Proteasome Inhibitor PS519 Reduces Infarction and Attenuates Leukocyte Infiltration in a Rat Model of Focal Cerebral Ischemia

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Background and Purpose—Reperfusion brain injury after cerebral ischemia is associated with a developing inflammatory response at the site of infarction. Proteasome inhibitors block nuclear factor-κB activation and provide anti-inflammatory effects in several animal models of peripheral inflammation. We tested the novel proteasome inhibitor PS519 in a rat model of transient focal ischemia to establish its pharmacodynamics as a neuroprotection treatment and related effects on leukocyte infiltration.

Methods—Rats were subjected to 2 hours of focal cerebral ischemia by means of the filament method of middle cerebral artery occlusion (MCAo). After either 22 or 70 hours of reperfusion, infarct size was measured and neurological function, electroencephalographic (EEG) activity, and/or neutrophil and macrophage infiltration was quantified. PS519 was administered in a single intravenous bolus at 2 hours after MCAo. In addition, the therapeutic window for PS519 was estimated by delaying treatment for 4 or 6 hours after MCAo.

Results—Dose-response analysis of infarct volume at 24 hours revealed that PS519 neuroprotection approached 60%, and clinical evaluations showed significant improvements in neurological function and EEG activity. Neutrophil infiltration at 24 hours was also significantly decreased in cortical and striatal infarcted tissue of PS519-treated rats. Delaying the PS519 treatment up to 4 hours continued to result in significant neuroprotection. In the 72-hour injury model, infarction was reduced 40% by PS519, and significant improvements in neurological function and EEG recovery were again measured. Considerable reductions in both neutrophil and macrophage infiltration were evident.

Conclusions—PS519 mitigates infarction and improves neurological recovery in brain-injured rats, an effect in part caused by a reduction in the leukocyte inflammatory response. (Stroke. 2000;31:1686-1693.)

Key Words: cerebral ischemia ■ enzyme inhibitors ■ neuroprotection ■ rats

The progression and extent of brain injury resulting from cerebral ischemia (ie, stroke) is related to several reperfusion mechanisms, many of which involve postinjury inflammatory response elements. These inflammatory mediators include an early neutrophil response, occurring even 4 hours after reperfusion, and a delayed macrophage infiltration that occurs several days later.1–4 Accompanying the early leukocyte response is a significant accumulation of other inflammatory elements such as interleukins, tumor necrosis factor-α (TNF-α), and cellular adhesion molecules.5,6 Interleukin-1β and TNF-α also stimulate the expression of cellular adhesion molecules on endothelial cells, which promote leukocyte adherence and their subsequent migration into brain parenchyma. This early inflammatory response is independent of necrosis and suggests that these elements are important in the progression and extent of brain infarction.7 That inflammatory elements exacerbate cerebral ischemic injury has been demonstrated by the significant neuroprotection observed after inhibition of leukocyte and cytokine actions.7–9 The induction of these cellular inflammatory elements is regulated by the transcription factor nuclear factor (NF)-κB, which is itself directly regulated by the ubiquitin-proteasome pathway.10,11 Proteasome inhibitors block activation of NF-κB by preventing the degradation IκBα and prevent TNF-α induction of several types of cell adhesion molecules.12 One such inhibitor is lactacystin.13 Recently, a novel, more potent, small-molecular-weight analog, PS519, was developed14 and shown to elicit cardioprotective effects15 and to exhibit anti-inflammatory actions in a variety of other inflammatory-related pathological conditions.16–18 PS519 is a highly selective and potent inhibitor of proteasome activity and stabilizes the levels of NF-κB.14

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This report describes the effect of PS519 to limit tissue damage in a rodent model of cerebral ischemia. Our results show that PS519 significantly reduced the size of infarction caused by middle cerebral artery occlusion (MCAo) and reperfusion and significantly improved neurological outcome and electroencephalographic (EEG) brain recovery. PS519 reduced the infiltration of neutrophils and macrophages into infarcted tissue.

