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The proteasome inhibitor VELCADE® reduces infarction in rat models of focal cerebral ischemia

Nils Henninger [a,*], Kenneth M. Sicard [b], James Bouley [a], Marc Fisher [a], Nancy E. Stagliano [c]

[a] *Department of Neurology, University of Massachusetts Medical School, Worcester, MA 01655, USA*
[b] *Center for Comparative NeuroImaging, Department of Psychiatry, University of Massachusetts Medical School, Worcester, MA 01655, USA*
[c] *Department of Global Medical Affairs, Millennium Pharmaceuticals Inc., Cambridge, MA 02139, USA*

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Abstract

The potential neuroprotective effects of VELCADE® were investigated in two different models of focal cerebral ischemia. For time-window assessment, male Wistar–Kyoto rats were treated with 0.2 mg/kg VELCADE® at 1, 2, or 3 h after the induction of permanent middle cerebral artery occlusion (MCAO) using the suture occlusion method (experiment 1). To evaluate effects in a different model, male Sprague–Dawley rats received 0.2 mg/kg VELCADE® after embolic MCAO (experiment 2). Infarct volume was calculated based on TTC-staining 24 h postischemia and whole blood proteasome activity was fluorometrically determined in both experiments at baseline, 1 and 24 h post-MCAO. In experiment 1, a dose of 0.2 mg/kg inhibited proteasome activity by 77% and infarct volume was reduced to $175.7 \pm 59.9\,mm^3$ and $205.9 \pm 83.9\,mm^3$ (1 and 2 h group, respectively; $p < 0.05$) compared to $306.5 \pm 48.5\,mm^3$ (control). Treatment at 3 h was not neuroprotective ($293.0 \pm 40.1\,mm^3$). After embolic MCAO, infarct volume was $167.5 \pm 90.7\,mm^3$ (treatment group) and $398.9 \pm 141.3\,mm^3$ (control; $p = 0.002$). In conclusion, VELCADE® treatment inhibited whole blood proteasome activity and achieved significant neuroprotection in two rat models of focal cerebral ischemia at various time points poststroke.
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Keywords: Embolic stroke; MCAO; Neuroprotection; Proteasome inhibition; Suture model

A significant proportion of strokes result from embolic occlusion in the cerebral vasculature with the progression and evolution of ischemic injury being related to several mechanisms, some of which are triggered through the activation of the pro-inflammatory transcription factor nuclear factor kappaB (NF-κB) [9,20,40]. A new strategy to impede NF-κB activity and inflammation in stroke is the selective inhibition of the ubiquitin-proteasome pathway (UPP) [16,36,37].

The UPP acts to dispose of dysfunctional or damaged proteins, regulates proteins that control cell division, and generates antigens for the immune system [16]. Proteasome inhibition results in a decrease of overall protein breakdown and the accumulation of short-lived proteins. Previous studies with proteasome inhibitors in rodent models of ischemia demonstrated limited tissue damage, which was correlated with attenuated expression of inflammatory NF-κB gene products and a reduction in brain leukocytes [4,11,24,35,39].

VELCADE® (*N*-pyrazinecarbonyl-L-phenylalanine-L-leucine boronic acid; Bortezomib, previously known as PS-341 or MLN-341, Millennium Pharmaceuticals Inc., Cambridge, MA, USA) is the only clinically approved proteasome inhibitor for relapsed, refractory multiple myeloma [6]. Although the fields of oncology and neurology are vastly different, both share activation of NF-κB as one mechanism of disease. With an understanding of its mechanism of action, a known toxicity profile in cancer patients and a knowledge of achievable doses in the clinic, we sought to evaluate the potential for acute use of VELCADE®, a potent and selective proteasome inhibitor, in reducing ischemia-induced cerebral injury.

All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. For experiment 1 (suture model, sMCAO) male Wistar–Kyoto (WK) rats (Charles River Laboratories, Kingston, NY) weighing 292 ± 21 g, and for experiment 2 (embolic model,

* Correspondence to: Center for Comparative NeuroImaging (CCNI), 303 Belmont Street, Worcester, MA 01604, USA. Tel.: +1 508 856 8185; fax: +1 508 856 8090.
E-mail address: Nils.Henninger@umassmed.edu (N. Henninger).

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eMCAO) male Sprague–Dawley (SD) rats (Taconic Farms, NY, USA) weighing 295 ± 37 g were studied. Animals were anesthetized with isoflurane (5% for induction, 2% for surgery, 1.5% for maintenance) in room air. PE-50 polyethylene tubing was inserted into the femoral artery for monitoring of mean arterial blood pressure (MABP) and for obtaining blood samples to measure blood gases (pH, PaO_2, $PaCO_2$, HCO_3^-, SaO_2), hematocrit, hemoglobin concentration, electrolytes (Na^+, K^+, Ca^{2+}) and plasma glucose at baseline and 90 min after MCAO. Body temperature was monitored continuously with a rectal probe and maintained at 37.0 ± 0.5 °C with a thermostatically controlled heating lamp. All experiments were performed in a blinded and randomized manner.

In experiment 1, the right internal carotid artery (ICA) and external carotid artery (ECA) were exposed through a midline incision of the neck. The ECA was isolated, and superior thyroid and the occipital arteries were ligated and transected. The distal portion of the ECA was ligated and transected to create an ECA stump with a length of approximately 5 mm. The pterygopalatine artery (PPA) was also ligated. Permanent focal cerebral ischemia was produced by inserting a 4-0 silicone-coated nylon suture with a thermically rounded tip through the ECA stump until a mild resistance indicated correct placement of the filament for complete MCAO [3].

