

ORIGINAL ARTICLE

# Sustained functional improvement by hepatocyte growth factor-like small molecule BB3 after focal cerebral ischemia in rats and mice

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Hepatocyte growth factor (HGF), efficacious in preclinical models of acute central nervous system injury, is burdened by administration of full-length proteins. A multiinstitutional consortium investigated the efficacy of BB3, a small molecule with HGF-like activity that crosses the blood–brain barrier in rodent focal ischemic stroke using Stroke Therapy Academic Industry Roundtable (STAIR) and Good Laboratory Practice guidelines. In rats, BB3, begun 6 hours after temporary middle cerebral artery occlusion (tMCAO) reperfusion, or permanent middle cerebral artery occlusion (pMCAO) onset, and continued for 14 days consistently improved long-term neurologic function independent of sex, age, or laboratory. BB3 had little effect on cerebral infarct size and no effect on blood pressure. BB3 increased HGF receptor c-Met phosphorylation and synaptophysin expression in penumbral tissue consistent with a neurorestorative mechanism from HGF-like activity. In mouse tMCAO, BB3 starting 10 minutes after reperfusion and continued for 14 days improved neurologic function that persisted for 8 weeks in some, but not all measures. Study in animals with comorbidities and those exposed to common stroke drugs are the next steps to complete preclinical assessment. These data, generated in independent, masked, and rigorously controlled settings, are the first to suggest that the HGF pathway can potentially be harnessed by BB3 for neurologic benefit after ischemic stroke.

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## INTRODUCTION

Hepatocyte growth factor (HGF) is a cytokine that participates in the regulation of angiogenesis, neurogenesis, organogenesis, and tissue reconstruction.<sup>1</sup> A broad range of cell types including fibroblasts, epithelial, and endothelial cells produce or respond to HGF via its cognate receptor c-Met.<sup>2</sup> Hepatocyte growth factor can act as a morphogen inducing transition of epithelial cells into a mesenchymal morphology and stimulates epithelial branching and tubulogenesis. These responses reflect HGF's role in organogenesis and tissue healing.<sup>3,4</sup> Hepatocyte growth factor is also cytoprotective as a consequence of its antiapoptotic and antifibrotic activity.<sup>5–7</sup> Preclinical models investigating HGF protein or gene therapy have demonstrated positive results in ischemia/reperfusion injury to the liver, heart, lung, and kidney.<sup>8–11</sup>

Hepatocyte growth factor and c-MET are also present in both the developing and adult mammalian brain.<sup>12,13</sup> Activation of the HGF/c-Met pathway may offer value to the acutely injured brain as a neurorestorative therapeutic. Hepatocyte growth factor has been repeatedly associated with increased neurogenesis, synaptogenesis, and angiogenesis and decreased lesion size in experimental brain ischemia.<sup>14–17</sup>

BB3 is a small molecule, discovered by phage display with molecular modeling, which emulates HGF properties *in vitro*. In preclinical models of renal and myocardial ischemic injury, therapeutic effects of BB3 were observed with drug administration starting 24 hours after insult (data not shown); an observation consistent with upregulation of the c-Met receptor in at-risk tissue.<sup>18</sup>

BB3 is currently under clinical investigation as a therapeutic for delayed graft function after kidney transplantation (NCT01286727). We hypothesized that BB3 will exert neurorestorative effects in the context of experimental focal ischemic stroke. A multiinstitutional consortium was established to systematically define BB3 efficacy in preclinical middle cerebral artery occlusion (MCAO) models respecting the Stroke Therapy Academic Industry Roundtable (STAIR) and Good Laboratory Practice guidelines.<sup>19,20</sup>

## MATERIALS AND METHODS

The Covance Animal Care and Use Committee (Raleigh, NC, USA) approved Experiment 1. The Duke University Animal Care and Use Committee (IACUC; Durham, NC, USA) approved Experiments 2, 4 to 7, and 9. The Angion Biomedica Corporation Animal Care and Use Committee

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(Uniondale, NY, USA) approved Experiments 3 and 10. The Massachusetts General Hospital Subcommittee on Research and Animal Care (Boston, MA, USA) approved Experiment 8. Experiments were performed according to IACUC guidelines, the National Institutes of Health guide for care and use of laboratory animals, and ARRIVE guidelines ([www.nc3rs.org.uk/arrive-guidelines](http://www.nc3rs.org.uk/arrive-guidelines)).

### BB3

Angion Biomedica Corporation provided BB3. BB3 was prepared by sonication in a vehicle consisting of five parts polyethylene glycol 300, one part Tween and four parts phosphate-buffered saline (PBS, V/V). Final concentration was 6 mg/mL.

### Experimental Designs

Experimental designs will first be outlined with detailed methodology to follow.

#### Experiment 1: Rat BB3 Blood–Brain Barrier Penetration

To assess the distribution of BB3 in the central nervous system, seven healthy male 8-week-old Long Evans rats (Harlan, Indianapolis, IN, USA) were given a single intravenous dose of 2 mg/kg  $^{14}\text{C}$ -BB3. At prespecified time points, the rats were exsanguinated and  $^{14}\text{C}$ -BB3 concentrations were measured in plasma, cerebellum, cerebral cortex, medulla, olfactory lobe, and spinal cord white and gray matter (one rat per time point).

#### Experiment 2: Physiologic Effects of a Single BB3 Dose After Rat Middle Cerebral Artery Occlusion

This experiment was performed to determine whether BB3 causes changes in physiologic values known to alter ischemic outcome, which might warrant control in subsequent experiments. Male rats (10 to 12 weeks of age) were subjected to either transient (temporary MCAO, tMCAO) or permanent (pMCAO) middle cerebral artery occlusion and placed in a harness system allowing continuous pericranial temperature and arterial blood pressure monitoring. Rats were randomly assigned to treatment with 0, 2, 6, or 12 mg/kg intraperitoneal BB3 ( $n=4$  to 6 per group) at 90 minutes after MCAO onset. For tMCAO, pericranial temperature was continuously controlled at  $37.5 \pm 0.2^\circ\text{C}$  during the 90 minutes ischemic interval and was then allowed to freely flux with continuous monitoring for 20 hours after treatment onset. Arterial blood pressure, blood gases/pH/glucose and hematocrit were serially recorded over 20 hours. For pMCAO, pericranial temperature was controlled at  $37.5 \pm 0.2^\circ\text{C}$  for 60 minutes after occlusion and then allowed to freely flux with physiologic monitoring as described for tMCAO for 20 hours. These rats were not subjected to outcome analysis.

