mg/kg (Fig 3). A one-way ANOVA indicated a significant effect of group and a post-hoc orthogonal contrast analysis revealed that the 10 and 20 mg/kg dose groups differed significantly from the vehicle group (P<.05), whereas the lower dose group did not. The statistical analysis also indicated that the magnitude of the protection observed in the 10 and 20 mg/kg bolus groups was not different. Thus, a bolus dose of 10 or 20 mg/kg was equally efficacious when followed by an infusion of 3.33 mg/kg per hour×6 hours, reducing infarct volume by 68±15% (n=12) and 43±14% (n=12), respectively (Fig 4).

Next, the infusion dose was varied while holding the bolus dose constant at 20 mg/kg (Fig 5). A one-way ANOVA indicated significant differences between groups and post-hoc orthogonal contrast analysis revealed that the medium and high infusion dose groups differed significantly from the vehicle group (P<.05), while the low dose group did not. The infusion doses of 3.33 and 6.67 mg/kg per hour were equally efficacious when preceded by a 20 mg/kg bolus dose, reducing infarct volume by 60±10% (n=12) and 44±17% (n=10), respectively. The lowest dose (1.67 mg/kg per hour) was ineffective in reducing infarct volume (3±18%, n=12). Therefore, the minimally effective dose for neuroprotection for MDL 28,170 was determined to be a bolus of 10 mg/kg followed by an infusion of 3.33 mg/kg per hour×6 hours for a cumulative dose of 30 mg/kg. Statistical analysis of this dosing study did not reveal any groups exhibiting partial efficacy. Therefore, the minimally effective dose determined in these studies also provides maximal efficacy.

**Therapeutic Window of Efficacy Using MDL 28,170**

The dose response analysis at 30 minutes postischemia revealed the importance of both the bolus dose and the infusion dose. Therefore, a conservative dose of 20 mg/kg plus 3.33 mg/kg per hour×6 hours was selected to determine the therapeutic window of opportunity for MDL 28,170 in the 3-hour ischemia model. As seen in Fig 6, the administration of MDL 28,170 can be delayed for 6 hours after the initiation of ischemia (ie, 3 hours after reperfusion) and still significantly reduce infarct volume at 24 hours. Data analysis using one-way ANOVA revealed a significant effect of group, and post-hoc comparisons using orthogonal contrast indicated that initiating dosing at 0.5, 3, 4, and 6 hours postischemia was similarly effective in reducing infarct volume, yielding neuroprotection of 64±9% (n=12), 80±9% (n=12), 67±12% (n=9), and 46±15% (n=11) of vehicle infarct volume, respectively. However, administration starting 8 hours after ischemia failed to significantly reduce infarct volume (11±26%, n=13). Mortality rates between vehicle- and drug-treated groups averaged 20.1±2.5% and 24.6±3.1%, respectively, and these were not statistically different (unpaired Student’s t test, P>2). The individual data points for all sections from each group in the window of opportunity study are shown in Fig 7.

**Discussion**

The present results systematically define the therapeutic window of opportunity for a CNS-penetrating calpain inhibitor...
The 6-hour window of opportunity for calpain inhibition has significant implications regarding the mechanism of ischemic cell death. Ischemic neuronal death can be arbitrarily divided into three phases: initiation, propagation, and commitment to cell death, with hypoxia and excitotoxicity being the major components of the initiation phase. The excitotoxic cell death is initiated by the hypoxia but it is independent of the hypoxia since NMDA-receptor antagonists reduce the volume of the infarct despite the hypoxic insult. Our studies suggest that calpain is most likely involved in the propagation phase. This propagation phase is initiated by hypoxia and excitotoxicity, but also appears to proceed independently of these two events since calpain inhibition is neuroprotective despite the hypoxic and excitotoxic insults. In support of this suggestion, it has been observed that calpain inhibitors protect neurons from several types of hypoxic and excitotoxic cell death in vitro. For example, MDL 28,170 has been shown to improve posthypoxic recovery in neuronal slice preparations14–16 and to protect cultured neurons from NMDA, AMPA, and kainate toxicity.17–19 The possibility that the calpain inhibitor–mediated neuroprotection might involve the inhibition of apoptotic processes has been suggested,20–25 but remains to be proven in ischemic cell death.