In experiment 2, embolic stroke was induced using a protocol modified from that previously described by Busch et al. [5]. Briefly, the right common carotid (CCA), ICA, and ECA were exposed, and the origin of the PPA was identified. ECA and PPA were permanently ligated, while the CCA was temporarily clipped for embolization. PE-10 tubing was inserted into the ECA proximal to its ligation, and one red blood clot (diameter = 0.35 mm, length = 36 mm) was injected into the ICA over ∼1 s at the bifurcation of the PPA and ICA. Isoflurane was reduced to 1.5%, the CCA clamp removed, and the rat left in this condition for 10 min. Thereafter, the catheter was removed, the ECA stump tied off and the incision closed.

The protocol for embolus (clot) preparation used herein was modified from a protocol originally described by Toomey et al. [33]. Whole blood (200 μL) was withdrawn from the rat 24 h prior surgery into an Eppendorf tube. The blood was promptly mixed with 1.0 NIH unit (10 μL) of human thrombin and 4.5 μL of 1 mol/L $CaCl_2$ for a final $CaCl_2$ concentration of 20 mmol/L. Within 5 s, a small portion of this mixture was drawn into an approximately 30.0-cm length of polyethylene catheter (PE-50) and allowed to clot at 37 °C for 2 h. At the end of this period, the clot was extruded from the catheter into a saline-filled petri dish and stored at 4 °C for 22 h. Prior to thromboembolism a 5–10 cm section of clot was placed into a separate petri dish containing deionized water and incubated for 5 min at room temperature. At the end of this treatment (osmotic shock), the clot was placed into a solution of isotonic saline and dissected into a single 36 mm section. This section (clot) was collected into a PE-10 catheter in a volume of 50 μL of saline. The interval between this final step and embolization was less than 5 min.

Lyophilized VELCADE® was resuspended in 10.5 mL of sterile 0.9% saline to a concentration of 0.33 mg/mL and used within 8 h of preparation. Prior to intravenous administration, VELCADE® was replenished with saline for a total injection volume of 1 mL and given as a bolus over 1 min. Sterile 0.9% saline served as the vehicle. In experiment 1, male WK rats ($n = 32$) were randomized into four groups of eight animals and received VELCADE® (0.2 mg/kg) and/or vehicle. Group 1 received vehicle at 60 and 180 min. Group 2 received VELCADE® at 60 min and vehicle at 120 min. Group 3 received vehicle at 60 min and VELCADE® at 120 min. Group 4 received vehicle at 60 min and VELCADE® at 180 min. In experiment 2, male SD rats ($n = 16$) randomly received either VELCADE® (0.2 mg/kg) or vehicle 30 min after inducing embolic stroke.

Blood samples were collected before MCAO as well as 1 and 24 h after VELCADE® or vehicle administration. Note that in experiment 1 data was only obtained from animals receiving VELCADE® at 1 h after sMCAO. Whole blood (100 μL) was collected through the tail vein to a heparin pre-coated Eppendorf tube and the sample immediately frozen to −80° C. Proteasome activity was determined using a FL600 fluorescence plate reader to follow kinetics at 37 °C ($\lambda_{ex} = 380$ nm, $\lambda_{em} = 450$ nm) as previously described [12]. Briefly, blood samples were lysed in 5 mM EDTA and then either assayed the same day or stored frozen in 40 mM HEPES containing 20% glycerol. Whole blood lysates were diluted in 10 mM HEPES, 1 mM EDTA, 10% glycerol, pH 8.0 to obtain approximately 3000 μg protein per mL. Thirty microliters of diluted lysate were transferred into 96-well fluorometric plates (Coming/Costar plate #3915) in triplicate and 100 μL chymotryptic assay buffer per well were added. The increase in fluorescence was measured for 25 min and the fluorescence units were fitted by linear regression to determine the rate of hydrolysis of the chymotryptic substrate. Calculations were done as with cuvet-based assay.

Animals were anesthetized 24 h after stroke onset with pentobarbital (150 mg/kg) and decapitated. Animals dying prematurely between 16 and 24 h after stroke onset were included in the data analysis [18]. Brains were removed and cut into six 2-mm coronal slices starting 1 mm from the frontal pole using a plastic rat brain matrix (Harvard Apparatus, Holliston, MA). Histological staining was performed using 2,3,5-triphenyltatrazolium chloride (TTC). The stained sections were then fixed in 10% buffered zinc formalin. After 48 h, the sections were photographed and infarct volumes were determined using ImageJ software (Rasband, http://rsb.info.nih.gov/ij/) by an investigator blinded to the treatment. To compensate for the effects of brain edema, a corrected infarct volume was calculated using the following formula: area of the intact contralateral (left) hemisphere—area of intact regions of the ipsilateral (right) hemisphere [3]. Infarct areas were then summed among slices and multiplied by slice thickness to give the total infarct volume, which was expressed as a percentage of the intact contralateral hemispheric volume.

Data are expressed as mean ± standard deviation. Infarct sizes and blood proteasome activity were compared among and within groups by *t*-test, analysis of variance (ANOVA) or repeated-measures ANOVA, with post hoc Bonferroni tests to correct for multiple comparisons, as appropriate. Mortality data were analyzed via Fisher exact or chi-square tests, as appro-

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