#### Experiment 3: BB3 Dose-Response Analysis in the Rat (Temporary Middle Cerebral Artery Occlusion)

Male Wistar rats (10 to 12 weeks) were subjected to 90 minutes tMCAO. Pericranial temperature was controlled ( $37.5 \pm 0.2^\circ\text{C}$ ) during tMCAO. Dose range selection was based on a large body of data demonstrating 2 mg/kg BB3 to be highly efficacious in peripheral tissue models of ischemic injury (data not shown) and the results of Experiment 2. Rats were randomly assigned to receive intraperitoneal vehicle ( $n=19$ ), 2 mg/kg BB3 ( $n=19$ ), or 6 mg/kg BB3 ( $n=21$ ). Treatment onset was 90 minutes after tMCAO reperfusion. Rats survived 14 days with daily intraperitoneal vehicle or BB3 treatment. Neurologic scores and cerebral infarct volume were then measured.

#### Experiment 4: BB3 Effects on Rat Temporary Middle Cerebral Artery Occlusion Outcome (Delayed Treatment Onset)

Male Wistar rats (10 to 12 weeks) were subjected to 90 minutes tMCAO. Pericranial temperature was controlled ( $37.5 \pm 0.2^\circ\text{C}$ ) during tMCAO and for 20 hours after reperfusion. Six hours after reperfusion onset, rats were randomly assigned to receive vehicle or BB3 ( $n=20$ /group) for 14 days. On the basis of Experiment 3, we selected a daily intraperitoneal BB3 dose of 6 mg/kg. Rats were housed under standard vivarium conditions for an additional 27 days and then subjected to neurologic evaluation and infarct size measurement.

#### Experiment 5: Effects of BB3 on Rat Permanent Middle Cerebral Artery Occlusion Outcome (Delayed Treatment Onset)

This experiment was identical to Experiment 4 with the exception that the MCA was permanently occluded by cautery and bisection in 10 to 12 week old male Wistar rats. Pericranial temperature was controlled at  $37.5 \pm 0.2^\circ\text{C}$  for 20 hours after MCAO onset. Rats were randomly assigned to BB3 (6 mg/kg per day intraperitoneal) beginning 6 hours after pMCAO onset ( $n=21$ ), or an equivalent volume of vehicle ( $n=21$ ) continued for 14 days. Neurological function and infarct size were measured 28 days after MCAO.

#### Experiment 6: Sex-Specific Effects of BB3 on Rat Temporary Middle Cerebral Artery Occlusion Outcome (Delayed Treatment Onset)

Female and male rats (10 to 12 weeks) were investigated contemporaneously, subjected to 90 minutes tMCAO, and randomly assigned to receive daily BB3 (6 mg/kg intraperitoneal) or vehicle beginning 6 hours after reperfusion onset for 14 days ( $n=20$ /group/sex). Pericranial temperature was controlled at  $37.5 \pm 0.2^\circ\text{C}$  during ischemia and for 20 hours after reperfusion. Neurological function and infarct size were measured 28 days after MCAO. Females were subjected to tMCAO in the estrus stage.<sup>21</sup>

#### Experiment 7: BB3 Effects on Permanent Middle Cerebral Artery Occlusion Outcome Outcome in Aged Rats (Delayed Treatment Onset)

Male Fischer 344 rats (Taconic Biosciences, Inc., Germantown, NY, USA, National Institute on Aging, 22 months of age) were first subjected to the standardized neurologic examination to determine baseline functional status. To provide the most rigorous test possible, we subjected these rats to the pMCAO protocol. To minimize surgical invasiveness and allow repeated blood pressure monitoring during recovery, a tail artery catheter was not used. Instead, tail artery mean arterial pressure (MAP) was measured noninvasively 15 minutes before MCAO onset, 1 hour after MCAO onset, and repeatedly during the 28-day recovery.

Aged rats were randomly assigned to receive daily BB3 (6 mg/kg intraperitoneal  $n=21$ ) or vehicle ( $n=19$ ) beginning 6 hours after pMCAO onset for 14 days. Pericranial temperature was controlled ( $37.5 \pm 0.3^\circ\text{C}$ ) for the first 20 hours after MCAO. Neurologic function and infarct size were measured 28 days after MCAO.

#### Experiment 8: BB3 Effect on Mouse Temporary Middle Cerebral Artery Occlusion Outcome (Acute and Delayed Treatment Onset)

Mice were subjected to 60 minutes tMCAO and then randomly assigned to intraperitoneal BB3 (6 mg/kg,  $n=20$ ) or vehicle ( $n=20$ ) beginning, for each group, either 10 minutes ( $n=10$ ) or 6 hours ( $n=10$ ) after reperfusion onset, and subsequently dosed daily for 14 days. Rectal temperature was controlled ( $37.0^\circ\text{C}$ ) during ischemia. Neurologic function was examined over 8 weeks after tMCAO. Cerebral lesion volume was then measured.

#### Experiment 9: BB3 Effects on Rat Post-Middle Cerebral Artery Occlusion Outcome Brain Water Content

Male Wistar rats (10 to 12 weeks) were subjected to tMCAO. Pericranial temperature was controlled ( $37.5 \pm 0.2^\circ\text{C}$ ) during ischemia and for 20 hours after reperfusion. Six hours after reperfusion, rats were randomly assigned to vehicle or BB3 treatment groups ( $n=10$  per group). Twenty-four hours after MCAO, the rats were re-anesthetized with isoflurane, decapitated, and brain water content was measured using the wet weight–dry weight method described previously.<sup>22</sup> To provide normal reference values, five naive rats, without ischemic insult, were anesthetized and brain water content was measured.

#### Experiment 10: Immunohistochemical Effects of BB3 On c-Met Phosphorylation and Synaptophysin Expression After Rat Temporary Middle Cerebral Artery Occlusion Outcome.