The ability to clearly define the role of calpain in ischemic cell death requires a specific calpain inhibitor that readily penetrates cells and the blood–brain barrier. Several commercially available inhibitors (including calpain inhibitors I and II, E64, and leupeptin) do not readily cross cell walls26 or likely the blood–brain barrier. The ex vivo assays used in these experiments provided a convenient system to measure the brain penetration of MDL 28,170 and its association with the active site of brain proteinases. Previously, results determined that MDL 28,170 provides dose dependent inhibition of brain protease activity at 1 hour postdosing in this assay, beginning at 10 mg/kg (M.D.L., J.R.K., S.M., unpublished observations, 1997). The present experiments demonstrate that MDL 28,170 rapidly crosses diffusion barriers associated with the brain and the cell wall to get to the enzyme active site. Maximal activity was noted at the first time point examined (30 minutes), and the half-life of activity was approximately 2 hours. This pharmacodynamic analysis correlates well with our pharmacokinetic analysis indicating a plasma half-life between 1 and 2 hours (data not shown).

The ex vivo brain penetration assay provides a valuable tool to define the pharmacodynamic properties of compounds that meet the kinetic requirements for this assay. It is especially useful for intracellular targets that are protected by both the blood–brain barrier and the cell wall barrier. Alternative analytical methods for measuring brain penetration, like high-performance liquid chromatography analysis of brain extracts or in vivo microdialysis of the extracellular space, are not capable of directly measuring intracellular concentrations of an active compound at the enzyme active site.

There are experimental factors that should be considered when interpreting the results of the ex vivo brain penetration assays. First, the assay is only effective for compounds that have a slow dissociation rate from the enzyme, since the homogenization and centrifugation steps provide opportunity for this dissociation to occur. This also means that one cannot determine absolute values for brain enzyme inhibition, but only relative rates compared with vehicle-treated controls. The second consideration is that this assay is not specific for calpain, since other proteases that target this substrate can contribute to the fluorescent signal. We believe that a significant amount of the activity is related to calpain because the protease activity in the brain extract is almost completely inhibited by an excess of MDL 28,170, and the peak of activity coelutes with authentic calpain (J.R.K., S.M., unpublished observation, 1997). How-
ever, other enzymes in the brain extracts likely contribute to the substrate hydrolysis and substantial experimentation will be required to fully define the calpain component of this homogenate. Finally, it should be noted that the Fischer rats used in the ex vivo studies were smaller than the Wistar rats used in the ischemia studies. Therefore, there could be pharmacokinetic differences in brain penetration due to strain and weight.

In addition to its potential therapeutic utility, MDL 28,170 is a useful pharmacological tool because of its potent calpain inhibition and relative specificity for this enzyme. The compound has very little CNS receptor–binding activity as established by a commercially available receptor screening service that demonstrated greater than 100-fold selectivity against 56 different receptors, including the family of excitatory amino acid receptors (PanLabs receptor screen, data not shown). Therefore, the pharmacology of MDL 28,170 predicts that there would be an absence of NMDA receptor–mediated side effects. It has also proven to be a predictable compound across several models. While the Kᵢ of the compound is 10 nmol/L, it required an intravenous dose of 10 mg/kg to give significant inhibition of brain protease activity at 1 hour after injection. This dose was similar to the minimally effective infusion dose in the stroke studies and matched the minimally effective bolus dose. This dose was similar to the minimally effective infusion dose in the stroke studies and matched the minimally effective bolus dose. Hong et al16 used a multiple bolus dosing paradigm and observed protection with a cumulative dose of 30 mg/kg in a focal ischemia model indicating that the effective dose range of MDL 28,170 is in the low mg/kg range.

