

# Delayed Treatment of Ischemia/Reperfusion Brain Injury Extended Therapeutic Window with the Proteasome Inhibitor MLN519

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**Background and Purpose**—Clinical development of novel neuroprotection therapies for the treatment of brain injury has been unsuccessful. One critical limitation is the lack of a viable therapeutic treatment window (TW). In this study, we evaluated the neuroprotection TW for the proteasome inhibitor MLN519 after ischemia/reperfusion brain injury in rats as related to its antiinflammatory mechanism.

**Methods**—Male Sprague-Dawley rats were subjected to 2 hours of middle cerebral artery occlusion (MCAo), followed by 70 hours of reperfusion and recovery. MLN519 was administered after injury (starting 6 to 12 hours after MCAo) to evaluate the full TW. Brain infarction, neuronal degeneration, neurological recovery, leukocyte infiltration, and inflammatory gene mRNA levels were assessed.

**Results**—Core infarct volume in vehicle-treated rats ( $216 \pm 25$  mm<sup>3</sup>) was reduced with delayed MLN519 treatments of 6, 8, or 10 hours after injury ( $45 \pm 13$ ,  $86 \pm 28$ , and  $150 \pm 27$  mm<sup>3</sup>, respectively,  $P < 0.05$ ) and was associated with reductions in neuronal and axonal degeneration. MLN519-treated rats had reduced brain mRNA levels of TNF- $\alpha$  (46%,  $P < 0.05$ ), ICAM-1 (58%,  $P < 0.05$ ), IL-6 (58%,  $P < 0.05$ ), and E-selectin (72%,  $P < 0.05$ ) at 24 hours after injury. Furthermore, MLN519 treatment reduced leukocyte infiltration by 32% to 80% ( $P < 0.05$ ) in ischemic brain regions.

**Conclusions**—Neuroprotection treatment with MLN519 provides an extended TW of up to 10 hours after ischemia/reperfusion brain injury, in part by attenuating the inflammatory response. As such, the delayed onset of brain inflammation after an ischemic injury offers a prime target for extending the neuroprotective TW with compounds such as MLN519, used either alone or possibly as an adjunctive therapy with thrombolytic agents. (*Stroke*. 2004;35:1186-1191.)

**Key Words:** middle cerebral artery occlusion ■ inflammation ■ brain injuries ■ proteasome

Despite aggressive research into developing neuroprotection treatments for brain injury, no drugs have proven successful in advanced clinical trials. Several concomitant factors have likely contributed to these failures, including poor preclinical pharmacodynamic evaluations, especially narrow treatment windows (TWs).<sup>1</sup> Inclusion of a surrogate marker of drug activity, evaluation of long-term recovery in both permanent and transient focal stroke models, improved patient selection criteria and trial design based on the preclinical modeling, and targeting an adequate clinical dose would also enhance the probability of success in future clinical trials.<sup>1</sup> Clearly, the development of preclinical (ie, animal) testing must focus on these factors to improve opportunities for successful transitions from preclinical to clinical studies in which secondary cell death mechanisms are the necessary target of delayed treatment effects. The current study was designed to measure the full therapeutic neuroprotection TW of the proteasome inhibitor MLN519 in a 72-hour recovery model of ischemia/reperfusion brain injury in the rat

using an optimal dosing schedule (initiated 6 to 12 hours after injury) that was chosen based on earlier dose-response studies with this compound.<sup>2-4</sup>

## Materials and Methods

### Surgical Procedures

Male Sprague-Dawley rats (270 to 330 g; Charles River Labs, Raleigh, Va) were used in all studies. All surgical procedures were performed using aseptic technique. Intravenous catheters and 2 epidural electroencephalographic (EEG) electrodes were chronically implanted into all animals, as described in detail elsewhere.<sup>2,3,5</sup> Body temperature was maintained normothermic ( $37 \pm 1^\circ\text{C}$ ) throughout all surgical procedures.

After 48-hour recovery, temporary focal ischemia was induced under 2% halothane anesthesia using the filament method of middle cerebral artery occlusion (MCAo) and reperfusion.<sup>6</sup> The filament remained in place for 2 hours, at which time the animal was re-anesthetized for approximately 5 minutes and the filament retracted.