Male Wistar rats (10 to 12 weeks) were subjected to tMCAO with pericranial temperature servo-controlled at  $37.5 \pm 0.2^\circ\text{C}$  during ischemia. Separate animals were used for each analytic procedure. For c-Met phosphorylation, rats ( $n=5$ /group) were randomly assigned to receive intraperitoneal BB3 (6 mg/kg) or vehicle at 2 and 23 hours after reperfusion. One hour after the second dose, brains were harvested and frozen ( $-80^\circ\text{C}$ ). For synaptophysin

expression, rats were randomly assigned to receive intraperitoneal vehicle ( $n=6$ ) or BB3 (6 mg/kg daily) for 14 ( $n=4$ ) or 28 ( $n=4$ ) days. All the rats were allowed to survive 28 days, after which synaptophysin was measured.

#### Rat Temporary Middle Cerebral Artery Occlusion Outcome Procedure

Wistar rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were housed in a temperature-controlled environment with an artificial light/dark cycle (12 hours). They were fasted from food but allowed free access to water for 12 hours before ischemia. Anesthesia was induced with 5% isoflurane in 30% O<sub>2</sub>, balance N<sub>2</sub>. The trachea was intubated and the lungs were mechanically ventilated. The inspired isoflurane concentration was decreased to 1.5% to 2%, and animals were prepared for MCAO using techniques previously described.<sup>23</sup>

The surgery was performed with aseptic technique. The tail artery was cannulated to monitor MAP and sample blood. A calibrated flexible thermistor was percutaneously implanted beneath the right temporalis adjacent to the skull and secured. Pericranial temperature ( $37.5\text{ }^{\circ}\text{C} \pm 0.2\text{ }^{\circ}\text{C}$ ) was continuously controlled by surface heating/cooling. The scalp was incised and the parietal bone thinned 2 mm posterior and 6 mm lateral to bregma. A guide was glued to the skull to position a laser Doppler flow (LDF) probe.

A skin incision was made and the right common carotid identified. The external carotid artery was isolated, ligated, and divided. The internal carotid was dissected distally until the origin of the pterygopalatine artery was visualized. After surgical preparation, a 20-minute interval was allowed for physiologic stabilization. Heparin (50 IU) was given intravenously to prevent arterial thrombosis.

To achieve MCAO, a 0.25-mm-diameter nylon filament coated with silicon (0.38 mm diameter) was inserted into the external carotid artery stump and advanced 19 mm to 20 mm from the carotid artery bifurcation until resistance was felt. Animals failing to meet a  $>60\%$  LDF reduction from baseline or having hemorrhage were *a priori* excluded. After filament insertion, a silicon harness was positioned on the torso. The thermistor lead was passed through a flexible coil attached to both the harness and a swivel commutator. Isoflurane was discontinued to minimize anesthetic exposure.<sup>24</sup>

The rats were transferred to an acrylic chamber allowing continuous control of pericranial temperature at  $37.5\text{ }^{\circ}\text{C} \pm 0.2\text{ }^{\circ}\text{C}$ . The trachea was extubated. At 85 minutes after MCAO onset, isoflurane was reintroduced, the filament and arterial catheter were removed, and wounds were infiltrated with 0.25% bupivacaine and closed with suture. Isoflurane was discontinued. The rat was returned to the acrylic chamber containing 30% O<sub>2</sub>/balance N<sub>2</sub> for 20 hours allowing pericranial thermoregulation to continue with free access to food and water. The thermistor and harness were removed and rats returned to their cages.

Arterial CO<sub>2</sub> and O<sub>2</sub> partial pressures and pH, glucose, hematocrit were measured 15 minutes before and 45 minutes after MCAO onset and 15 minutes after reperfusion. MAP was continuously monitored until 15 minutes after reperfusion.

#### Rat Permanent Middle Cerebral Artery Occlusion Outcome Procedure

Procedures were similar to those used for tMCAO with the following exceptions. A right subtemporal craniectomy was performed and the dura opened. The MCA was separated from the meninges, cauterized, and bisected just proximal to the lenticulostriate artery. Bupivacaine (0.25%) was instilled and the wound closed with suture. Rats were awakened and pericranial temperature was thermoregulated for 20 hours at  $37.5\text{ }^{\circ}\text{C} \pm 0.2\text{ }^{\circ}\text{C}$ . Rats were then returned to their cages.

#### Rat Neurologic Scoring System

We used a system that evaluates general status (spontaneous activity, body symmetry, gait), simple motor deficit (forelimb asymmetry, circling, hind limb placement), complex motor deficit (vertical screen climbing, beam walking), and sensory deficit (hind limb, trunk, vibrissae, and face touch).<sup>25–27</sup> The score given to each animal at the completion of testing was the sum of the four individual scores with 0 being the minimum (best) score and 48 the maximum possible (worst) score.

#### Measurement of Rat Cerebral Infarct Volume

Rats were anesthetized with 5% isoflurane and decapitated. The brains were removed, frozen at  $-40\text{ }^{\circ}\text{C}$  in 2-methylbutane, and stored at  $-80\text{ }^{\circ}\text{C}$ . Serial quadruplicate 20- $\mu\text{m}$  thick coronal sections were taken at 800- $\mu\text{m}$  intervals over the rostral-caudal extent of the infarct. The sections were dried and stained with hematoxylin and eosin. An image analyzer digitized a section from each 800- $\mu\text{m}$  interval. Each image was stored as a 1,280  $\times$  960 calibrated pixel matrix and displayed on a video monitor. The noninfarcted ipsilateral cerebral cortex, noninfarcted ipsilateral subcortex, contralateral cerebral cortex, and contralateral subcortex were cursor outlined. The area within each region ( $\text{mm}^2$ ) was determined by automated pixel counting. Ipsilateral noninfarcted cortex and subcortex areas were subtracted from the corresponding contralateral regions of interest values to estimate the area of ischemic tissue damage. Infarct volumes ( $\text{mm}^3$ ) were computed as running sums of subtracted infarct areas multiplied by the known interval (e.g., 800  $\mu\text{m}$ ) between sections over the rostral-caudal extent of the infarct calculated as an orthogonal projection.<sup>28</sup> Cortical and subcortical infarct volumes were added to obtain total infarct volume.

