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Recovery from ischemic brain injury in the rat following a 10 h delayed injection with MLN519☆

Anthony J. Williams*, Todd M. Myers, Scott I. Cohn, Keith M. Sharrow, Xi-Chun M. Lu, Frank C. Tortella

Department of Applied Neurobiology, Division of Psychiatry and Neuroscience, Walter Reed Army Institute of Research, 503 Robert Grant Ave, Room 2A40, Silver Spring, MD 20910, USA

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Abstract

In the present study, we evaluated delayed treatment effects of the proteasome inhibitor and anti-inflammatory agent MLN519 (initiated 10 h post-injury) to improve recovery following ischemic brain injury in rodents. Male rats were exposed to 2 h of middle cerebral artery occlusion (MCAo) and treated with MLN519 (1.0 mg/kg, i.v. @ 10, 24, and 48 h post-occlusion) or vehicle. By 2 weeks post-injury, 60% (6/10) of vehicle animals survived, which was improved (although non-significantly) to 78% (7/9) following MLN519 treatment. The percent loss of tissue in the ipsilateral brain hemisphere (at 2 weeks) was significantly reduced from 27±4% (vehicle) to 15±4% (MLN519). MLN519 treated animals also lost significantly less body weight (39%) and showed significant improvement in overall neurological function across the 2-week recovery period. However, no significant treatment effects were observed to reduce foot-fault deficits (balance beam) or improve recovery of operant performance (active avoidance test). Overall, delayed treatment with MLN519 provided significant improvement in 3 of 6 test metrics (histopathology, body weight, and neurological dysfunction) supporting improved outcome for brain-injured subjects.
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Keywords: Ischemic brain injury; MCAo; MLN519; Neuroprotection; Proteasome; Stroke

1. Introduction

To date, clinical trials have met with limited success for the treatment of ischemic brain injury. The induction of reperfusion following stroke with recombinant tissue plasminogen activator is the only approved therapy but treatment is limited to the first 3 h post-injury (DeGraba and Pettigrew, 2000; Janardhan and Qureshi, 2004). Unfortunately, most stroke victims do not receive medical care within 3 h indicating the continued need for development of novel compounds for the treatment of ischemic brain injury.

Extensive basic research studies within the past decade have provided a wealth of information on the mechanisms involved in the promotion of brain injury (Lipton, 1999; Bramlett and Dietrich, 2004). In particular, research has focused on altering delayed cell death mechanisms including the induction of inflammation and delayed apoptotic cell death (Janardhan and Qureshi, 2004). In fact, treatment with anti-inflammatory compounds that target the ubiquitin proteasome system (UPS) have shown excellent pre-clinical efficacy for the treatment of brain injury with a wide therapeutic window of opportunity for initiation of treatment (Wojcik and Di Napoli, 2004). The UPS is an important cellular regulatory system involved in non-lysosomal proteolysis. Although a major role of the UPS is to degrade misfolded or damaged proteins, this system is also used by cells to activate transcriptional regulatory proteins such as nuclear factor κB (NF-κB). In particular, NF-κB is involved

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* Corresponding author. Tel.: +1 301 319 9461; fax: +1 301 319 9905.
E-mail address: anthony.williams@na.amedd.army.mil (A.J. Williams).

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in the upregulation of several pro-inflammatory genes that promote the neuro-inflammatory response of the brain to injury (Elliott et al., 2000; Elliott and Ross, 2001).

Recent experimental studies have indicated that the 20S proteasome inhibitor MLN519 has a neuroprotective function in in vivo models of brain injury related to an anti-inflammatory effect (Phillips et al., 2000; Zhang et al., 2001; Berti et al., 2003; Williams et al., 2003, 2004). In particular, an optimal treatment regiment with MLN519 has indicated a therapeutic window out to 10 h post-injury to reduce brain infarction and improve functional recovery as assessed out to 72 h following middle cerebral artery occlusion in rats (Williams et al., 2004). The objective of the current study was to extend the evaluation period and verify the neuroprotective effects of MLN519 out to 2 weeks following MCAo injury in rats.

2. Materials and methods

2.1. Design

Male Sprague–Dawley rats (270–330 g; Charles River Labs, Raleigh, VA) were subjected to a 2 h MCAo injury or sham surgery followed by a 2 week recovery period. All procedures were approved by the Walter Reed Army Institute of Research Animal Care and Use Committee, and all research was conducted in compliance with the animal welfare act, Guide for the Care and Use of Laboratory Animals (National Research Council) and other federal statutes and regulations relating to animals and experiments involving animals. Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Four groups of animals were evaluated; MLN519/MCAo, MLN519/sham, vehicle/MCAo, vehicle/sham. A bolus injection of MLN519 or vehicle was administered to each animal at 10, 24, and 48 h following MCAo or sham surgery. Body weight was recorded pre-MCAo, and again at 24 h, 48 h, 72 h, 7 days, 11 days, and 14 days post-injury. Body temperature was recorded using a rectal probe pre-MCAo, during ischemia at 30 min and 2 h (prior to reperfusion), and again during the reperfusion period at 4 h, 6 h, 24 h, 48 h, 72 h, 7 days, 11 days, and 14 days post-injury. On the 14th day animals were deeply anesthetized, euthanized, and brain tissue was collected for analysis. Drug treatment was randomized and all behavioral testing (neurological scoring, balance beam testing, and active avoidance behavior) and histopathological analysis performed by an experimenter blind to the treatment group.