Materials and Methods

General Surgery Procedures
All surgical procedures were carried out under aseptic conditions in identified surgery suites and were performed in accordance with the Laboratory Animal Care and Use Committee, Walter Reed Army Institute of Research. Initially, rats (male Sprague-Dawley; weight 260 to 320 g) were anesthetized with 5% halothane in oxygen. During surgery, anesthesia was maintained with 2% to 3% halothane in oxygen, and animals were kept normothermic (37±0.5°C) with the use of a homeothermic blanket control unit system (Harvard Apparatus). After surgery, a Vetco thermal barrier and heating lamps were used to maintain normal body temperature.

The animals were surgically implanted with right external jugular vein catheters (PE 50/silastic) filled with heparinized saline (10 Units/mL), and 2 cortical EEG recording electrodes (0-80 stainless steel screws) were permanently attached to the skull and positioned over the right (ie, injured) frontal and parietal cortex. For the physiology studies, a polyurethane catheter (MRE-25; Braintree Scientific) was also implanted into the right femoral artery.

Physiological Parameters
Mean arterial blood pressure and heart rate were continuously monitoring in awake, freely moving rats by means of a Digi Med blood pressure analyzer (MicroMed). Femoral artery blood (85 μL) was analyzed for pH and gases with an ABL5 Blood Gas System (Radiometer A/S). For rats undergoing MCAo, blood was drawn and analyzed before MCAo as a baseline measurement and then again at 30, 120, 150, and 240 minutes after MCAo. The blood sampling at 120 minutes was done immediately before reperfusion surgery and vehicle or PS519 administration. Blood sampling at 150 minutes was done immediately after reperfusion and before MCAo administration. The blood sampling at 120 minutes was done immediately before reperfusion surgery and vehicle or PS519 administration. Blood sampling at 150 minutes was done immediately after reperfusion and before MCAo administration. The blood sampling at 120 minutes was done immediately before reperfusion surgery and vehicle or PS519 administration. Blood sampling at 150 minutes was done immediately after reperfusion and before MCAo administration. The blood sampling at 120 minutes was done immediately before reperfusion surgery and vehicle or PS519 administration. Blood sampling at 150 minutes was done immediately after reperfusion and before MCAo administration.

EEG activity was measured in each rat under anesthesia immediately before the initial 2-hour injection and again at 24 or 72 hours. Neurological scores (NS) were derived by means of a cumulative 10-point grading scale: 4 points if there is a reduction in the resistance to a contralateral push (across a corrugated cardboard surface); 3 points if contralateral circling is evident; 2 points for the appearance of contralateral shoulder adduction; and 1 point for contralateral forelimb flexion, when suspended vertically by the tail. Therefore, rats exhibiting all neurological signs of impairment receive an NS of 10. Importantly, rats not exhibiting an NS of ≥7 at 2 hours after MCAo were excluded from the study.

Brain Activity
EEG activity was measured in each rat under anesthesia immediately before MCAo, during the filament insertion, immediately before reperfusion at 2 hours after occlusion, and again at the 24- or 72-hour end point before euthanasia. This enabled us to establish an experimental exclusion criterion (ie, a diminution in EEG amplitude by ≥80% at 2 hours after occlusion compared with preconditioning amplitude) and to determine the effect of PS519 to improve cortical EEG activity in injured rats. Changes in EEG amplitude were quantified with the use of spectral analysis and data reported as percent EEG recovery compared with the 2-hour prereperfusion sample.

Infarct Analysis
Seven coronal sections (2 mm thick) were taken from the region beginning 1 mm from the frontal pole and ending just rostral to the corticocerebellar junction. The sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC), computer imaged, and analyzed for quantification of ischemic cerebral damage and infarct as described in detail by Britton et al. Hemispheric infarct size, calculated as percentage of core infarcted tissue referenced to the contralateral unjured cerebral hemisphere, was also obtained to exclude the possible contributing effect of hemispheric edema to infarct volume. Core injury was defined as brain tissue (cortex or subcortical area) completely lacking TTC staining.