#### Mouse Temporary Middle Cerebral Artery Occlusion Outcome and Outcome Analysis

Spontaneously breathing male mice (C57/BL6, 8 to 9 weeks, Charles River Laboratories International, Wilmington, MA, USA) were anesthetized with 1.5% isoflurane in 70% N<sub>2</sub>O/30% O<sub>2</sub>. Rectal temperature was maintained at 37  $^{\circ}\text{C}$  using a servo-controlled heating pad. Temporary middle cerebral artery occlusion was induced in the right hemisphere by inserting a 7-0 suture through the external carotid artery. Mice were removed from anesthesia 5 minutes after occlusion and re-anesthetized for suture removal 60 minutes after insertion. Laser Doppler flow was monitored over the MCA area. Failure of LDF to decrease  $>80\%$  of baseline or hemorrhage during surgery were *a priori* exclusion criteria. After tMCAO, mice were placed in a temperature-controlled environment with easy access to food and water, and administered daily subcutaneous saline supplements (1 mL/day) and ampicillin (1 mg/day) for up to 7 days. Four sensorimotor tests were performed pre-ischemia, and 1, 3, 6, and 8 weeks after tMCAO. The adhesive-removal test measured the time to remove tape from the contralateral forepaw (up to 300 seconds);<sup>29</sup> grid-walk measured forepaw placement accuracy on a 20  $\times$  30 cm grid plate (% foot-faults/total steps);<sup>30</sup> corner test measured the direction of rear-and-turn movement when the animal faced a wedge with a 30  $^{\circ}$  corner (% ipsilateral/total turns);<sup>31</sup> cylinder test measured the % forepaw use ((ipsilateral – contralateral)/total) in rearing when placed in a vertical transparent cylinder.<sup>32</sup>

At 8 weeks, brains were harvested as described for the rat experiments with sections collected every 500  $\mu\text{m}$  and stained with hematoxylin and eosin. Using image analysis, hemisphere and ventricle areas were measured and integrated along the anteroposterior axis. Hemispheric volume was calculated in  $\text{mm}^3$  by subtracting the ventricle volume from each hemisphere.

#### Immunohistochemistry

To measure c-Met phosphorylation, sections (20  $\mu\text{m}$ ) were warmed to room temperature for 30 minutes. The tissue was immersed in  $-20\text{ }^{\circ}\text{C}$  100% methanol for 40 minutes, repeated, and then in  $-20\text{ }^{\circ}\text{C}$  acetone for 30 minutes. Samples were dried for 20 minutes. For antigen retrieval, samples were washed twice with PBS for 3 minutes at room temperature, heated to 100  $^{\circ}\text{C}$  in 10 mM citrate buffer (pH 6.0) for 20 minutes, cooled to room temperature, PBS washed as above, and blocked in 2% bovine serum albumin in PBS with 2 mM EDTA for 45 minutes. Rabbit anti-phospho-c-Met (phospho-Met (Tyr1234/1235)(D26) XP, Cell Signaling Technology, Danvers, MA, USA), diluted 1/50 in fresh blocking buffer, was added and incubated overnight at 4  $^{\circ}\text{C}$ . Alkaline phosphatase conjugated anti-rabbit secondary antibody was diluted 1/100 in blocking buffer and incubated on samples for 2 hours at room temperature. Samples were washed twice in PBS for 5 minutes and incubated with alkaline phosphatase substrate (Vector Blue, #SK-5300, Vector Labs, Burlingame, CA, USA) at room temperature, then washed with tap water for 1 minute, rinsed with deionized water, and air dried at room temperature before mounting. Images were captured and quantified using Bioquant Nova Prime V6.90.10 (Nashville, TN, USA).

To measure synaptophysin serial sections taken from the dorsal cortex peri-infarct region at bregma  $\pm 1\text{ mm}$  were washed twice with PBS for 3 minutes at room temperature followed by heating to 100  $^{\circ}\text{C}$  in 10 mM TE (Tris-EDTA, pH 9.0) for 20 minutes, for antigen retrieval. Samples were

**Table 1.**  $^{14}\text{C}$ -BB3 concentrations after a single intravenous dose of 2 mg/kg

Time (hours)	Blood	Cerebellum	Cerebrum	Medulla	Olfactory lobe	Spinal cord	Spinal cord (gray matter)	Spinal cord (white matter)
0.25	1,440	729	571	744	831	1,050	773	1,790
1	427	154	144	160	172	219	189	301
4	135	30	28	29	27.7	30	ND	ND
24	42	13	13	16	10.3	BLQ	ND	ND
72	27	ND	ND	ND	ND	BLQ	ND	ND
168	25	ND	ND	ND	ND	BLQ	ND	ND
336	ND	ND	ND	ND	ND	ND	ND	ND

Abbreviations: BLQ, below the limit of quantification; ND, none detected. Values = mean (ng/g tissue).

cooled to room temperature, washed twice with Tris-buffered saline for 3 minutes with shaking and blocked with 2% normal goat serum in Tris-buffered saline (150 mM NaCl and 20 mM Tris-HCl (pH 8.0)) with 2 mM EDTA (blocking buffer) for 60 minutes. Primary monoclonal antibody to synaptophysin (EMD Millipore, Darmstadt, Germany) was diluted 1/100 in fresh blocking buffer and incubated overnight at 4 °C. Fluorescein isothiocyanate conjugated secondary antibody (goat anti-mouse, Santa Cruz, Biotechnology, Inc., Dallas, TX, USA; 1/100 dilution) was added and samples incubated in blocking buffer for 2 hours at room temperature, washed twice for 10 minutes with shaking in Tris-buffered saline, mounted, and kept at 4 °C protected from light. Images were captured on Leica Confocal SPS-2500 System and quantified using Leica Metamorph software (Nashville, TN, USA).

### Statistical Analysis

A computerized randomizer (<http://randomizer.org>),<sup>33</sup> made group assignments for all experiments except Experiment 8 for which randomization was conducted by blindly drawing assignments from a box.

All experiments were conducted in a fully masked fashion. Surgeons were unaware of group assignment. A separate individual conducted treatment injections. Group allocation was concealed from all individuals conducting outcome analysis. In most cases, individuals who conducted outcome analysis were different from those who performed surgery or treatment. When the same individuals who performed another task conducted outcome analysis, concealment was achieved by recoding the histologic slides.