One caveat to the specificity of MDL 28,170 is activity against cathepsin B. It has been synthetically difficult to separate cathepsin B activity from calpain activity, although MDL 28,170 does provide a 2.5-fold separation for these enzymes (Kᵢ for MDL 28,170=25 and 10 nmol/L for cathepsin B and calpain, respectively). Both enzymes share a very similar catalytic domain, and peptide aldehydes that are active against calpain are also active against cathepsin B. However, no clear role for lysosomal cathepsin B has been established in ischemic neuronal death.27 The activity of MDL 28,170 has also been evaluated against several additional enzymes using spectrophometric or spectrophotometric methods. The compound exhibits >100-fold specificity for calpain relative to trypsin, plasmin, kallikrein, α-chymotrypsin, caspase-1 (interleukin-1β convertase), cathepsin D, cathepsin H, HIV protease, TPA, angiotensin–converting enzyme, nitric oxide synthetase, epidermal growth factor receptor kinase, calcineurin phosphatase, and protein kinase C.

The current experiments suggest additional experimentation in two areas to fully understand the magnitude of calpain inhibitor–mediated neuroprotection. First, the phenomenon of delayed neurodegeneration is well established in global ischemia models,28 and has been reported recently in a focal ischemia model.29 Therefore, it is possible that calpain inhibition may delay death in those neurons that have been irreparably damaged. Second, the TTC method for measuring infarct volume is a macrosopic method that does not permit the evaluation of cell death on a microscopic scale. Even conventional histological and immunohistochemical detection methods can lack the sensitivity necessary to detect subtle ischemic neuronal death.30 Thus, there may have been scattered cell death in the drug-treated animals that was not within the detection limits of the TTC staining. However, the TTC method provides a quantitative determination of ischemic cell death that is identical to results obtained with conventional histological methods (ie, hematoxylin and eosin) when used within 48 hours of the ischemic challenge.31–33 Although the present data clearly demonstrate the neuroprotective effect of MDL 28,170, an evaluation of isolated, scattered cell death or delayed cell death will require a more detailed histological analysis. The therapeutic potential of calpain inhibitors is not confined to ischemic neuronal damage because calpain has been implicated in several disorders in which tissue destruction and/or protease activation contributes to the pathology of the disease. For example, inappropriate or excessive calpain activation has been implicated in cataracts, myocardial ischemia, muscular dystrophy, Alzheimer’s disease, and platelet aggregation.34 The present observations strongly suggest that an appropriately designed calpain inhibitor may be a useful therapeutic agent, especially in the treatment of acute stroke.

References

Editorial Comment

Determination of the therapeutic window in ischemic brain injury is of great importance because of excessive delays commonly experienced between stroke onset and therapeutic intervention. If the entire ischemic region evolved into infarction within 1 to 2 hours, there would be little opportunity to successfully intervene.1 The therapeutic window for reperfusion alone to reverse lethal injury in the penumbral zone appears to be about 3 to 4 hours.2

It is commonly believed that cytoprotective therapy in combination with reperfusion will salvage more tissue than either therapy alone. However, when considering the complex cascade of events that eventually precipitates cell death, it is reasonable to think that the therapeutic window for pharmacological reversal of cytotoxic events will not be the same for all cytoprotective agents. For example, a 2-hour time window has been demonstrated for an NMDA antagonist,3 and a 3-hour window exists for basic fibroblast growth factor.4

Remarkably, in the present study Markgraf and coworkers demonstrated a 6-hour therapeutic window for calpain inhibition in a rat model of focal stroke. Although MDL 28,170 is a peptide, it readily penetrated the brain and inhibited calpain activity. Because previously, potent, selective, calpain inhibitors that rapidly penetrated the brain were unavailable, the therapeutic window for calpain inhibition was unclear. The major pharmacological activity of MDL 28,170 appeared to be exerted through inhibition of calpain; however, it is possible that other enzymes involved in ischemic cell death may have been inhibited as well. One of the most important features of these findings is the potential clinical value of using calpain inhibitors to treat cerebral ischemia. The expanded time window may prove to be of significant therapeutic benefit.

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