2.2. General surgical procedures

During all surgical procedures animals were maintained under gas anesthesia delivered through a face-mask (5% induction followed by 2% maintenance with isoflurane) and body temperature was maintained normothermic (37±1 °C) by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA). Chronic indwelling cannulas were placed into the right jugular vein of all animals 5 days prior to MCAo or sham surgery for i.v. drug injections (Tortella et al., 1999). Food and water were provided 'ad libitum' pre- and post-surgery and the animals were individually housed under a 12 h light/dark cycle.

2.3. Middle cerebral artery occlusion (MCAo)

Temporary focal brain ischemia was induced using the filament method of MCAo and reperfusion as described elsewhere (Tortella et al., 1999). Briefly, the right external carotid artery was isolated and its branches coagulated. A piece of 3-0 uncoated monofilament nylon suture with rounded tip was introduced into the internal carotid artery via the external carotid artery and advanced (approximately 22 mm from the carotid bifurcation) until a slight resistance was observed, thus occluding the origin of the middle cerebral artery (MCA). The MCAo procedure averaged 15 min in duration. The rats were allowed to recover from anesthesia for 2 h and were then re-anesthetized (approximately 5 min in duration) for removal of the endovascular suture to allow reperfusion of blood to the MCA.

2.4. Neurological scoring

A neurological examination (derived from (Bederson et al., 1986) was performed on each rat immediately prior to MCAo and again at 2 h (prior to reperfusion), 24, 48, and 72 h post-occlusion. Neurologic Scores (NS) were derived using a 10 point sliding scale. Each animal was examined for reduced resistance to lateral push (score=4), open field circling (score=3), shoulder adduction (score=2), and contralateral forelimb flexion (score=1) when held by the tail (Tortella et al., 1999). Rats extending both forelimbs toward the floor and not showing any other signs of neurological impairment were scored 0. Using this procedure, maximal neurological deficit was measured as an NS=10. In the present study, all rats subjected to MCAo either exhibited a neurological score of 10 when examined 2 h post-ischemia, immediately prior to reperfusion, or were excluded from the study.

2.5. Balance beam testing

A 135 cm long tapered balance beam (Dragonfly Inc., Ridgeley, WV) (Williams et al., 2005) was used to assess non-compensatory foot-fault deficits (based on a design by Schallert et al., 2002). The balance beam included a tapered surface of 5.5 cm width at the starting end and 1.5 cm width at the opposite, finishing end. Along each side of the beam, a 2 cm wide ledge was positioned 2 cm below the beam surface, which served as a 'crutch' for impaired animals to walk on, avoiding compensatory changes in

posturing and weight distribution by the animal (Schallert et al., 2003). Each ledge was also equipped with mechanical sensors and digital recorders to detect the number of left or right foot-faults (steps on the crutch). A 15.0×5.5 cm platform was positioned at the starting end of the beam as a staging area for the animal to initiate the task and a black box placed at the finishing end as a reinforcer. Rats were trained prior to the MCAo or sham surgery until each animal was able to successfully ambulate along the beam without foot-faults. If necessary, a mild tail pinch was used to motivate the animal. Foot-faults were recorded prior to MCAo or sham surgery and again following MCAo at 7, 11, and 14 days post-surgery. The number of left and right foot-fault deficits were summed separately from three consecutive runs.

2.6. Active avoidance behavior

Cognitive behavior was evaluated by active avoidance testing in Hamilton–Kinder avoidance chambers (Clark et al., 2003). Rats were trained to avoid an unconditional stimulus (US, e.g. a brief mild electric shock, 1.0 mA direct current) by moving into an adjacent compartment within 5 s after a conditional stimulus (CS, i.e. light) presentation. To avoid or escape the US, the rat was required to move to the adjacent compartment. If the animal failed to escape, the CS and US automatically terminated after 15 s. Each animal performed 51 trials per daily session. The number of avoidance and escape responses, their respective latencies, and intertrial responses (a measure of spontaneous locomotor activity) were recorded.