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## Neuroprotection Studies

MCAo/reperfusion was performed on each animal ( $n=6$  to 8/group), followed by a delayed injection of MLN519 (1.0 mg/kg, intravenous) starting at 6, 8, 10, or 12 hours after occlusion. Additional injections of MLN519 (1.0 mg/kg, intravenous) were administered at 24 and 48 hours to all animals. Rectal temperature was monitored throughout the recovery period (ie, 2, 4, 6, 24, 48, and 72 hours). A 2-minute EEG recording was taken from anesthetized animals before MCAo and again at 2 hours (immediately before reperfusion). At the conclusion of the experiment (72 hours after MCAo), rats were euthanized by decapitation, and the brain tissue was removed for histological evaluation.

## Infarct Analysis

From each rat brain, analysis of cerebral ischemic damage included total and core infarct volume as well as hemispheric infarct size using triphenyl tetrazolium chloride (TTC) staining.<sup>5</sup> Computer-assisted image analysis was used to calculate infarct volumes from 7 coronal brain sections (2 mm thick), as described in detail elsewhere.<sup>7</sup> Core injury was defined as brain tissue completely lacking TTC staining, whereas total injury volume included all compromised brain regions as compared with the contralateral brain hemisphere. Hemispheric swelling (edema) was expressed as the percent increase in size of the ipsilateral (injured) brain hemisphere over the contralateral (uninjured) hemisphere.

## Neurological Examination

A neurological examination was performed on each rat immediately before MCAo, before reperfusion at 2 hours, and again at 24, 48, and 72 hours. Neurologic scores (NS) were derived using a 10-point scale. Each animal was examined for reduced resistance to lateral push (score=4), open field circling (score=3), and shoulder adduction (score=2) or contralateral forelimb flexion (score=1) when held by the tail (modified from<sup>8</sup>). NS of 10 indicated maximal neurological deficit.

## Histology

TTC-stained brain slices (fixed in 10% formalin) were subsequently paraffin-embedded using routine histological procedures as described previously.<sup>2</sup> Briefly, paraffin-embedded tissue was cut into 6- $\mu$ m sections from the second, third, and fourth coronal brain sections. Brain sections were stained with hematoxylin and eosin (H&E) or FluoroJade. Twelve 40 $\times$  microscope fields from both striatal and cortical brain regions of each H&E slide were used to count infiltrating leukocytes (neutrophils and macrophages) by an observer blinded to the treatment group.<sup>3</sup> Degeneration of neurons and axons was analyzed using FluoroJade staining.<sup>3</sup> Slides were de-waxed and placed in 0.06% potassium permanganate for 15 minutes. Slides were then washed in deionized water and incubated in 0.001% FluoroJade (Histo-Chem Inc).

## Molecular Studies

Separate groups of rats ( $n=6$  to 8 /group) received an intravenous injection of vehicle (50% polypropylene glycol [PPG], 50% saline) or MLN519 (1.0 mg/kg), but only at 6 hours after MCAo. At either 24 or 72 hours after injury, rats were euthanized and brain tissue was immediately collected. A 3-mm coronal section was taken from the area perfused by the MCA, starting 5 mm from the frontal pole. Approximately 100 mg of tissue was collected from the ipsilateral (injured) and contralateral (uninjured) hemispheres. Sham animals underwent identical procedures including surgery, but the filament was not inserted into the MCA.

## TaqMan Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Samples were processed as previously described.<sup>9</sup> Briefly, the tissue samples were homogenized in TRIzol reagent (Life Technologies) and total RNA was extracted from the tissue. Reverse-transcriptase reactions were performed using the RNA PCR Core Kit (Perkin

Elmer). Primer and probe sequences used for each gene are the same as previously reported.<sup>9</sup> Amplification was performed using the ABI PRISM 7700 sequence detection system (PE Applied Biosystems). All samples were performed in duplicate.