Histopathology for Analysis of Neutrophil and Macrophage Infiltration
Some of the TTC-stained brain slices (stored in 10% formalin) were subsequently prepared for paraffin embedding by the use of routine histological procedures. Briefly, the tissue was dehydrated with gradient alcohol concentrations, cleared in Hemo-De (a xylene substitute; Fisher Scientific, Inc), and embedded with paraffin wax with the use of an automatic tissue processor (Tissue Tek; Miles Inc). Slices (6 μm) were cut from the second through fifth coronal sections and stained with hematoxylin and eosin. Standard tissue dehydration and clearing for paraffin embedding removed the TTC stain. A section corresponding to approximate coronal section 9.2 interaural, 0.2 bregma was selected for evaluation of neutrophil and macrophage infiltration. Before histological analysis, the injured hemisphere of the slice was visually demarcated into parietal and insular cortices and the underlying striatum. Neutrophils and macrophages were counted in 12 random fields within ischemic regions of each area under light microscopy at ×40 magnification, and only intact, extravascular leukocytes were included. No macrophages were seen in brain slices of rats at 24 hours after MCAo, so only

Drug Administration
Vehicle (50% 1,2-propanediol in saline) or PS519 injections were given ~2 hours after MCAo, immediately after onset of reperfusion. For the 24-hour recovery studies, vehicle (n=13) or PS519 (0.003 to 0.3 mg/kg, n=6 to 9 per dose) was injected as a single intravenous bolus. For the 72-hour experiments, vehicle (n=7) or PS519 (0.1 mg/kg, n=5) was injected intravenously at 2 hours after injury. In other studies, the injection of PS519 was delayed 4 or 6 hours after MCAo.
A stock concentration of PS519 was prepared fresh daily in 1,2-propanediol, then mixed 50:50 with saline immediately before intravenous administration. Importantly, the doses of PS519 used in the present study were chosen from previous experiments examining inhibition of 20S proteasome activity in circulating rat white blood cells in a dose-related manner 1 hour after intravenous bolus injections (unpublished data).
neutrophil counts were recorded. Both neutrophils and macrophages were counted in brains of rats at 72 hours after MCAo.

**Results**

### 2-Hour MCAo in Vehicle-Treated Rats

MCAo with 22-hour reperfusion resulted in significant core infarction within the temporal/parietal cortex and underlying striatum of the ipsilateral (injured) hemisphere. Ischemic damage generally extended from the most rostral forebrain brain section to the final caudal section and was greatest in the area around the bregma (Figure 1). Total core infarct volumes averaged $282 \pm 20 \text{mm}^3$ (Figure 2), of which 80% represented cortical infarction ($226 \pm 15 \text{mm}^3$) and 20% striatal infarction ($56 \pm 6 \text{mm}^3$). At 2 hours after MCAo, neurological function (NS $= 10.0 \pm 0.6$) and EEG activity (Table 1) were severely impaired; at 24 hours, there was only marginal recovery measured in vehicle rats. Histological analysis of the ischemic cortex and underlying striatum revealed the presence of neutrophils ($27 \pm 2$ and $14 \pm 1$, respectively) but not macrophages (Figure 3).

At 72 hours after MCAo, the mean core infarct volume was slightly smaller than that observed at 24 hours, whereas neurological function showed significant spontaneous improvements (Table 2). However, similar to the 24-hour injury, EEG activity at 72 hours was not improved in these rats. At 72 hours after injury, both neutrophils and macrophages were present in injured cortical and striatal tissues (Figure 4).