2.7. Histopathological analysis

From each rat brain, quantitation of cerebral damage was achieved using a TTC (2,3,5-triphenyl tetrazolium chloride) staining method as described in detail elsewhere (Tortella et al., 1999). In brief, brain sections were taken from seven coronal sections (2 mm thick), beginning 1 mm from the frontal pole and ending just rostral to the cortico-cerebellar junction. The posterior surface of each TTC stained forebrain section was digitally imaged (Loats Associates, Westminster, MD) to allow demarcation of regions of interest (i.e. infarct region or total area of left and right hemisphere). Sequential integration of the respective areas from each slice yielded respective total volumes (mm^3). The amount of tissue lost (i.e. due to cavitation and phagocytic evacuation of injured tissue) was calculated as the difference between the contralateral and ipsilateral hemispheric volume. We also calculated the amount of viable brain tissue (i.e. TTC-positive tissue) equal to the difference between the ipsilateral hemispheric volume and infarct volume. All volume data were normalized to the respective contralateral hemisphere to account for variation in overall brain size and presented as a percent hemispheric volume.

2.8. Drug preparation and administration

MLN519 was obtained from Millenium Pharmaceuticals (Cambridge, MA). MLN519 (1.0 mg/kg, i.v.) was delivered in a vehicle of 50/50 physiological saline and polypropylene glycol (bolus injection of l.0 ml/kg) at 10, 24, and 48 h following induction of MCAo.

2.9. Data analysis

Kaplan–Meier survival curves were used to compare survivability between treatment groups. Histological endpoints were evaluated by independent *t*-tests between treatment groups. Repeated measures were evaluated by ANOVA followed by a post hoc *t*-test analysis (with a Bonferroni correction for multiple comparisons) between matched vehicle and MLN519 treated groups or Dunnetts test to compare repeated measures to baseline values. Pearson's correlation was used to evaluate the relationship between endpoint parameters.

3. Results

3.1. Sham animals

Ten rats were subjected to sham surgery ($n=5$ per treatment group). All sham rats survived to 14 days post-surgery with no histopathological brain damage as assessed by TTC staining (not shown). ANOVA did not indicate significant deficits in neurological outcome, balance beam, or active avoidance behavior post-injury. Animals maintained body temperature throughout the recovery period (ANOVA, $P>0.05$ across both Time and Treatment) with average temperatures ranging from 36.6–37.7 °C. A significant change in body weight was measured across Time ($P<0.001$) but not Treatment ($P=0.077$) with no significant Time * Treatment interaction ($P=0.855$). Both

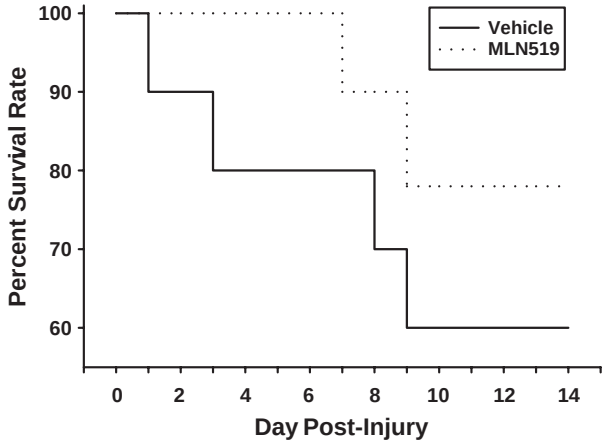

Fig. 1. Survival curves following MCAo injury. Overall survival rate was 60% (6/10) for vehicle as compared to 78% (7/9) for MLN519 treated rats (log-rank, $P=0.371$).

treatment groups had an initial body weight loss of 23–29 g from baseline values (i.e. vehicle=286±8 g and MLN519=281±5 g pre-sham surgery) on day 1 following sham surgery ($P=0.014$) that recovered and was significantly higher than baseline values at day 11 (i.e. vehicle=319±10 g, MLN519=331±9 g, $P<0.01$) and day 14 (i.e. vehicle=341±10 g, MLN519=348±10 g, $P<0.001$).

3.2. Survival

Twenty-six rats were used for the MCAo studies. One animal did not recover from the reperfusion surgery. Five animals died prior to the initial injection at 10 h post-injury, 3 of which were observed to have intracranial hemorrhage upon post-mortem examination of brain tissue. One rat was excluded (neuroscore<10) at 2 h post-injury. Of the remaining 19 animals, Kaplan–Meier survival curves (Fig. 1) indicated an improved (though non-significant) survival outcome in the MLN519 treated group following MCAo (log-rank, $P=0.371$). Overall, 78% (7/9) of MLN519 treated animals survived to 14 days post-injury compared to 60% (6/10) of vehicle treated animals. From 0–7 days post-injury all MLN519 treated animals survived and 20% of vehicle animals were lost. From 7–14 days post-injury a similar rate of mortality was observed in both groups (~20%). Of all animals that did not survive, weight loss was progressive and the animals were never observed to recover body weight at any post-injury time point, which may be related to the observed mortality.