## Data Analysis

Data were presented as the mean $\pm$ standard error of the mean. Quantitation of data was evaluated by analysis of variance (ANOVA). Post-hoc Tukey analysis was used to compare differences between groups, or Bonferroni analysis (adjusted for multiple comparisons) was used to compare MLN519 to vehicle treatment;  $P<0.05$  was considered significant. Two animals were eliminated from the study based on the following inclusion criteria (as recorded at 2 hours after injury): (1) a  $>80\%$  decrease in EEG and (2) a maximal NS of 10.<sup>2,5</sup>

## Compound

MLN519 was received from Millennium Pharmaceuticals. The compound was dissolved in a solution of 50% PPG in physiological saline immediately before injection and administered intravenously without handling or disturbing normal animal behavior.

## Results

### Brain Pathology After 2-Hour MCAo/72-Hour Recovery

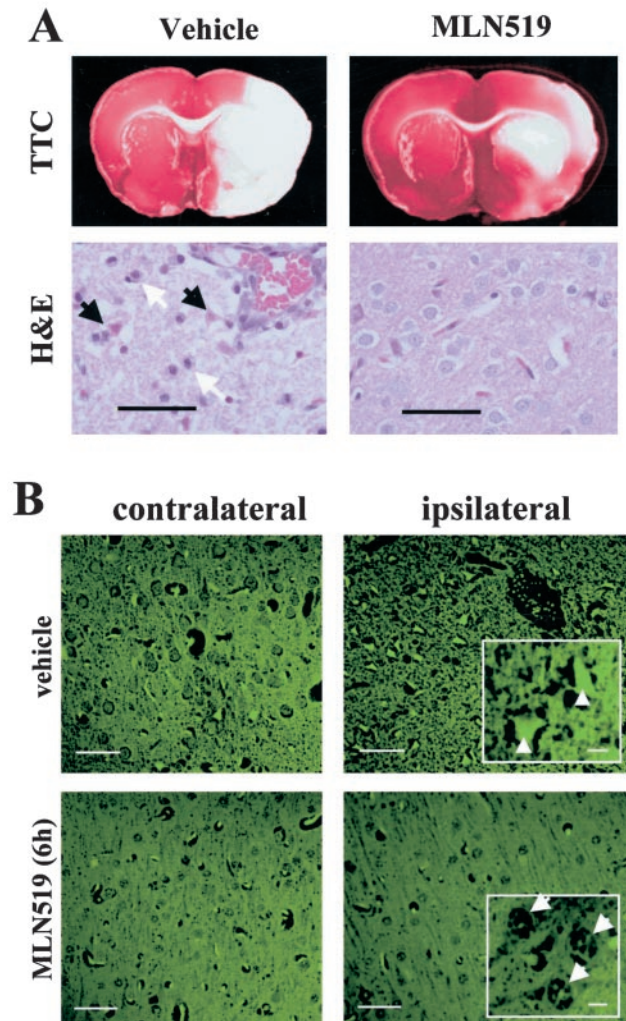
Vehicle-treated rats exhibited striatal and cortical infarctions ipsilateral to the site of injury as indicated from TTC-stained brain sections (Figure 1A). Specifically, ischemic regions included the temporal, parietal, and piriform cortices, as well as the caudoputamen, internal capsule, and, rarely, the anterior thalamus or hypothalamus. Regions of infarct visible from TTC-stained tissue were also identified in sequential H&E slides as hypereosinophilic, polygonal, shrunken cells present in the pale staining neuropil (Figure 1A).

### Neuronal Degeneration (FluoroJade Staining)

Positive FluoroJade immunofluorescence staining was used to indicate neuronal degeneration (Figure 1B). In the contralateral parietal cortex of both vehicle- and MLN519-treated rats, normal neuronal morphology was present, as indicated in cortical layers II/III, and was comparable to normal or sham-injured animals (not shown). In contrast, in the ipsilateral parietal cortex of vehicle-treated animals, severe neuronal degeneration was present as indicated by the strong fluorescence labeling of neurons and loss of axonal processes. FluoroJade positive cells were largely absent from the parietal cortex after a 6-hour delayed treatment of MLN519. In rats treated with MLN519 at 10 hours after MCAo, a mix of both normal and degenerate neuronal morphology was observed (not shown).

