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A Selective Cathepsin B/L Inhibitor Improves Recovery after Experimental Intracerebral Hemorrhage

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Abstract

Object—This study investigates a potential novel application of a selective cathepsin B and L inhibitor in experimental intracerebral hemorrhage (ICH) in rats.

Methods—Adult male Wistar rats ($n = 40$) received an ICH by stereotactic injection of 100 μ L of autologous blood or sham via needle insertion into the right striatum. The rats were treated with a selective cathepsin B/L inhibitor (CP-1) or sterile saline intravenously at 2 and 4 hours after injury. Modified neurological severity score (mNSS) and corner turn tests were performed at 1, 4, 7, and 14 days after ICH. The rats were sacrificed at 3 and 14 days post-ICH for immunohistology to analyze tissue loss, neurogenesis, angiogenesis, and apoptosis.

Results—CP-1-treated animals demonstrated significantly reduced apoptosis as well as tissue loss compared to controls ($p < 0.05$ for each). Neurological function as assessed by mNSS and corner turn tests showed improvement after CP-1 treatment at 7 and 14 days ($p < 0.05$). Angiogenesis and neurogenesis parameters demonstrated improvement after CP-1 treatment compared to controls ($p < 0.05$) at 14 days.

Conclusion—This study is the first report of treatment of ICH with a selective cathepsin B/L inhibitor. Cathepsin B/L inhibition has been shown to be beneficial after cerebral ischemia, likely by its upstream regulation of the other prominent cysteine proteases, calpains and caspases. While ICH may not induce a major component of ischemia, the cellular stress in the border zone may activate these proteolytic pathways. The observation that cathepsin B/L blockage is efficacious in this model is provocative for further investigation.

Keywords

Intracerebral hemorrhage; cysteine protease inhibitors; cathepsin B; cathepsin L

Spontaneous intracerebral hemorrhage (ICH) represents 10–15% of all strokes, and it exerts a much worse prognosis than injury caused by ischemic stroke.⁹ The size of the hematoma formed in the brain parenchyma from the rupture of a cerebral vessel is a significant predictor of poor outcome.⁴⁰ Inflammation and toxicity triggered by extravascular blood products increase the cerebral damage observed in the early phases after hematoma

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Disclosure

The authors do not report any conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

formation and also lead to worsening edema around the clot.⁴¹ Effective surgical and drug approaches for clinical practice have been elusive.^{9,10,14}

Cathepsins B and L are intracellular/lysosomal and secreted cysteine proteases that have been implicated in the process of neuronal cell death after experimental global and focal cerebral ischemia and after experimental spinal cord injury.^{1,6,11,16,17,34,38} The levels of these cathepsins are significantly increased and relocate from the lysosomes to the cytoplasm following global cerebral ischemia.. Cathepsin B is one of the causative factors of microglia-induced neuronal apoptosis.^{32,34} In our previous study, CP-1, a non-toxic cysteine protease inhibitor which is selective for cathepsins B and L, but not the calpains or caspases, was shown to be effective at reducing infarct volume and improving functional scores when administered intravenously to rats after 2 hours of middle cerebral artery occlusion (MCAO) and reperfusion.³⁵

The role of cathepsins in ICH has not been studied. However, results from models of cerebral ischemia suggest that inhibition of cathepsin B and L may be efficacious in preventing neuronal cell death. In this study, we investigated the ability of CP-1 to promote functional and histological recovery after ICH.

Material and Methods

Animal Surgical Procedures

All studies were approved by the Institutional Animal Care and Use Committee. 40 male Wistar rats (Charles River; Wilmington, MA) weighing between 270 and 320 g were selected for this study. An ICH lesion was induced by injecting 100 μ l of nonheparinized autologous blood, which was obtained from the femoral vein and placed into a 1cc syringe with a 26½G needle, into the right striatum (3.5 mm lateral to midline, 0.5 mm anterior to bregma, depth 5.5 mm below the surface to midline) at a steady infusion rate of 10 μ l per minute. Sham-injured animals were subjected to the same manipulations as ICH rats but no blood was injected.