3.3. Histopathology

MCAo injured animals exhibited infarction throughout the MCA territory in cortical and subcortical tissue along with a distinct loss of hemispheric volume in the ipsilateral (injured) brain hemisphere that was reduced with MLN519 as compared to vehicle treatment (Fig. 2A). In general, the parietal, temporal and piriform cortices and subcortical regions of the caudate putamen indicated extensive infarction or tissue loss. However, the parietal cortex (forelimb motor and somatosensory regions) and caudate putamen of three MLN519 treated animals remained intact. In comparison, only one vehicle animal was observed to have minimal damage to the parietal cortex although this animal did have extensive infarction in subcortical regions.

Quantitation of infarction and hemispheric volume indicated that MLN519 treated animals had a 38% smaller infarction ($P=0.099$) and 45% less tissue loss in the injured

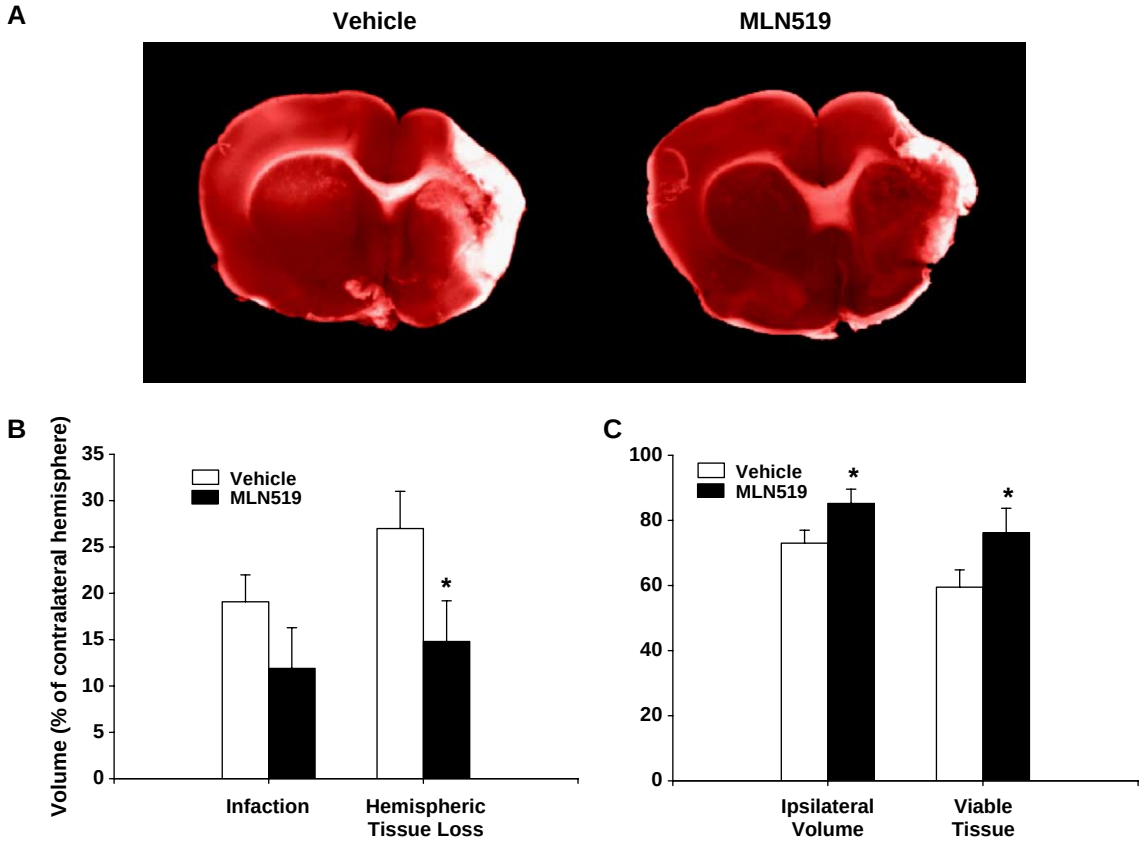

Fig. 2. Representative TTC-stained coronal brain slices from vehicle and MLN519 treated rats at 14 days post-injury (panel A). MLN519 treatment was associated with a 38% reduction of infarction and 45% less hemispheric tissue loss in the ipsilateral (injured) brain hemisphere (panel B) and had 17% more hemispheric brain volume and 22% more viable brain tissue in the ipsilateral hemisphere (panel C). *$P<0.05$ between vehicle and MLN519 groups. Values are represented as mean±S.E.M.