### Physiological Parameters

MCAo significantly ($P < 0.05$) increased mean arterial blood pressure by $\approx 25\%$ and heart rate by $\approx 12\%$ to $23\%$ within the first 120 minutes, which remained elevated in both vehicle-treated and PS519-treated rats through 4 hours. In contrast, blood pH and pCO$_2$ were not significantly different from normal, pre-MCAo levels. There was, however, a slight decrease in pO$_2$ at 2.5 to 4 hours after MCAo in PS519-treated rats ($P < 0.05$). MCAo also caused a transient, mild hyperthermia at 30 to 120 minutes after occlusion that was not changed by PS519 and returned to baseline by 24 hours. These results are summarized in Table 3. In normal, uninjured rats, PS519 did not appear to alter any of the recorded physiological parameters up to 6 hours after injection (data not shown).

### PS519 Reduces Ischemic Infarction at 24 hours

PS519 reduced the core infarcted areas as seen in the forebrain profiles (Figure 1). All but the lower 2 doses of

![Figure 1. Forebrain profiles of infarction caused by transient MCAo and 24-hour recovery. Vehicle or PS519 was administered intravenously at 2 hours after MCAo. Data represent mean core infarct area $\pm$ SEM (n=6 to 13).](image1)

![Figure 2. Scatterplot and histogram demonstrating dose-response effect of PS519 to decrease core infarct volumes after 2-hour transient MCAo and 24-hour recovery; dots indicate respective core infarct volume of each animal per group and histogram indicates mean volume response $\pm$ SEM. $^*P < 0.05$, $^**P < 0.005$ compared with vehicle controls (Student’s t test).](image2)

![Figure 3. Neutrophil counts within infarct tissue of cortex (A) and striatum (B) of injured hemisphere at 24 hours after MCAo. Data represent mean $\pm$ SEM (n=3). $^*P < 0.05$, Student’s t test compared with vehicle controls.](image3)

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**TABLE 1. Effect of PS519 on EEG Recovery and Neurological Function at 24 Hours After 2-Hour MCAo and Reperfusion**

<table>
<thead>
<tr>
<th>PS519, mg/kg IV, at 2 h After MCAo</th>
<th>n</th>
<th>% EEG Recovery</th>
<th>Neurological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>1.4±1.7</td>
<td>8.0±0.6 (0)</td>
</tr>
<tr>
<td>0.003</td>
<td>8</td>
<td>7.3±5</td>
<td>6.7±1.2 (3)</td>
</tr>
<tr>
<td>0.01</td>
<td>6</td>
<td>24±9†</td>
<td>4.5±1.4 (3)*</td>
</tr>
<tr>
<td>0.03</td>
<td>7</td>
<td>28±9†</td>
<td>6.6±1.3 (3)</td>
</tr>
<tr>
<td>0.10</td>
<td>7</td>
<td>10±3*</td>
<td>5.4±1.2 (3)*</td>
</tr>
<tr>
<td>0.30</td>
<td>7</td>
<td>11±6</td>
<td>4.4±1.0 (4)†</td>
</tr>
</tbody>
</table>

Values are mean $\pm$ SEM. Numbers in parentheses adjacent to neurological scores represent the number of rats per group that exhibited >50% improvement (score is <5) in 0 to 10 graded scale.

$^*P < 0.05$, $^†P < 0.005$ compared with vehicle controls (Student’s t test).
PS519 were effective at reducing core infarct area in all forebrain sections. PS519 doses 0.03 to 0.1 mg/kg were the most effective, whereas 0.01 mg/kg achieved its neuroprotective effect only in brain regions caudal to the bregma (Figure 1). Rats treated with 0.01 to 0.3 mg/kg of PS519 had significantly reduced volumes of infarction ranging from $183 \pm 42$ mm$^3$ to $138 \pm 30$ mm$^3$ (Figure 2), respectively. Normalization of the data to the infarction measured in the group of vehicle-treated rats established that maximal neuroprotection approached 50% to 60% at the 3 highest doses of PS519 tested (61 ± 14%, 57 ± 11%, and 51 ± 11%, respectively; *P*, 0.005). Importantly, the percent neuroprotection calculated with the use of the volume of the uninjured, contralateral hemisphere thereby accounts for hemispheric swelling. The percent change in hemispheric volume at these PS519 neuroprotective doses was 61 ± 14%, 55 ± 12%, and 46 ± 11%, respectively (*P*, 0.005). Therefore, the similarity in neuroprotection levels based on core infarct volume and those based on hemispheric volume indicates that the protective effects of PS519 were not mediated in part by a reduction in brain edema.