For two-group outcome rat experiments, a power analysis was calculated using data for rat MCAO and 6 hours delayed treatment onset reported previously for Mn porphyrins.<sup>27</sup> Assuming  $\beta=0.80$  and s.d. of 61 mm<sup>3</sup> for total infarct volume in the vehicle group, 16 rats/group was calculated to be the minimum to detect infarct volume reduction of 37% by BB3.

Data from Experiment 1 were analyzed qualitatively. Rat infarct volumes, neurologic scores, and brain water content were examined for normality of distribution and homogeneity of variance and then compared using one-way analysis of variance (*post hoc* Tukey honest significant difference) or the unpaired Student's *t*-test, as appropriate. Simple linear regression tested an association between neurologic score (dependent variable) and total cerebral infarct volume (independent variable). To conserve statistical power, male and female rats were analyzed independently in Experiment 6. The Fisher Exact test was used to compare between group mortality rates in Experiment 7. In Experiment 8, data were analyzed by two-way repeated measures analysis of variance followed by Fisher's least significant difference test (Prism v6, GraphPad Software, La Jolla, CA, USA). Statistical analysis in the remaining experiments was performed using JMP Pro 9.0.0, SAS, Cary, NC, USA. Statistical significance was assumed when  $P < 0.05$ . Values are presented as mean  $\pm$  s.d.

## RESULTS

### Experiment 1: BB3 Crosses the Blood-Brain Barrier

$^{14}\text{C}$ -BB3-derived radioactivity was detected in all measured brain and spinal cord regions after a single intravenous injection. Peak concentrations were present at 0.25 hours after injection, which declined over time. Relative to radioactivity levels found in the organs of excretion and nongastrointestinal tract tissues, CNS

tissue concentrations were lower, however, these data indicate that BB3 rapidly distributes into the brain (Table 1).

### Experiment 2: Physiologic Effects of a Single BB3 Dose After Rat Middle Cerebral Artery Occlusion Outcome

Physiologic values were examined in both tMCAO and pMCAO models as a function of BB3 dose. There was no effect of BB3 dose on MAP, hematocrit, PaCO<sub>2</sub>, PaO<sub>2</sub>, or arterial blood pH or glucose (data not shown). However, for both tMCAO and pMCAO, post-ischemic pericranial hypothermia was evident before the start of treatment. BB3 dose-dependently enhanced hypothermia (Figure 1). Most noteworthy was the reduction of temperature to 33 °C with 12 mg/kg, which caused us to discontinue the study of this dose. Given the effects of anesthesia and BB3, pericranial temperature was controlled (37.5 °C) during MCAO for an additional 20 hours in most subsequent experiments. Because BB3 had no effect on MAP, MAP was not monitored during the treatment phase in subsequent experiments, except for in Experiment 7 (aged rats).

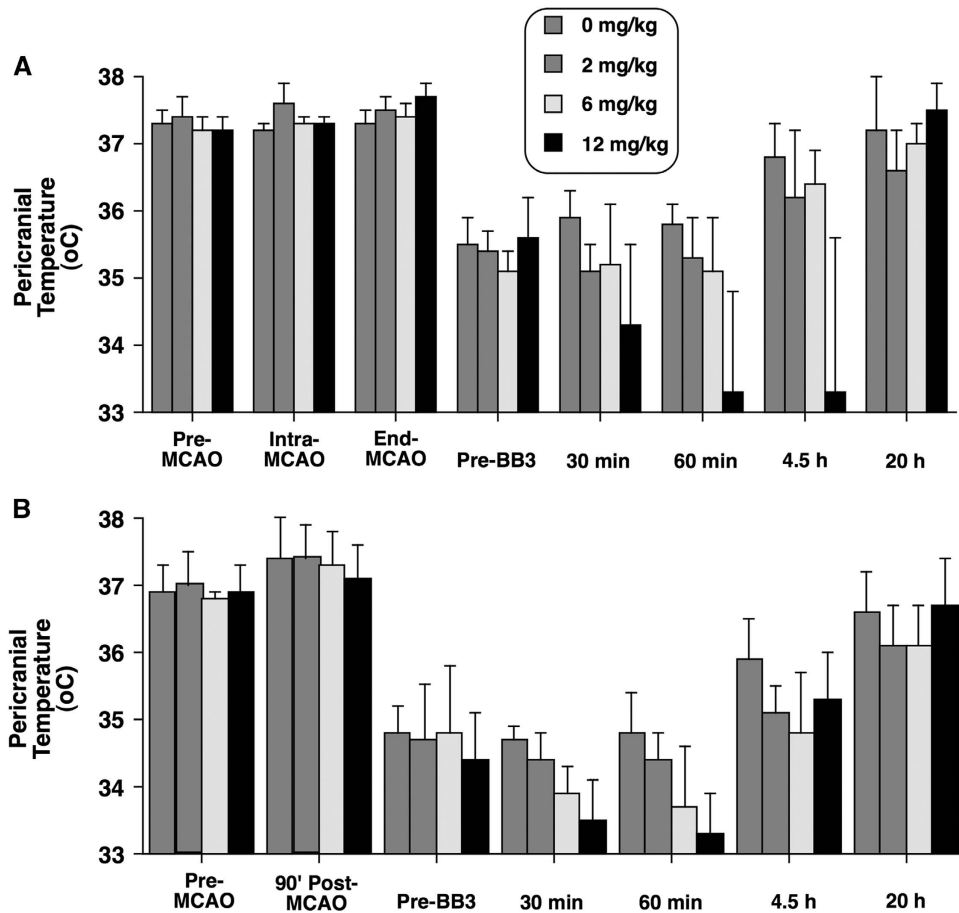
### Experiment 3: BB3 Dose-Response Analysis In Rat (Temporary Middle Cerebral Artery Occlusion Outcome)