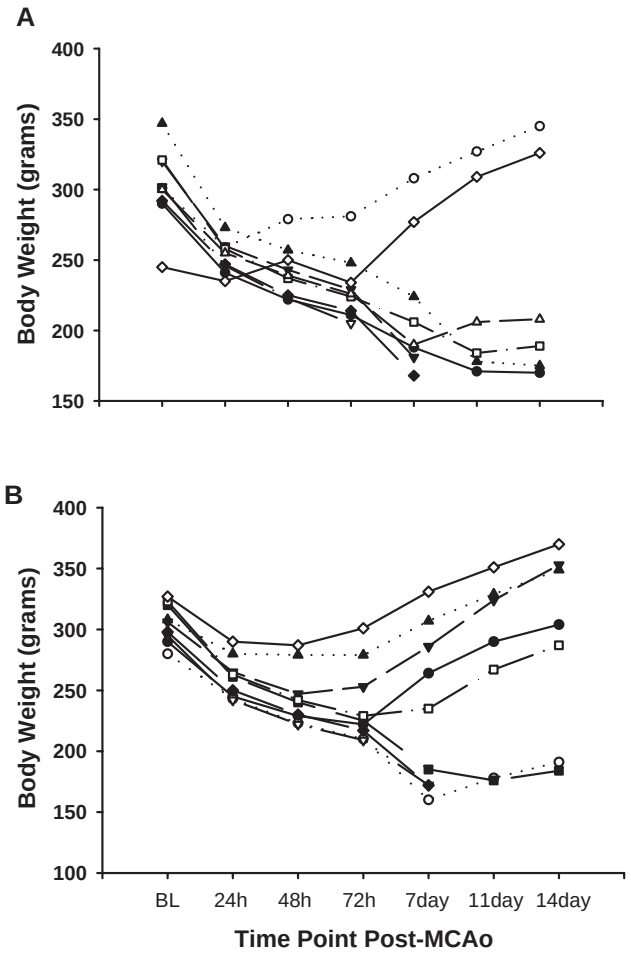

Fig. 3. MLN519 treated animals gained significantly more body weight post-injury than vehicle treated animals (ANOVA, $P<0.05$). Only 2 of 6 vehicle treated rats began to recover body weight after day 3 post-injury (panel A) as compared to 5 of 7 MLN519 treated rats (panel B).

brain hemisphere ($P=0.032$) (Fig. 2B). Subcortical infarct volume was reduced 45% from 37±6 mm^3 (vehicle) to 21±7 mm^3 (MLN519) ($P=0.053$ between groups) and cortical infarct volume was reduced 27% from 79±10 mm^3 (vehicle) to 58±21 mm^3 (MLN519) ($P=0.190$ between groups). MLN519 treated animals also had a 14% larger ipsilateral brain volume ($P=0.032$) along with 22% larger volume of viable brain tissue in the ipsilateral hemisphere ($P=0.049$) (Fig. 2C). No significant difference was measured in the total volume of the contralateral (i.e. uninjured) brain hemisphere between groups (Vehicle=871±17 mm^3 and MLN519=882±14 mm^3, $P=0.614$).

3.4. Body temperature

ANOVA of body temperatures in MCAo injured animals indicated a significant change in temperature across Time ($P=0.007$) but not Treatment ($P=0.536$). Average body temperature was elevated by 1.1–1.2 °C ($P<0.001$) from baseline values (i.e. 36.8±0.2 °C pre-MCAo) at 2 h post-occlusion and returned towards baseline values thereafter with average body temperatures ranging from 36.7 to 38.0 °C across time points and treatment groups (data not shown). Additionally, no significant Treatment*Time interaction was measured in body temperature across groups (ANOVA, $P=0.495$).

3.5. Body weight

Fig. 3 illustrates the changes in body weight recorded for each MCAo injured animal over the 2 week recovery period for both the vehicle and MLN519 treatment groups. ANOVA of MCAo injured animals indicated a significant change in body weight across both Time ($P=0.026$) and Treatment ($P=0.042$) with no significant Time*Treatment interaction ($P=0.7433$). Overall, 5 of 7 surviving MLN519 treated animals showed significant recovery of body weight beyond day 3 (Fig. 3A) as compared to 2 of 6 surviving vehicle-treated animals (Fig. 3B). A strong correlation was also measured between body weight at day 14 post-injury and both percent infarction ($r=0.794$, $P<0.001$) and percent of tissue loss in the ipsilateral hemisphere ($r=0.859$, $P<0.001$).

3.6. Neurological recovery

MCAo injured animals demonstrated significant and sustained neurological deficits post-injury that were reduced with MLN519 as compared to vehicle treatment (Fig. 4). ANOVA indicated a significant change in neuroscores across both Time ($P<0.001$) and Treatment ($P=0.040$) with no significant Time*Treatment interaction ($P=0.355$). All animals exhibited a maximal neurological deficit at 2 h post-injury (NS=10). MLN519-treated animals exhibited 0–50% lower (improved) neuroscores at each post-injury