### Effects of PS519 on Brain Activity and Neurological Function at 24 hours

Rats treated with neuroprotective doses of PS519 typically showed improved cortical EEG activity and neurological function at 24 hours compared with vehicle-treated rats (Table 1). Rats treated with all but the lowest (0.003 mg/kg) and highest (0.3 mg/kg) doses of PS519 had significant increases in percent EEG recovery compared with control rats, whereas all doses of the drug showed a trend to improve EEG recovery at 24 hours. Likewise, examination of neurological function at 24 hours in PS519-treated rats revealed significant improvements in NS. Importantly, all rats treated with PS519 had at least one third of their group exhibiting 50% improvement in neurological function, whereas no rats in the vehicle-treated group achieved such recovery.

### Effects of PS519 on Cortical and Striatal Injury at 24 hours

The degree of infarction was also determined separately in the cortical and striatal regions of each rat treated with 0.1 mg/kg PS519 (data not shown). The amount of infarction in cortical tissue for vehicle-treated and PS519-treated rats was $226 \pm 15$ mm$^3$ and $101 \pm 25$ mm$^3$, respectively, which represents a 55 ± 11% neuroprotection. Similar neuroprotection of striatal tissue was also seen in PS519-treated rats, that is, 64 ± 13%, in which core infarct volumes averaged $20 \pm 7$ mm$^3$ versus $56 \pm 6$ mm$^3$ in the vehicle-treated group.

### PS519 Reduces Neutrophil Infiltration Into Ischemic Brains at 24 Hours

Figure 3 describes neutrophil counts in the ischemic cortex (A) and striatum (B). Rats (n=3) treated with vehicle had significantly more neutrophils in these areas than those treated with PS519 (*P*<0.05). No macrophages were seen in ischemic brains of vehicle-treated or drug-treated rats.

### Effect of Delayed PS519 Treatment on Percent Neuroprotection at 24 Hours

The effect of delaying treatment with 0.10 mg/kg PS519 was examined to determine if neuroprotection could still be afforded after reperfusion had been established for prolonged periods (Figure 5). Significant neuroprotection was measured when treatment was delayed 2 or 4 hours ($57 \pm 11\%$ and $43 \pm 12\%$, respectively), and similar improvements were seen in NS and EEG recovery (data not shown). In rats treated at 6 hours after occlusion, only limited neuroprotection was measured.

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**Table 2. Effect of PS519 (0.1 mg/kg) on Total Core Infarct Volume, EEG Recovery, and Neurological Function at 72 Hours After 2-Hour MCAo and Reperfusion**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Infarct Volume, mm$^3$</th>
<th>Percent Neuroprotection</th>
<th>Percent EEG Recovery</th>
<th>Neurological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>231 ± 20</td>
<td>0.0 ± 6</td>
<td>6.3 ± 5.5</td>
<td>2.4 ± 0.9</td>
</tr>
<tr>
<td>PS519</td>
<td>5</td>
<td>138 ± 25*</td>
<td>40 ± 11*</td>
<td>61 ± 20*</td>
<td>1.0 ± 0.0*</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*P,< 0.05 compared with vehicle controls (Student’s t test).
Neuroprotective Effects of PS519 at 72 Hours After MCAo

In these experiments, PS519 was administered intravenously at 2 hours after occlusion, and neuroprotective efficacy was determined at 72 hours. At 72 hours, the rats treated with PS519 had infarct volumes 40\% smaller than the control animals (Table 2). Likewise, significant improvements in neurological function and EEG recovery were also seen (Figure 4).