Cysteine protease inhibitors

CP-1, Cbz-Phe-Ser(OBzl)-CHN₂ (Cbz, carbobenoxyl; OBzl, O-benzyl), is lipophilic and its molecular mass is 500.5. This inhibitor is chosen based on its relative selectivity for cathepsins B and L. The compound was synthesized and purified according to the method of Shaw et al.³⁶ After synthesis, CP-1 was chromatographed on silica gel with chloroform/methanol (49:1, v/v) and recrystallized from boiling ethyl acetate (mp 125–126 °C). The purity of the compound was checked by High-performance liquid chromatography (HPLC), Thin-layer chromatography (TLC), elemental analysis, infrared spectroscopy, proton nuclear magnetic resonance (¹H NMR) and mass spectrometry.⁴ The effectiveness of CP-1 *in vitro* was determined by protease inhibition kinetic measurements on purified cathepsin B, cathepsin L, calpain 1 and calpain 2 as described previously.^{2,3,7,8}

The dosing of CP-1 after experimental ICH was based on *in vivo* dose-response studies that we had previously performed in Wistar rats subjected to focal cerebral ischemia.^{3,35} CP-1 at 0.35 mg/kg significantly reduced cathepsin B activity in the brain *in vivo*.¹ In the current study, CP-1 was initially dissolved in 100% dimethyl sulfoxide (DMSO), and was blended into a serial dilution of 0.1 mM in 1% DMSO in sterile saline. CP-1 was given intravenously (0.175 mg/kg) at 2 and 4 hours after surgery (total dosage =0.35 mg/kg, while control animals received a same volume infusion of 1% DMSO in sterile saline.

Experimental Groups

This study was randomly divided into four groups: 1) CP-1 group: ICH rats (n = 16) were treated with the selective cathepsin B/L inhibitor; 2) Control group: ICH rats (n = 16) were treated with 1% DMSO in sterile saline; 3) Sham-CP-1 group: Sham rats (n = 4) were treated with CP-1; and 4) Sham-control group: Sham rats (n = 4) were treated with sterile saline. Six rats in the first group and six rats in the second group were killed at Day 3 for TUNNEL staining. The rest of the animals received daily injections of bromodeoxyuridine (BrdU) 100 mg/kg (Sigma, St. Louis, MO) intraperitoneally starting at 24 hours after injury and subsequently for 13 consecutive days. These rats were evaluated for neurological function as described below. After 14 days post-ICH or post-sham procedure the rats from groups 1–4 were sacrificed for immunohistology.

Neurological Functional Studies

Functional outcomes were measured using both the modified neurological severity score (mNSS)¹² and the corner turn test⁴³ by an observer blinded to the individual treatment status of the animals. Functional outcomes for each rat were tested at Days 1, 7 and 14 after ICH. The mNSS is used to assess motor, sensory, balance and reflex skills, with higher scores implying greater neurological injury, while the corner turn test measures the number of times that an animal turns to the right or left when placed in a corner.

Histology and Immunohistochemistry

At 14 days postoperatively, the animals were anesthetized with an intraperitoneal injection of ketamine and xylazine and subsequently perfused transcardially with PBS followed by 4% paraformaldehyde. Brain tissues were excised, fixed in formalin and sliced into a series of 2-mm-thick blocks. Each block was processed and embedded in paraffin. A series of adjacent 6- μ m-thick coronal sections were cut with a microtome from each block. A total of 7 sections from each animal were processed and stained with hematoxylin and eosin (H&E). These sections were tracked using an image analysis system (Data Translation, Marlboro, MA). The intact striatal area of the ipsilateral hemisphere was subtracted from the striatal area of the contralateral hemisphere, and the tissue loss volume was presented as a volume percentage of the lesion compared with the contralateral striatum.