There was a main effect for treatment group on neurologic scores 14 days after MCAO ( $F=6.47$ ,  $P=0.003$ ). *Post hoc* analysis showed an effect for 6 mg/kg BB3 ( $12 \pm 2$ ,  $P=0.002$ ), but not 2 mg/kg BB3 ( $14 \pm 3$ ,  $P=0.59$ ) versus vehicle ( $14 \pm 2$ ). A main effect could not be detected for cortical (vehicle =  $72 \pm 52$  mm<sup>3</sup>, BB3 2 mg/kg =  $74 \pm 42$  mm<sup>3</sup>, BB3 6 mg/kg =  $44 \pm 45$  mm<sup>3</sup>,  $F=2.40$ ,  $P=0.10$ ), subcortical (vehicle =  $48 \pm 57$  mm<sup>3</sup>, BB3 2 mg/kg =  $32 \pm 16$  mm<sup>3</sup>, BB3 6 mg/kg =  $24 \pm 19$  mm<sup>3</sup>,  $F=1.90$ ,  $P=0.16$ ) and total cerebral infarct volumes (vehicle =  $120 \pm 76$  mm<sup>3</sup>, BB3 2 mg/kg =  $106 \pm 56$  mm<sup>3</sup>, BB3 6 mg/kg =  $68 \pm 61$  mm<sup>3</sup>,  $F=3.01$ ,  $P=0.06$ ).

### Experiment 4: Delayed BB3 Treatment Onset Improves Neurologic Function in Rat Temporary Middle Cerebral Artery Occlusion Outcome

Fifty-nine rats were randomized to experimental groups. Eight did not meet the 60% LDF reduction and were excluded (three vehicle and five BB3). Seven died during recovery (vehicle = 4, BB3 = 3). Thus, 23 vehicle and 21 BB3 rats were used for final analysis.

There were no substantive differences between groups for physiologic values (Table 2). Neurologic scores at 28 days were different (vehicle =  $12 \pm 4$ , BB3 =  $6 \pm 4$ ,  $P < 0.0001$ , Figure 2A). No difference was detected between groups for cortical (vehicle =  $132 \pm 61$  mm<sup>3</sup>, BB3 =  $94 \pm 74$  mm<sup>3</sup>,  $P=0.07$ ), subcortical (vehicle =  $48 \pm 19$  mm<sup>3</sup>, BB3 =  $41 \pm 30$  mm<sup>3</sup>,  $P=0.32$ ) or total (vehicle =  $181 \pm 69$  mm<sup>3</sup>, BB3 =  $134 \pm 93$  mm<sup>3</sup>,  $P=0.07$ ) infarct volumes. Neurologic score was associated with total infarct size (neurologic score =  $2.18 + 0.04 \times$  total infarct size;  $R^2=0.57$ ,  $P < 0.0001$ ).



**Figure 1.** Pericranial temperature values for Experiment 2. Rats were subjected to either temporary (A) or permanent (B) middle cerebral artery occlusion (MCAO) with pericranial temperature controlled at  $37.5 \pm 0.2^\circ\text{C}$  during the ischemic insult (90 minutes in the pMCAO experiment) and treated with 0, 2, 6, or 12 mg/kg BB3 at 90 minutes after ischemia onset. Pericranial temperature was measured over the subsequent 20 hours without efforts to control temperature so as to determine the uncontrolled response to ischemia and BB3 treatment. Surgery and anesthesia caused a delayed post-ischemic hypothermia, which was transiently enhanced by BB3. Values = mean  $\pm$  s.d.

#### Experiment 5: Delayed BB3 Treatment Onset Improves Neurologic Function in Rat Permanent Middle Cerebral Artery Occlusion Outcome

Two rats in each treatment group died during the recovery period. Peri-ischemic physiologic values were similar to those reported in Table 2, with no differences between groups. The 28-day neurologic scores were improved by BB3 (vehicle =  $13 \pm 1$ , BB3 =  $10 \pm 1$ ,  $P=0.007$ , Figure 2B). There also was a difference between groups for cortical infarct volume (vehicle =  $138 \pm 40 \text{ mm}^3$ , BB3 =  $106 \pm 51 \text{ mm}^3$ ,  $P=0.04$ ). Subcortical (vehicle =  $35 \pm 16 \text{ mm}^3$ , BB3 =  $33 \pm 18 \text{ mm}^3$ ,  $P=0.69$ ) and total (vehicle =  $173 \pm 49 \text{ mm}^3$ , BB3 =  $139 \pm 65 \text{ mm}^3$ ,  $P=0.08$ ) infarct volumes were not significantly different. Neurologic score was associated with total infarct size (neurologic score =  $7.565 + 0.26 \times$  total infarct size;  $R^2=0.181$ ,  $P=0.008$ ).

#### Experiment 6: BB3 Delayed Treatment Onset Improves Neurologic Function In Both Female And Male Rats After Temporary Middle Cerebral Artery Occlusion Outcome

In both females and males, physiologic values were similar to those reported in Table 2 and were similar between treatment groups. In females, LDF was decreased by  $68 \pm 7\%$  and  $67 \pm 5\%$  in the vehicle and BB3 groups, respectively ( $P=0.70$ ). One rat in each group died during the recovery interval. The 28-day neurologic scores in surviving rats were improved by BB3 ( $12 \pm 5$  versus  $7 \pm 5$ ,

$P=0.02$ ). A difference was not detected between vehicle and BB3 groups for cortical ( $108 \pm 44 \text{ mm}^3$  versus  $94 \pm 53 \text{ mm}^3$ ,  $P=0.40$ ), subcortical ( $36 \pm 17 \text{ mm}^3$  versus  $33 \pm 15 \text{ mm}^3$ ,  $P=0.55$ ), or total ( $144 \pm 51 \text{ mm}^3$  versus  $127 \pm 61 \text{ mm}^3$ ,  $P=0.37$ ) infarct volumes, respectively.

In males, LDF was decreased by  $65 \pm 6\%$  and  $64 \pm 5\%$  in the vehicle and BB3 groups ( $P=0.73$ ), respectively. Two vehicle and three BB3 rats died during recovery. The 28-day neurologic scores in surviving rats were improved by BB3 (vehicle =  $12 \pm 5$  versus BB3 =  $9 \pm 4$ ,  $P=0.03$ ). Cortical ( $120 \pm 56 \text{ mm}^3$  versus  $129 \pm 57 \text{ mm}^3$ ,  $P=0.61$ ), subcortical ( $34 \pm 19 \text{ mm}^3$  versus  $35 \pm 15 \text{ mm}^3$ ,  $P=0.96$ ), and total ( $154 \pm 70 \text{ mm}^3$  versus  $164 \pm 61 \text{ mm}^3$ ,  $P=0.66$ ) infarct volumes were not different between vehicle and BB3 groups, respectively.