<table>
<thead>
<tr>
<th>Time After MCAo</th>
<th>Before MCAo</th>
<th>30 min</th>
<th>120 min*</th>
<th>150 min</th>
<th>240 min</th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>98.3±4.4</td>
<td>132.0±3.0</td>
<td>131.5±2.5</td>
<td>118.5±4.9</td>
<td>119.9±6.5</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>PS519</td>
<td>104.2±0.4</td>
<td>130.2±3.8</td>
<td>138.4±3.0</td>
<td>132.8±5.3</td>
<td>134.6±2.3</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>282±33.8</td>
<td>365±54.0</td>
<td>291±54.4</td>
<td>374±23.2</td>
<td>349±23.4</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>PS519</td>
<td>398±16.9</td>
<td>451±19.5</td>
<td>429±8.2</td>
<td>440±6.8</td>
<td>424±14.0</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Blood pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>7.43±0.02</td>
<td>7.38±0.01</td>
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<td>7.43±0.01</td>
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<td>...</td>
</tr>
<tr>
<td>PS519</td>
<td>7.43±0.00</td>
<td>7.43±0.00</td>
<td>7.45±0.01</td>
<td>7.43±0.01</td>
<td>7.42±0.03</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>pO2, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>94.0±7.5</td>
<td>92.3±5.6</td>
<td>86.3±2.7</td>
<td>97.7±12.9</td>
<td>98.7±16.7</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>PS519</td>
<td>90.7±2.0</td>
<td>93.0±17.6</td>
<td>87.0±10.2</td>
<td>72.0±1.7</td>
<td>79.0±1.7</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Rectal temperature, °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>36.3±0.1</td>
<td>37.7±0.2</td>
<td>37.6±0.2</td>
<td>36.4±0.4</td>
<td>36.2±0.3</td>
<td>34.5±0.4</td>
<td>...</td>
</tr>
<tr>
<td>PS519</td>
<td>36.4±0.1</td>
<td>37.9±0.4</td>
<td>38.3±0.3</td>
<td>37.8±0.5</td>
<td>36.3±0.2</td>
<td>35.5±0.8</td>
<td>...</td>
</tr>
</tbody>
</table>

Values are mean±SEM, n=3 to 7 per group.
*Vehicle or PS519 was injected intravenously at 120 minutes.

*Figure 5. Effect of delaying treatment with PS519 on percent neuroprotection at 24 hours after MCAo. Data represent mean±SEM (n=7 to 13). *P<0.05, **P<0.005, Student’s t test compared with vehicle controls.

Discussion

This study examined the in vivo neuroprotective efficacy of the 26S proteasome inhibitor PS519 and its therapeutic role in an early and late inflammatory response after transient focal brain ischemia in rats. We have demonstrated that PS519, administered after injury as a single intravenous bolus, significantly reduced core infarction in both 24- and 72-hour recovery models and determined that neuroprotection coincided with significant reductions measured in brain leukocyte accumulation. Importantly, neuroprotection produced by PS519 consistently coincided with therapeutic improvements in the neurological function and EEG activity of the injured rats.

The intraluminal filament model of MCAo used in this study produces experimental stroke by temporary occlusion of the MCA with subsequent reperfusion of blood at controlled time points. Because it is noninvasive (no craniec- tomy) and temporary (i.e., reperfusion), this transient model of
ischemia may be more clinically relevant to stroke than animal models of permanent occlusion or those requiring craniectomy. In human cases of cerebral embolism, recirculation often occurs after focal infarction, and in other cases thrombolytic agents may therapeutically initiate reperfusion. However, the importance of cerebral reperfusion to recovery is of some controversy. Although reperfusion serves to correct cellular energy deficits and metabolic imbalances at the site of injury, it can cause pathophysiological problems that may exacerbate the underlying ischemic damage including key cellular inflammatory. Studies in rat models of stroke indicate that the presence of cytokines and adhesion molecules precedes the presence of neutrophils, and although reperfusion may not be required for these elements to play a role in the development of injury, it does promote an earlier presence of neutrophils in the ischemic tissue.