The brain tissue residing between +0.1 – 0.86 mm of the bregma on the third block was the most severely injured and therefore every 40th coronal section from the third block for a total of 6 sections was specifically selected for immunohistochemical staining with the same antibody. Immunohistochemical staining was used for BrdU (1:100; Boehringer Mannheim, Indianapolis, IN), TUJ1 (1:5,000; ABCO, Berkeley, CA), Synaptophysin (1:1000; Millipore, Billerica, MA), and vWF (1:400, Dako, Carpinteria, Calif), as previously described.^{13,27} Double staining was performed to colocalize the BrdU-reactive cells expressing TUJ1 or VWF, as previously described.^{13,27} All immunostainings were performed at the same time with the omission of primary antibody and the use of pre-immune serum for quality control of the immunoassay procedure. To identify cellular apoptosis, terminal deoxynucleotidyl nick-end labeling (TUNEL) was performed using an ApopTag peroxidase in situ apoptosis detection Kit (Millipore, Billerica, MA).

Quantification—For semiquantitative measurements, all slides were digitized under a 20 $\times$ objective lens (Olympus BX40; Olympus Optical Co, Tokyo, Japan) by using a 3D-CCD color video camera (model DXC-970MD; Sony Corp, Tokyo, Japan) interfaced with an image analysis system (Imaging Research, Inc., St. Catharine's, ON, Canada). The numbers of BrdU-positive cells and the percentage of TUJ1-positive signals were counted in the SVZ; the percentage of synaptophysin-positive signals compared with the corresponding contralateral side and the numbers of apoptosis cells were calculated in the subcortical

striatum; and the perimeters of vWF positive vessels were measured in the peri-hemorrhagic striatum. The cells with BrdU (brown stained) that colocalized to the nucleus (hematoxylin stained) were counted as BrdU-positive cells, and the cells stained with Tunnel that showed dark brown, rounded or oval apoptotic bodies were counted as Tunnel-positive cells.

Statistical Analysis

Statistical analysis of neurological functional scores, areas of ICH-related tissue losses, and immunohistochemical results were all performed using the independent Student t-test. Data were presented as the mean $\pm$ standard error, and p values < 0.05 were considered significant. All measurements were performed by observers blinded to individual treatments.

Results

Neurobehavioral tests

Figure 1 presented the results for the cornering and mNSS tests. All ICH rats displayed similar and marked neurological impairments at Day 1 after injury compared to the sham-operated groups. Before the first week post-ICH there were still no significant differences observed between the control group and CP-1 group. At 1 and 2 weeks post-ictus cornering scores demonstrated that the CP-1 group improved significantly compared to the control group. Similarly, improvement of neurological function assessed using mNSS was significant at 1 and 2 weeks post-ICH compared to the control group; however, no significant differences were observed in mNSS and corner turn test results between the sham-control group and sham-CP1 group during the two weeks of experiments.

Tissue loss measurements

Using this model of ICH, we have demonstrated consistent results measured by MRI and immunohistological methods. After breakdown or resorption of the hematoma, there is an area of tissue loss in this model.^{24,33} The areas of striatal tissue loss as a percentage of the normal contralateral side obtained from H&E histology were as follows: sham-control group, $1.56 \pm 0.85\%$; sham-CP-1 group, $1.48 \pm 1.09\%$; control group, $30.67 \pm 2.46\%$; and CP-1 group, $23.22 \pm 1.61\%$ (Fig. 4). Statistically, the CP-1 group showed significantly reduced tissue loss compared to control group at 2 weeks after ICH.

Cell Proliferation and Differentiation

Endogenous cell proliferation and differentiation are believed to contribute to neurological functional recovery after ICH. To test if post-ICH treatment with CP-1 may have an effect on this process, BrdU and TUJ1 immunostainings were performed. The ipsilateral subventricular zone (SVZ) demonstrated significant increase in the number of newly formed cells in the CP-1-treated groups at 2 weeks post-ICH, compared to the control group ($p < 0.05$) (Fig. 2). CP-1 treatment similarly significantly enhanced the TUJ1 expression over the control group. A small number of BrdU-positive cells were colabeled with TUJ1, suggesting that only limited, newly formed cells represent neurogenesis. In each hemispheric section of the sham-operated rats and in the contralateral hemisphere of ICH rats, there were limited positive signals for BrdU and TUJ1 stainings, and no significant difference between the two sham groups.