### Brain Infarct Volume (TTC Staining)

Average core infarct volume for the vehicle-treated animals was  $216\pm 25$  mm<sup>3</sup> ( $27\pm 5\%$  hemispheric infarction). MCAo injury also induced an increase in brain volume of  $5\pm 1\%$  in the vehicle-treated group. MLN519-treated groups exhibited significantly lower core infarct volumes [ $F(4, 34)=7.08$ ,  $P<0.001$ , ANOVA] with delayed injection times of 6 hours (79% reduction,  $P<0.05$ ), 8 hours (60% reduction,  $P<0.05$ ), and 10 hours (31% reduction,  $P<0.05$ ), but not 12 hours (Figure 2). Concurrent with the reduction in core

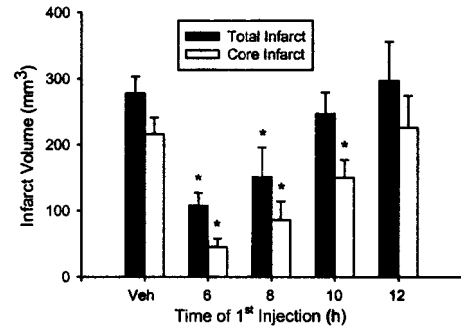


**Figure 1.** Representative coronal brain sections from vehicle-treated and MLN519-treated (injections starting 6 hours after MCAo) animals (72 hours after MCAo). (A) TTC-stained and H&E-stained sections are compared. Black arrows indicate dying neurons and white arrows indicate typical inflammatory macrophages; bar=50  $\mu$ m. (B) FluoroJade-stained sections between the contralateral and ipsilateral parietal cortex are compared; bar=50  $\mu$ m. High-magnification insets indicate degenerate neurons in vehicle-treated animals (FluoroJade positive cells, white arrowheads) and normal neuronal morphology in MLN519-treated animals (FluoroJade negative cells, white arrows); bar=10  $\mu$ m.

infarct, total infarct volume was also reduced [ $F(4, 34)=4.84$ ,  $P=0.04$ , ANOVA]. In all groups, the total infarct volume included a surrounding peri-infarct region of tissue that encompassed a volume ranging from 62 to 97  $\text{mm}^3$  (total minus core infarct volume). No significant reduction in edema/brain swelling was measured, with average increases in ipsilateral brain volume ranging from 3% to 5% in MLN519-treated animals.

### Neurological Recovery

Significant neurological impairment was observed at 24 hours after injury, with a gradual but significant recovery over 72 hours across all treatment groups [ $F(4, 104)=48.02$ ,  $P<0.001$ , ANOVA]. Neurological testing of vehicle-treated



**Figure 2.** Effect of delayed treatments with MLN519 to reduce infarction. A single delayed injection was administered at 6, 8, 10, or 12 hours after MCAo, followed by additional injections at 24 and 48 hours after MCAo. Values are mean $\pm$ SEM. \* $P<0.05$  as compared with vehicle.

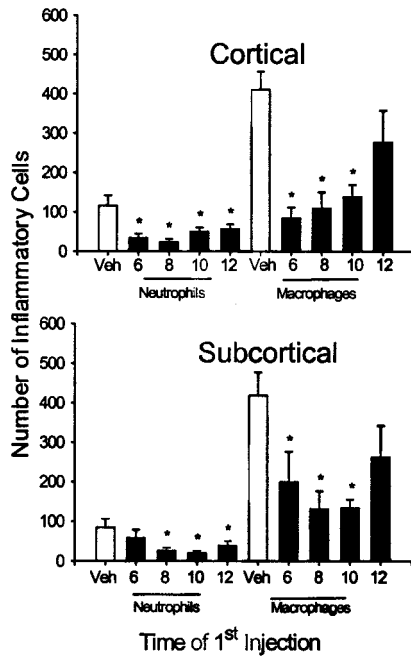
animals revealed scores of  $8.00\pm 21.29$ ,  $4.43\pm 1.48$ , and  $2.71\pm 0.81$  (24, 48, and 72 hours, respectively) out of a maximal deficit score of 10. Analysis of variance also revealed a significant difference between treatment groups [ $F(4, 104)=5.38$ ,  $P=0.001$ , ANOVA]. Post-hoc analysis revealed that treatment with MLN519 delayed by 6 hours induced significant improvements of  $3.43\pm 1.15$ ,  $1.00\pm 0.38$ , and  $0.71\pm 0.18$  (24, 48, and 72 hours, respectively) ( $P<0.05$ , Bonferroni). Compared with the corresponding vehicle groups, neurological deficits were also reduced from 20% to 39% in all other MLN519 treatment groups, albeit not reaching statistical significance ( $P>0.05$ , Bonferroni).