#### Experiment 7: BB3 Delayed Treatment Onset Improves Neurologic Function in Aged Rats After Permanent Middle Cerebral Artery Occlusion Outcome

Aged rats were clearly senescent with four dying spontaneously before entry to the protocol and two showing clinical evidence of anemia (pallor). These rats were not included. A total of 40 rats were studied, with 21 randomized to BB3 and 19 to vehicle. All rats received a neurologic score of  $\leq 2$  on the day before pMCAO onset (vehicle =  $1 \pm 1$ , BB3 =  $1 \pm 1$ ,  $P=0.79$ ). Two vehicle and six BB3 rats died during the 24-day recovery interval ( $P=0.24$ ). All

**Table 2.** Peri-ischemic physiologic values for Experiment 4 (temporary middle cerebral artery occlusion outcome)

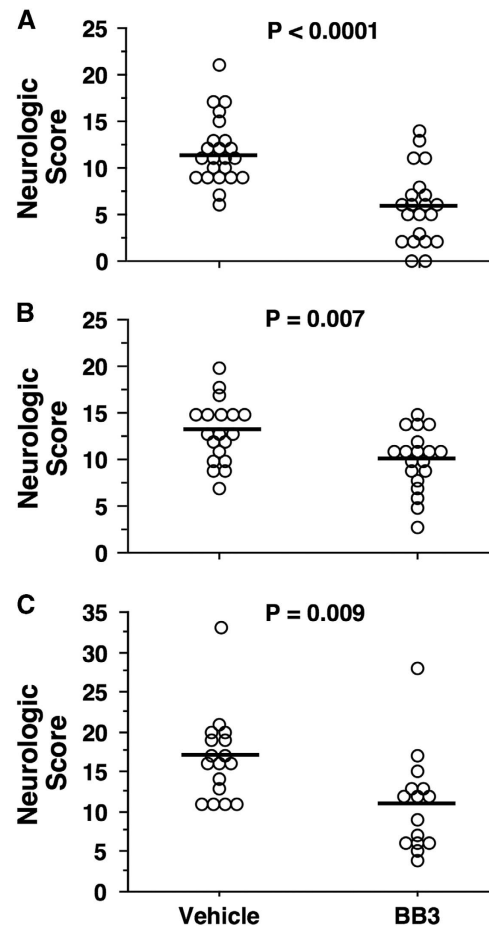
	Vehicle (n = 23)	BB3 (n = 21)
<i>Pre-ischemia</i>		
Body weight (g)	266 ± 22	269 ± 19
MAP (mm Hg)	81 ± 16	74 ± 14
Hematocrit (%)	44 ± 2	44 ± 1
Blood glucose (g/dL)	147 ± 29	153 ± 27
Arterial pH	7.40 ± 0.04	7.40 ± 0.05
PaCO <sub>2</sub> (mm Hg)	37 ± 4	38 ± 6
PaO <sub>2</sub> (mm Hg)	139 ± 13	144 ± 24
Pericranial temperature (°C)	37.5 ± 0.1	37.5 ± 0.2
<i>Intraischemia</i>		
MAP (mm Hg)	79 ± 15	72 ± 20
Hematocrit (%)	43 ± 2	43 ± 2
Blood glucose (g/dL)	145 ± 30	144 ± 25
Arterial pH	7.39 ± 0.05	7.39 ± 0.04
PaCO <sub>2</sub> (mm Hg)	38 ± 4	38 ± 4
PaO <sub>2</sub> (mm Hg)	140 ± 29	147 ± 27
Pericranial temperature (°C)	37.3 ± 0.4	37.4 ± 0.2
% LDF change	70 ± 6	67 ± 6
<i>Post-ischemia (15 minutes)</i>		
MAP (mm Hg)	77 ± 12	76 ± 17
Blood glucose (g/dL)	148 ± 27	143 ± 27
Hematocrit (%)	43 ± 2	43 ± 2
Arterial pH (mm Hg)	7.39 ± 0.04	7.39 ± 0.04
PaCO <sub>2</sub> (mm Hg)	38 ± 5	39 ± 4
PaO <sub>2</sub> (mm Hg)	145 ± 25	143 ± 25
Pericranial temperature (°C)	37.4 ± 0.3	37.4 ± 0.2
<i>4 Weeks post-ischemia</i>		
Body weight (g)	353 ± 55	358 ± 64

Abbreviations: LDF, laser Doppler percent change from pre-ischemia baseline; MAP, mean arterial pressure; PaCO<sub>2</sub>, arterial blood carbon dioxide partial pressure; PaO<sub>2</sub>, arterial blood oxygen partial pressure. Values = mean ± s.d. There were no statistically significant differences between groups.

deaths occurred at ≥ 7 days after ischemia onset. The cause could not be determined. There was no effect of group assignment on MAP over the 28-day recovery period ( $F = 0.334$ ,  $P = 0.57$ ), nor was there an interaction between group and MAP ( $F = 0.70$ ,  $P = 0.71$ ). BB3 improved neurologic score (vehicle =  $17 \pm 5$ , BB3 =  $11 \pm 6$ ,  $P = 0.009$ , Figure 2C). Importantly, the magnitude of neurologic deficit in the vehicle treatment group was greater than that observed in the younger animals described above. Cortical ( $43 \pm 33$  mm<sup>3</sup> versus  $35 \pm 27$  mm<sup>3</sup>,  $P = 0.47$ ), subcortical ( $26 \pm 13$  mm<sup>3</sup> versus  $17 \pm 12$  mm<sup>3</sup>,  $P = 0.07$ ), and total infarct volumes ( $68 \pm 42$  mm<sup>3</sup> versus  $52 \pm 37$  mm<sup>3</sup>,  $P = 0.26$ ) were not different between vehicle and BB3 groups, respectively. Neurologic score was associated with total infarct size (neurologic score =  $9.12 + 0.08 \times$  total infarct size;  $R^2 = 0.26$ ,  $P = 0.003$ ).