Angiogenesis

Angiogenesis is described as the sprouting of new blood vessels from pre-existing vessels. Cerebral vascular perimeters (e.g., von Willibrand factor) were significantly increased in the CP1 treatment group compared with the control group ($p < 0.05$) (Fig. 3). Double immunostaining revealed a subpopulation of cells that express a vascular marker while still

proliferating, suggesting that this subpopulation of cells positive for vascular phenotype is newly formed during the recovery stage.

Apoptosis

TUNEL staining showed apoptotic cells with typical dark brown, rounded or oval apoptotic bodies (Fig. 4). Scattered apoptotic cells were present throughout the damaged tissue, the vast majority of which were located along the boundary zone of the hematoma. The number of apoptotic cells is significantly reduced in the ipsilateral hemisphere in the CP-1 treatment groups compared to the control group ($p < 0.05$).

Discussion

Accumulating evidence has suggested a prominent role for cathepsins B and L in the pathogenesis of brain ischemia.^{1,22,25,30,35} Cathepsins B and L have been shown to be upregulated in microvessels and adjacent glial cells or neurons after brain ischemia.¹⁸ Broad specificity cysteine protease inhibitors and selective cathepsins B and L inhibitors have been shown to promote neurological recovery following stroke.^{35,39} While ICH may not have a major component of ischemia, the cellular stress along the border zone may activate these proteolytic pathways. The findings from our current study suggest that early treatment with CP-1 after ICH improves neurological function and reduces tissue loss, supporting the hypothesis that cathepsins B and L play an important role in ICH.

The underlying damaging effects of cathepsins following brain injury are partially associated with apoptosis and necrotic cell death.^{34,42} Microglial-secreted cathepsin B induces neuronal apoptosis and activates the proinflammatory caspases 11 and 1.^{19,25,32} Furthermore, cathepsin B can cleave the Bcl-2 family member Bid, which may lead to cytochrome-c release from the mitochondria and subsequent caspase activation.²¹ Cathepsin L is also implicated in apoptosis.²⁶ In line with these findings, the selective inhibitor for cathepsins B/L reduced the number of apoptotic cells at 2 weeks post-ICH, which may contribute to neurological recovery. Regarding necrotic cell death, we described previously that cathepsin B/L inhibition reduces loss of tissue after cerebral ischemia: one of the postulated mechanisms being reduction of the lysosome-mediated cell destruction.³⁴ In the cerebral hemorrhage model 10–20% of cell death is due to necrosis from direct injury from the clot. This suggests that CP-1 may improve post ICH recovery by interfering with the cathepsin B/L-induced apoptosis as well as the necrotic cell death pathway.^{10,20,41}

Neurogenesis has been demonstrated in animal models and adult human brain after ICH,^{5,13} and this may contribute to brain repair and functional recovery^{13,44} either by cell replacement or by indirect mechanisms such as cytoprotective or trophic factors. Inflammatory processes, which also play a vital role in secondary brain injury following the initial hematoma post ICH, may block neurogenesis.^{15,29} Cathepsins represent one of the important factors involved in necrosis and the inflammatory cascade,⁴² and inhibition of cathepsins B and L by CP-1 in the current study enhanced endogenous neurogenesis after ICH. Therefore, we postulate that CP-1 may facilitate endogenous neurogenesis because it interferes with the inflammatory enzymatic cascades. Cystatin C, a potent endogenous inhibitor of lysosomal proteinases including cathepsins B and L, has also been shown to stimulate self-renewing, multipotent neural stromal cells in vitro and neurogenesis in vivo.³¹ CP-1 may be functioning with a similar mechanism as cystatin C in regards to neurogenesis.