Neuroprotection therapy targeting this inflammatory response could represent an effective primary or adjunctive treatment for ischemic brain injury. Selective reduction in leukocyte infiltration has been achieved in ischemic brains of rats after in vivo treatment with specific leukocyte antibody and/or interleukin-1 receptor antagonist. Proteasome inhibition would also reduce the activation of a broad range of inflammatory mediators by blocking the activation of NF-κB and consequently reduce their transcriptional upregulation. Our results showed that PS519 reduced neutrophil infiltration into the ischemic cortex and striatum by 70% and by 63%, respectively. With the use of an identical MCAo model, Chopp et al. have shown that treating rats with anti-Mac-1 antibodies also significantly reduced neutrophil accumulation in ischemic cortex and subcortex. Furthermore, Matsuo et al. reported reductions in cortical and striatal neutrophils when ischemic rats were pretreated with an antineutrophil monoclonal antibody. Both reports showed a positive correlation between the reduction in ischemic injury with the subsequent reduction in neutrophil infiltration. In the present study, PS519 significantly decreased core infarction by ~60%, which was consistent with a large reduction (60% to 70%) in leukocyte infiltration at 24 hours. Interestingly, PS519 also reduced myocardial neutrophil accumulation by ~70% in a model of cardiac ischemia. This effect was attributed to the PS519-induced attenuation in P-selectin expression in the ischemic tissue. To date, we have not determined if reductions in cytokines and adhesion molecules are associated with the PS519-induced reduction in neutrophil invasion and infarction in MCAo-injured brain tissue. However, a reduction in inflammatory mediators with another proteasome inhibitor has been reported in an inflammatory model of rheumatoid arthritis.

In addition to neutrophils, significant levels of macrophages were observed within infarcted brain tissue at 72 hours after injury. Again, PS519 significantly reduced neutrophil accumulation into the ischemic tissue of the injured cortex and striatum while macrophage invasion was also reduced. This delayed-macrophage inflammatory response after temporary MCAo coincides with other reports and with the concept of a "second phase" of inflammation. While the earlier "first phase" of the inflammatory response is characterized by the presence of polymorphonuclear cells (ie, neutrophils), the second phase is characteristic of monocyte and macrophage invasion. The anti-inflammatory actions of proteasome inhibitors may be of therapeutic importance in the temporal change from the early to late stages of inflammation, which is believed initiated by cytokines and chemokines. Additionally, it has been reported that macrophages of the delayed, second inflammatory phase release additional cytokines.

NF-κB translocation is thought to play a significant role in both the early and late phases of inflammation. Schneider et al. reported that after 2 hours of MCAo and 20 hours of reperfusion in rats, activated NF-κB translocation was evident in the striatum, and after 72 hours of reperfusion, translocation was evident in the penumbral cortex. We reported twice as many neutrophils and macrophages in the ischemic cortex compared with the striatum at 72 hours, which may be related to a greater level of NF-κB activity in the cortex at 72 hours. In agreement, Bethea et al. have reported significant levels of activated NF-κB in macrophages 72 hours after ischemic injury. Of relevance is the report that the antioxidant LY23167 reduced neuronal injury after 72 hours of global ischemia, which was related to inhibition of persistent but not transient activation of NF-κB. This delayed elevation of NF-κB supports its likely involvement in the late, second phase of inflammation, which would make it a target for proteasome inhibition therapy. The detrimental role of NF-κB in ischemic injury is further supported by reports that both glutamate and many oxidative free radicals induce NF-κB activity. Therefore, treatment with NMDA antagonists, antioxidants, or other neuroprotective agents in combination with PS519 may be beneficial in achieving synergistic effects as well as reducing the adverse actions often associated with higher doses of a single-agent therapy. Also, a second, delayed injection of PS519 at 48 to 60 hours after injury (or as adjunctive therapy) may yield a better, more significant reduction in leukocyte infiltration at 72 hours.