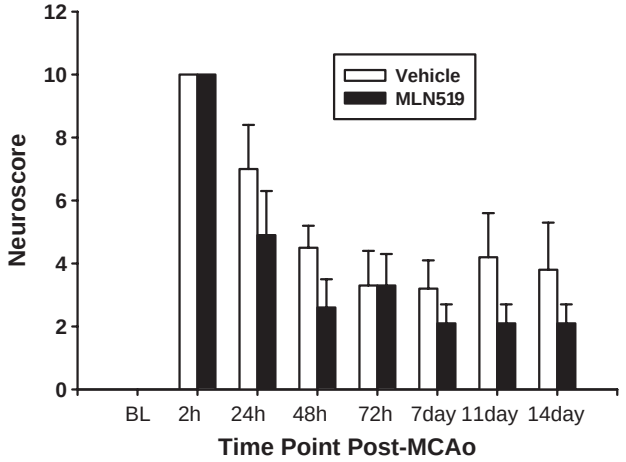

Fig. 4. MLN519 treated animals has significantly fewer neurological deficits post-injury as compared to vehicle treated animals across time post-injury (ANOVA, $P<0.05$) as exhibited by a 0–50% reduction in neuroscore at each post-injury day although no significant changes were observed at any individual time point (Dunnetts, $P>0.05$). Values are represented as mean±S.E.M.

day tested although post hoc analysis did not reveal a significant treatment effect at any individual day ($P>0.05$ at each time point).

3.7. Balance beam

ANOVA of MCAo injured animals indicated a significant change in the number of contralateral foot-faults across Time ($P=0.018$) but not Treatment ($P=0.459$), with no Time*Treatment interaction ($P=0.671$). Prior to injury, the number of baseline foot-faults were minimal (vehicle=0.3±0.2, MLN519=0.4±0.2). However, following MCAo the number of foot-faults was significantly increased ($P=0.008$) at day 7 post-injury (vehicle=13.7±5.4, MLN519=7.3±4.0) with partial recovery by post-injury days 11 (vehicle=7.3±3.2, MLN519=6.9±3.6) and 14 (vehicle=4.7±1.8, MLN519=4.9±2.5).

3.8. Active avoidance

ANOVA revealed no significant differences in the total duration spent in shock across Time ($P=0.485$), Treatment ($P=0.058$), or Time*Treatment interaction ($P=0.481$) following MCAo injury. The effect of MCAo was still apparent in both groups, however, when compared to the drug-appropriate sham controls (ANOVA, $P<0.05$). Specifically, both treatment groups spent considerably more time in shock than did the sham animals out to day 2 post-injury ($P<0.05$ at each time point), which remained elevated, but not significantly, beyond day 3. Similarly, no significant treatment effects were observed in the total number of avoidance, escape, or intertrial responses.

4. Discussion

In the current study, MCAo injured rats showed improved recovery following a 10 h delayed treatment with the proteasome inhibitor MLN519 as evaluated out to 2 weeks post-injury. These results are in support of previous studies, which have evaluated the neuroprotective effects of MLN519 out to 72 h post-occlusion in this same injury model (Phillips et al., 2000; Williams et al., 2003, 2004). Specifically, a 1.0 mg/kg dose of MLN519 has been shown to significantly reduce brain injury with a therapeutic window of 6–10 h post-injury (Williams et al., 2004) as related to an attenuation of NF-κB induced cytokine and cell adhesion molecule expression (Berti et al., 2003; Williams et al., 2003) and reduction in both neutrophil and macrophage infiltration into the injured rat brain (Phillips et al., 2000; Williams et al., 2003). Furthermore, the dosing schedule used here has been shown to reduce blood proteasome levels by 80–90% in MCAo-injured rats (Williams et al., 2003) as well as in healthy adult volunteers without notable side effects (Shah et al., 2002). These previous experimental studies have indicated that targeting the delayed NF-kB mediated neuro-inflammatory response, through proteasome inhibition, offers a favorable therapeutic window for neuroprotection treatment of ischemic brain injury.

TTC staining of rat brains at 2 weeks post-injury indicated distinct injury to the ipsilateral cortex and basal ganglia with a marked loss of overall hemispheric brain volume (i.e. cavitation), similar to previous reports with this model (Clark et al., 1993, 1994). MLN519 treatment was associated with a significant reduction (45%) in the amount of brain tissue lost due to cavitation of the infarct. Furthermore, there was an overall increase in the amount of viable (i.e. non-injured) brain tissue in the ipsilateral hemisphere. The loss of brain tissue resulting from MCAo was also associated with changes in physiological status (body weight and temperature) and functional outcome (neurological status, balance beam performance, and active avoidance behavior), which were influenced, in part, by MLN519 treatment. The selective effect on outcome may have been related to the nature of the injury and the specific brain regions injured and/or salvaged following MLN519 treatment.

Overall, MCAo-induced injury was associated with a significant mortality, loss of body weight, and a transient hyperthermia (~1.0 °C at 2 h post-injury). MLN519 treatment improved both survival rate and recovery of body weight following MCAo. Initially, all injured animals lost 8–10% of their pre-injury body weight by 24 h post-injury with recovery of body weight in 71% of MLN519 treated rats and in only 33% of vehicle treated rats by day 14. In contrast, MLN519 had no effect on body temperature as compared to vehicle treatment, indicating no confounding role of hypo- or hyperthermia on injury outcome between groups (Busto et al., 1987; Zhang et al., 1993).