### Leukocyte Infiltration (H&E Staining)

At 72 hours, H&E staining showed the presence of neutrophils and macrophages in the ischemic regions of brain-injured animals (Figure 1A, H&E), with rare detection in the contralateral hemispheres of normal or sham animals. A significant difference in leukocyte counts was measured between treatment groups [ $F(4, 123)=11.84$ ,  $P<0.001$ , ANOVA], as well as a significantly higher number of macrophages over neutrophils at the 72-hour time point [ $F(4, 123)=70.62$ ,  $P<0.001$ , ANOVA]. However, no significant differences were measured between cortical and subcortical brain regions [ $F(4,123)=0.04$ ,  $P=0.847$ , ANOVA]. Post-hoc analysis revealed significant reductions ( $P<0.05$ , Bonferroni) in leukocyte counts from each of the MLN519 treatment groups as compared with vehicle (Figure 3). Average macrophage counts for vehicle-treated animals were  $411\pm 46$  (cortical) and  $419\pm 59$  (subcortical) cells per injured hemisphere, with reductions ranging from 32% to 80% (cortical) and 37% to 69% (subcortical) after delayed treatment with MLN519. Average neutrophil counts for vehicle-treated animals were  $116\pm 27$  (cortical) and  $85\pm 21$  (subcortical) cells per injured hemisphere, with reductions ranging from 51% to 79% (cortical) and 32% to 76% (subcortical) after delayed treatment of MLN519.

### Core Body Temperature

Average body temperature in all animals ranged from  $36.5^\circ\text{C}$  to  $37.6^\circ\text{C}$  before MCAo surgery. Analysis of variance revealed no significant differences between treatment groups at any time point [ $F(4, 208)=1.64$ ,  $P=0.165$ ]; however, there



**Figure 3.** Neutrophil and macrophage counts from cortical and subcortical injured brain hemispheres in vehicle-treated and MLN519-treated rats. Values are mean±SEM. \**P*<0.05 compared with corresponding vehicle.

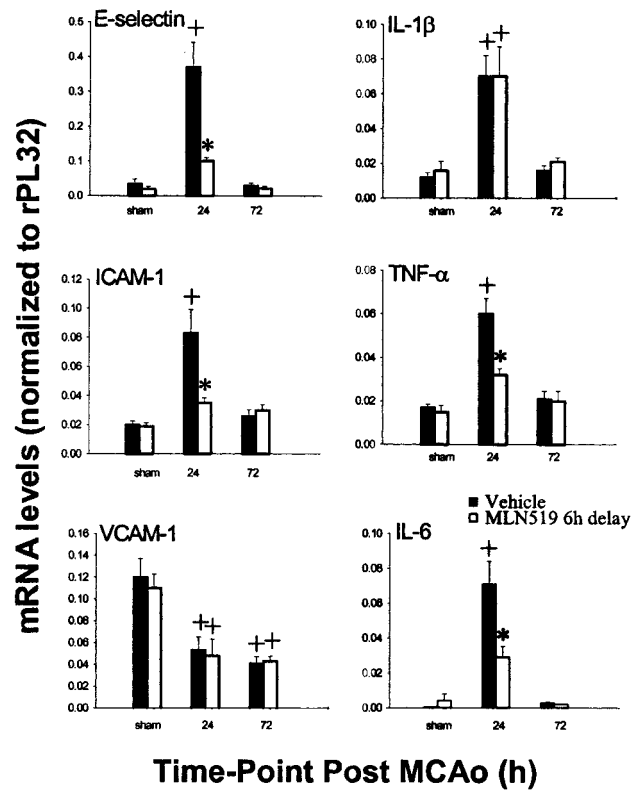
were statistically significant changes over time [F(5, 208)=21.39, *P*<0.001]. In general, after MCAo, a slight increase in mean body temperature of 0.2°C to 1.0°C was measured at 2 hours after injury (*P*<0.01, Tukey) immediately before reperfusion. Body temperature was normalized by 6 to 24 hours after injury. A slight decrease in mean temperature of 0.9°C to 1.1°C (*P*<0.01, Tukey) was measured at 48 hours, which persisted through the final 72 hours (0.5°C to 1.9°C, *P*<0.01, Tukey) as compared with preinjury baseline values. Importantly, these changes in body temperature were similar to previous reports with this model<sup>2,5,7</sup> and were measured across all groups (nontreated and treated).