Although the full mechanism of action of PS519 was not elucidated in the present study, it is clear that the anti-ischemic effect is mediated through a reduction in the number of invading neutrophils and macrophages. The diapedesis of both cell types is critically regulated by the expression of cell adhesion molecules on the ischemic endothelium, and in vitro studies with PS519 we have shown that it can significantly reduce the expression of cell adhesion in human endothelial cells stimulated with TNF-α (data not shown). It is also possible that PS519 is acting directly on ischemic neurons and glia in the injured brain. However, in preliminary studies with radiolabeled PS519, we did not see any evidence of brain penetration of this compound when given at times when blood-brain barrier integrity is weakest, for example, at 2 hours and 24 hours after injury (data not shown). Hence, it appears likely that the neuroprotective effects of PS519 described herein involve nonneuronal mechanisms, primarily in the vasculature within the ischemic area.

The possible role of the proteasome in ischemia has been supported by several lines of evidence describing its involvement in the activation NF-κB. We believe this
report to be the first describing proteasome inhibitors eliciting an in vivo neuroprotective profile in cerebral ischemia. Agents acting at various levels of the ischemia cascade have been tested in various clinical trials for treatment of acute stroke.33 To date, only recombinant tissue plasminogen activator has been approved, but its clinical use is limited because of its tendency to induce intracranial hemorrhage,34 whereas all other neuroprotection drug trials in stroke have failed.35 With PS519, significant neuroprotection and improved recovery was achieved with a single postinjury injection in both reperfusion models, approaching 60% at 24 hours and 40% at 72 hours. Furthermore, 43% neuroprotection was still obtained even when the treatment with PS519 was delayed to 4 hours after MCAo, indicating a better therapeutic window than some other agents.36–38

In conclusion, we have described a neuroprotective action of PS519 in a rat model of transient forebrain ischemia (ie, stroke) that is dose-dependent and attributed, in part, to significant reductions in both an early and late leukocyte invasion of injured tissue. Infarct reduction was achieved with an acute, single-dose treatment regimen and was accompanied by improvements in neurological function and EEG activity. Critically, successful neuroprotection was obtained even when treatment was delayed 4 hours after injury. Collectively, these results support our contention that proteasome inhibitors such as PS519, possibly through blockade of NF-κB activation, can provide a significant means of protection in many ailments involving a notable inflammatory response. A full preclinical safety assessment will be reported elsewhere to support the planned phase I clinical safety trials for its development as an antistroke agent.

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References

One of the most probable reasons for the failure of many clinical trials with experimental drugs in focal stroke is the lack of an adequate time window for therapeutic intervention. Most of the drugs that have been clinically tested in *Stroke* thus far were found to be inactive in experimental focal stroke models if given 2 or more hours after occlusion. Attempts are currently being made by several groups to find agents that can reduce infarct size when administered 3 or more hours after occlusion. In the present study by Phillips et al, the time window for therapeutic intervention was expanded to at least 4 hours after occlusion by a novel approach using the proteasome inhibitor PS519. These findings could signal a significant advance on the horizon in the ability of stroke patients to be successfully treated with drugs. The authors propose that the proteasome inhibitor blocked NFκB activation and reduced the production of adhesion molecules that are necessary for neutrophil invasion. Clearly, PS519 reduced infarct size and prevented neutrophil invasion into the infarcted area and thereby targeted the inflammatory response. Although activated NFκB is known to stimulate the production of adhesive proteins, there also are ways of activating NFκB that are independent of the proteasome. It would have been useful had the authors measured NFκB activation to confirm, in the present case, that proteasome inhibition actually reduced the activation of NFκB. In agreement with the present study, however, Stephenson et al have shown that NFκB is activated after focal stroke and an antioxidant that reduces stroke damage prevented the activation of NFκB.

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