MCAo injury was also associated with significant functional impairments including contralateral neurological deficits and an increased number of foot-faults on a balance beam test. In particular, at 2 h post-injury, all animals exhibited a maximal neurological deficit indicating a successful MCAo injury. However, MLN519 treatment was associated with a significant overall reduction in neurological deficit scores as compared to the vehicle group, although no treatment effects were observed to reduce foot-fault deficits. The improvement in neurological score may be related to sparing of forelimb motor and somatosensory cortical regions in 3 of 7 MLN519 treated animals as compared to only 1 in 6 of the vehicle treated animals. Balance beam deficits were reduced at day 7 post-injury in MLN519 treated animals although the effect was not significant between treatment groups, this could be related to the sparing of cerebellar structures that coordinate balance.

It should also be noted that both treatment groups exhibited a measurable degree of spontaneous recovery in neurological deficits and balance beam performance over time. Spontaneous recovery of neurological function may

involve several possible mechanisms such as restoration of biochemical homeostasis or resolution of diaschisis (Johansson, 2000; Carmichael et al., 2004; Finger et al., 2004). For the balance beam task, compensatory changes in body posture and weight distribution to rely on unimpaired limbs are also observed following a unilateral brain injury (Schallert et al., 2002). In the current studies, a modified balance beam was used that included ledges along both sides of the beam that served as a 'crutch' for the impaired limb to limit compensatory motor learning (Schallert et al., 2003). However, despite measurable deficits in balance beam performance, even out to 14 days post-injury, no significant treatment effects with MLN519 were observed. Recent reports have indicated that balance beam tasks are not always sensitive to neuroprotective therapies although a reduction of brain infarct volume and improved neurological outcome were measured (Zausinger et al., 2000). In fact, this same comprehensive study of neurological impairments induced by MCAo injury (Zausinger et al., 2000) indicated that the neurological test developed by Bederson et al. (1986), as used in the current study, is the most sensitive and strongest correlative to the degree of histological brain injury.

Along with the observed functional deficits, MCAo injury induced delayed shuttle-box responses to avoid or escape an aversive stimulus (i.e. mild foot shock), although significant deficits were only measured out to day 2 post-injury as compared to sham (non-injured) animals. In the current study, no treatment effects were observed with MLN519 to improve the active avoidance response. Other studies (Kato et al., 2000) have indicated that acquisition of a shuttle-box avoidance response (i.e. post-injury learning of the task) is sensitive to neuroprotective treatment, which may be a superior method for assessing MCAo-induced deficits and recovery. In the current study, low shock levels were used to minimize stress to the animal. Although sufficient to motivate an avoidance response, as evidenced in sham animals, the task may not have been challenging enough (i.e. not a strong enough foot shock) to assess neuroprotective recovery. Another interpretation is that MCAo-induced neurological deficits interfered with motor performance of the task (rather than the sensory component) and the degree of neuroprotection offered by MLN519 was not sufficient to overcome these deficits in the active avoidance behavior test. A standard model for assessing neuroprotection treatment on higher-order behavioral/cognitive outcome following MCAo injury in rats has yet to be established (Bland et al., 2000; DeGraba and Pettigrew, 2000).

In conclusion, MLN519 treatment of focal ischemia/reperfusion brain injury improved overall outcome as measured by significant reduction in brain pathology and improved neurological outcome and weight gain. Other tasks, including balance beam and shuttle-box avoidance, were not affected or were not sensitive to the neuroprotective therapy provided by MLN519. These data would suggest that lesion volume and neurological recovery are the strongest indicators of neuroprotection treatment with MLN519. As such, the current study supports previous studies with this compound indicating the favorable therapeutic window for treating ischemic brain injury and improving outcome. In particular, the neuroprotective effects of MLN519 have been verified out to 2 weeks following ischemic brain injury in rats and may translate into successful clinical efficacy for the treatment of ischemic brain injury where the treatment window following onset of injury is a critical therapeutic determinant.

References

Bederson J, Pitts L, Tsuji M, Nishimura M, Davis R, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 1986;17:472–6.

Berti R, Williams A, Velarde L, Elliott P, Adams J, Yao C, et al. Effect of the proteasome inhibitor MLN519 on expression of inflammatory molecules following middle cerebral artery occlusion and reperfusion in the rat. Neurotox Res 2003;5:505–14.

Bland ST, Schallert T, Strong R, Aronowski J, Grotta JC, Feeney DM. Early exclusive use of the affected forelimb after moderate transient focal ischemia in rats: functional and anatomic outcome. Stroke 2000;31 1144–52.

Bramlett HM, Dietrich WD. Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. J Cereb Blood Flow Metab 2004;24:133–50.

Busto R, Dietrich WD, Globus MY, Valdes I, Scheinberg P, Ginsberg MD. Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury. J Cereb Blood Flow Metab 1987;7:729–38.