### Changes in Expression of Inflammatory Gene mRNA

Significant changes occurred in the expression of all genes studied (Figure 4, *P*<0.001, ANOVA). In vehicle-treated animals, the expression of all genes (except VCAM-1) was significantly upregulated at 24 hours after injury as compared with sham levels (*P*<0.05 for all groups, Tukey), with a return to within baseline values by 72 hours. After MLN519 treatment, significant reductions in the 24-hour peak upregulation of E-selectin (72%), ICAM-1 (58%), TNF-α (46%), and IL-6 (58%) were measured. In contrast, no significant differences were measured in IL-1β and VCAM-1 mRNA levels between vehicle- and MLN519-treated animals at either time point tested. VCAM-1 levels gradually decreased in both treatment groups with a 56% to 68% reduction by 24 to 72 hours after injury.

### Discussion

Neuronal injury after an ischemic attack is an evolving process that can continue for days after injury.<sup>10,11</sup> Strong



**Figure 4.** Postinjury expression of cytokine and adhesion molecule mRNA from vehicle-treated and MLN519-treated animals. Y-axis values represent rPL32 normalized mRNA levels. Values are mean±SEM. \**P*<0.05 compared with corresponding vehicle. +*P*<0.05 compared with sham.

evidence now exists indicating that active, delayed injury processes are involved in, and ultimately determine, the eventual degree of cell survival after injury.<sup>12</sup> The inflammatory response is a complex and multi-step delayed injury process that includes inflammatory gene upregulation, release of chemotactic agents into the blood stream, and activation/recruitment of peripheral leukocytes. Diapedesis of inflammatory cells into the injured brain begins with an initial phase of neutrophil infiltration, which peaks at 24 hours after injury, followed by macrophage infiltration at 72 hours after injury.<sup>11</sup> Inflammatory cells not only dispose of cellular debris but also are a major source of postinjury toxins, including reactive oxygen species and pro-inflammatory cytokines.<sup>13</sup> Although phagocytic evacuation of dead cells is part of the natural healing process, it has been purported that overactivation can potentially stress cells adjacent to the core infarcted region.<sup>13,14</sup>

One mechanism for reducing inflammation after injury is to shut down the inflammatory gene response of cells. Activation of the transcription factor NF-κB is largely responsible for upregulation of a host of inflammatory genes after ischemic brain injury.<sup>9</sup> Proteasome inhibition is one method of selectively blocking the activation of NF-κB by inhibiting its release from the inhibitory κB molecule.<sup>15,16</sup> Previous studies with the proteasome inhibitor MLN519 have directly related the neuroprotective effects to reversible proteasome inhibition, reduction of NF-κB activation, and asso-

ciated inflammatory gene upregulation, as well as inhibiting the infiltration of inflammatory cells into the ischemic rat brain.<sup>2,3,17</sup> Furthermore, no apparent behavioral toxicity has been associated with MLN519 treatment, including no significant alteration of body temperature or physiological parameters in MCAo-injured animals,<sup>2,3</sup> or in a recent phase I clinical trial.<sup>4</sup> A therapeutic benefit for MLN519 has also been demonstrated in the embolic clot model of ischemic/reperfusion injury in rats, in which it was shown not only to extend the TW of tissue plasminogen activator but also to reduce infarction when administered alone 4 hours after injury.<sup>18</sup> Critically, this same study also demonstrated that the therapeutic benefit was associated with a reduction in the brain inflammatory response. Other studies with MLN519 have indicated an effective treatment of myocardial reperfusion injury in pigs as related to a reduction in NF- $\kappa$ B activity.<sup>19</sup>