Neurological functional recovery has been attributed to synaptic reorganization after stroke and brain trauma.^{13,27} Synaptophysin expression is an indicator of synaptogenesis.²⁸ In our study, synaptophysin density is significantly increased in the boundary zone around the

hematoma region 2 weeks after CP-1 treatment, which suggests that CP-1 treatment may either directly protect the synapses from damage or enhance new synaptic formation.

An interesting finding is that CP-1 treatment at the acute period promotes endogenous angiogenesis following ICH. This may relate to the neuroprotective effect of CP-1, whereby cell survival and recovery is promoted by cathepsin B/L inhibitor during the first day after injury. Cathepsin cysteine proteases, however, have been reported to be effectors of invasive growth and angiogenesis during multistage tumorigenesis.²³ Moreover, deficiency of these cysteine proteases can impair microvessel growth. The main effect of cathepsins is to penetrate the extracellular matrix to form vessel sprouts. After MCAO, new capillary buds start to appear in the ischemic bed 3 days post-injury, and angiogenesis is induced after 7 days of focal cerebral ischemia.³⁷ Following acute treatment of ICH in our study, CP-1 is expected to limit the initial cathepsin surge after injury. However, cathepsin activity is restored subacutely by the creation of new enzyme, thereby being available to promote neurological recovery with angiogenesis at the later timepoints. Whether CP-1 has a direct effect on angiogenesis after ICH cannot be determined by the current study.

Conclusion

This is the first study demonstrating that post-ICH administration of a cysteine protease inhibitor selective for cathepsins B and L significantly reduces tissue loss and improves neurological function in the rat. One likely mechanism is neuroprotection as demonstrated by decreased neuronal apoptosis following CP-1 treatment, substantiating the involvement of cathepsins B and/or L in neuronal cell death after ICH. This suggests that the selective inhibitor improves cellular survival along the ICH border independently from direct effects on the calpain or caspase enzyme system. In addition to the observed neuroprotective effect of CP-1, the ICH region also shows post-treatment evidence of neuronal recovery with neuroplasticity and angiogenesis.

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NSS Test-ICH

Corner Test-ICH

Fig. 1.

CP-1 improves neurological outcome. Quantitative bar graph results of NSS and corner turn test of control and treatment groups are presented in the left and middle panels, respectively. Statistical significance levels are: * $p < 0.05$ CP-1 group compared with control group. # $p < 0.05$ control group compared with sham control group. @ $p < 0.05$ sham control group compared with sham CP-1 group.

Fig. 2. Immunostaining of the control and treatment sections showing the quantitative immunoreactivities of BrdU and TUJ1. Quantitative immunoreactivities for both groups are presented as bar graphs on the right side of each panel. Colocalization of BrdU and TUJ1 in a subpopulation of cells near the injured region of the combination group is shown on the upper panel. Arrows indicate cells positively stained for both BrdU and TUJ1.

Fig. 3.

Representative immunostaining and quantitative immunoreactivities of γ WF and BrdU in the ipsilateral hemisphere at 2 weeks after ICH. Upper panels show BrdU (left) and γ WF (center) immunostaining and colocalization of BrdU- γ WF (right) for a subpopulation of cells located near the injured site for a CP-1-treated animal. Arrows indicate cells that stained positively for both BrdU and γ WF. γ WF results are shown in the lower panels for control (left) and CP-1 (center) treatment groups, respectively. Quantitative immunoreactivities for both groups are presented as bar graphs in the lower right panel.

Fig. 4.

Immunostaining for TUNEL and synaptophysin along the boundary area around the hematoma and tissue loss at 2 weeks after ICH. The number of apoptotic cells is reduced in the CP-1 treatment group compared to the control group. A higher percentage per area of cells positively reacting to synaptophysin is seen in the sections derived from CP-1-treated versus control rats. Quantitative immunoreactivities for both groups are presented as bar graphs on the right side of each panel. H&E staining showing representative sections and bar graphs of quantitative striatal tissue loss percentages in the ICH region relative to the contralateral normal region of four groups are shown in the bottom panels.