Carmichael ST, Tatsukawa K, Katsman D, Tsuyuguchi N, Kornblum HI. Evolution of diaschisis in a focal stroke model. Stroke 2004;35:758–63.

Clark R, Lee E, Fish C, White R, Price W, Jonak Z, et al. Development of tissue damage, inflammation and resolution following stroke: an immunohistochemical and quantitative planimetric study. Brain Res Bull 1993;31:565–72.

Clark R, Lee E, White R, Jonak Z, Feuerstein G, Barone F. Reperfusion following focal stroke hastens inflammation and resolution of ischemic injured tissue. Brain Res Bull 1994;35:387–92.

Clark MG, Vasilevsky S, Myers TM. Air and shock two-way shuttlebox avoidance in C57BL/6J and 129X1/SvJ mice. Physiol Behav 2003;78:117–23.

DeGraba TJ, Pettigrew LC. Why do neuroprotective drugs work in animals but not humans? Neurol Clin 2000;18:475–93.

Elliott P, Ross J. The proteasome: a new target for novel drug therapies. Am J Clin Pathol 2001;116:637–46.

Elliott P, Shah I, Lees K, Pien C, Tortella F, Adams J. Proteasome inhibition—a novel target for anti-stroke therapy. Restor Neurol Neurosci 2000;16:156.

Finger S, Koehler PJ, Jagella C. The Monakow concept of diaschisis: origins and perspectives. Arch Neurol 2004;61:283–8.

Janardhan V, Qureshi AI. Mechanisms of ischemic brain injury. Curr Cardiol Rep 2004;6:117–23.

Johansson BB. Brain plasticity and stroke rehabilitation The Willis lecture. Stroke 2000;31:223–30.

Kato M, Iwata H, Okamoto M, Ishii T, Narita H. Focal cerebral ischemia-induced escape deficit in rats is ameliorated by a reversible inhibitor of monoamine oxidase-A: implications for a novel animal model of post-stroke depression. Biol Pharm Bull 2000;23:406–10.

Lipton M. Ischemic cell death in brain neurons. Physiol Rev 1999; 79:1431–568.

Phillips J, Williams A, Adams J, Elliott P, Tortella F. Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia. Stroke 2000;31:1686–93.

Schallert T, Woodlee M, Fleming S, editors. Disentangling multiple types of recovery from brain injury. Stuttgart: Medpharm Scientific Publishers; 2002.

Schallert T, Fleming S, Woodlee M. Should the injured and intact hemispheres be treated differently during the early phases of physical restorative therapy in experimental stroke or parkinsonism? Phys Med Rehabil Clin North Am 2003;14:S27–46.

Shah I, Lees K, Pien C, Elliott P. Early clinical experience with the novel proteasome inhibitor PS-519. Br J Clin Pharmacol 2002;54:269–76.

Tortella F, Britton P, Williams A, Lu X, Newman A. Neuroprotection (focal ischemia) and neurotoxicity (electroencephalographic) studies in rats with AHN649, a 3-amino analog of dextromethorphan and low-affinity *N*-methyl-D-aspartate antagonist. J Pharmacol Exp Ther 1999;291: 399–408.

Williams AJ, Hale SL, Moffett JR, Dave JR, Elliott PJ, Adams J, et al. Delayed treatment with MLN519 reduces infarction and associated neurologic deficit caused by focal ischemic brain injury in rats via antiinflammatory mechanisms involving nuclear factor-kappa B activation, gliosis, and leukocyte infiltration. J Cereb Blood Flow Metab 2003;23:75–87.

Williams AJ, Berti R, Dave JR, Elliot PJ, Adams J, Tortella FC. Delayed treatment of ischemia/reperfusion brain injury: extended therapeutic window with the proteosome inhibitor MLN519. Stroke 2004; 35:1186–91.

Williams AJ, Hartings JA, Lu XC, Rolli ML, Dave JR, Tortella FC. Characterization of a new rat model of penetrating ballistic brain injury. J Neurotrauma 2005;22:313–31.

Wojcik C, Di Napoli M. Ubiquitin–proteasome system and proteasome inhibition: new strategies in stroke therapy. Stroke 2004;35:1506–18.

Zausinger S, Hungerhuber E, Baethmann A, Reulen H, Schmid-Elsaesser R. Neurological impairment in rats after transient middle cerebral artery occlusion: a comparative study under various treatment paradigms. Brain Res 2000;863:94–105.

Zhang RL, Chopp M, Chen H, Garcia JH, Zhang ZG. Postischemic (1 hour) hypothermia significantly reduces ischemic cell damage in rats subjected to 2 hours of middle cerebral artery occlusion. Stroke 1993; 24:1235–40.

Zhang L, Zhang ZG, Zhang RL, Lu M, Adams J, Elliott PJ, et al. Postischemic (6-hour) treatment with recombinant human tissue plasminogen activator and proteasome inhibitor PS-519 reduces infarction in a rat model of embolic focal cerebral ischemia. Stroke 2001;32:2926–31.