It has been previously shown that treatment with MLN519 is well tolerated at doses that reversibly reduce 20S proteasome levels by up to 80% in healthy human volunteers, although MLN519 is rapidly cleared from the vasculature (likely into blood cells and endothelium).<sup>4</sup> Based on previous neuroprotection dose–response studies<sup>2,3</sup> and studies in brain injured rats demonstrating a transient reduction in blood proteasome levels by 80% to 90%,<sup>3</sup> an optimal dose of 1.0 mg/kg of MLN519 was chosen for this study. Using the same ischemia/reperfusion model but extending survival time to 3 days, the current study indicated a therapeutic treatment window of up to 10 hours to significantly reduce brain injury. These results indicated a significant improvement in the TW seen here (ie, 10 hours in the delayed 72-hour recovery model) as compared with earlier studies in which a more acute recovery model was used (ie, 4-hour TW with the 24-hour recovery model<sup>3</sup>). Relevant to this observation was the fact that limited macrophage presence was measured at 24 hours after injury as opposed to the predominance of neutrophils at this early time point.<sup>3</sup> The increased TW observed in the current study was probably caused, in part, by the profound increase in macrophage infiltration into the ischemic brain occurring from 24 to 72 hours after injury, which was significantly attenuated with MLN519 treatment.

In conjunction with the reduction of infarction, MLN519 also reduced gross neuronal degeneration throughout the ischemic region, as verified using FluoroJade.<sup>20</sup> A significant degree of spontaneous neurological recovery ( $\approx$ 65%) was also measured over the 72-hour recovery period in the vehicle-treated animals. However, neurological improvement was measured in all MLN519 treatment groups, albeit only statistically significant with the 6-hour delayed treatment group. Currently, we are evaluating the effects of delayed MLN519 treatment on functional recovery from both transient and permanent MCAo injury at extended survival time points. In particular, preliminary data from our laboratory indicates that MLN519 treatment (1.0 mg/kg, initiated 10 hours after MCAo with additional injections administered once per day for 14 days) reduced the number of foot-fault deficits (beam-walk task) evaluated 14 days after MCAo injury (unpublished observations).

MLN519 was very effective in reducing inflammatory cell infiltration, even with delayed treatments. In particular, significant reductions in neutrophil and macrophage counts were detected regardless of the delayed treatment time or brain region. Previous studies revealed that the effect of MLN519 was linked to a downregulation of cell adhesion molecules, particularly in endothelial cells.<sup>17</sup> This cell-specific effect of MLN519 to reduce inflammatory gene expression may reflect the relatively poor penetration of MLN519 across the blood–brain barrier,<sup>2</sup> indicating that the crucial target of this type of therapy may be at the level of the blood–brain barrier to interfere with inflammatory cell infiltration. It should also be noted that the effects of MLN519 might be acting directly on peripheral leukocytes to inhibit their movement into the brain. In the current study, the 6-hour delayed injection of MLN519 was also effective at reducing E-selectin and ICAM-1 cell adhesion expression by  $>50\%$ , along with a significant reduction of cytokine expression (IL-6 and TNF- $\alpha$ ). All 4 of these genes are involved in the inflammatory recruitment of peripheral blood cells into the injured brain.<sup>13</sup>

In conclusion, these results demonstrate a TW for MLN519 of up to 6 to 10 hours after injury, depending on the outcome measure evaluated. These neuroprotective properties correlated to reductions in levels of inflammatory gene expression and reduced leukocyte infiltration into the injured brain. Importantly, the TW was shown to be extended after a longer recovery period after injury as compared with previous studies. These results along with findings from other published studies would suggest that inflammatory cell infiltration plays a significant role in secondary brain injury and is an effective target for neuroprotective intervention.

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