#### Experiment 8: BB3 Is Efficacious in a Mouse Middle Cerebral Artery Occlusion Outcome Model

All mice met the LDF flow reduction criterion of 80% from baseline. No animals were excluded because of hemorrhage. Mortality rates over the 8-week recovery interval were 50%, 60%, 30%, and 20% for the vehicle 10-minute, BB3 10-minute, vehicle 6-hour, and BB3 6-hour groups, respectively. We pooled the vehicle 10-minute and vehicle 6-hour groups to improve statistical power. This provided 12 vehicle, four BB3 10-minute, and eight BB3 6-hour animals for final outcome analysis. Outcome data are presented in Figure 3. There was no difference among groups for LDF values or 8-week brain atrophy/tissue loss. Surviving mice



**Figure 2.** Neurologic scores 28 days after temporary (A) and permanent (B) middle cerebral artery occlusion (MCAO) in rats 10 to 12 weeks of age. (C) Shows 28-day neurologic scores in 22-month old male Fischer 344 rats subjected to permanent MCAO. Open circles indicate individual rat values. Horizontal bars indicate group mean values. A score of 0 indicates no deficit.

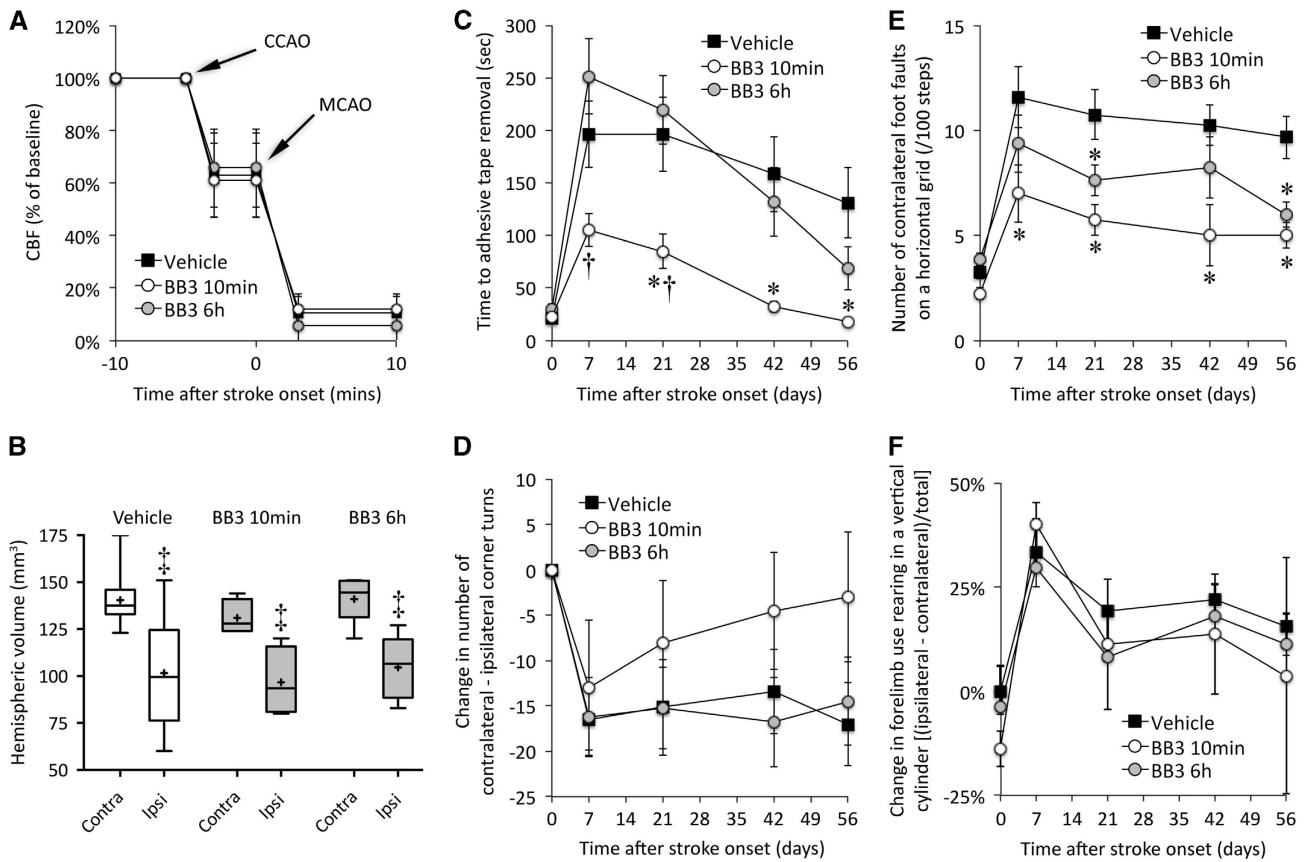
treated with BB3 at 10 minutes after MCAO were superior to the BB3 6-hour and vehicle groups in the tape-removal test. Corner test performance tended to be superior in the BB3 10-minute versus vehicle group. Both BB3 10-minute and BB3 6-hour mice had superior performance on the grid-walk compared with vehicle. There was no difference for the cylinder test.

#### Experiment 9: Delayed BB3 Treatment Does Not Affect Stroke-Induced Edema

There was a main effect in the right (ipsilateral) hemisphere ( $P < 0.001$ ) for ischemia, but not BB3. Both vehicle ( $81.7 \pm 1.0\%$ ,  $P = 0.002$ ) and BB3 ( $82.4 \pm 1.2\%$ ,  $P < 0.001$ ) groups had greater water content than naive rats ( $79.6 \pm 0.3\%$ ). There were no differences among groups in the contralateral hemisphere (naive =  $79.5 \pm 0.4\%$ , vehicle =  $79.0 \pm 0.4\%$ , BB3 =  $79.9 \pm 0.4\%$ ,  $P = 0.23$ ).

#### Experiment 10: BB3 Increases Post-Ischemic c-Met Phosphorylation and Synaptophysin Expression After Rat Temporary Middle Cerebral Artery Occlusion Outcome

BB3 increased phosphorylation (as a primary event in receptor signaling) of its cognate receptor c-Met 3.3-fold in penumbral tissue at 24 hours after ischemia ( $P = 0.047$ , Figures 4A to 4C).



**Figure 3.** Results from mouse temporary middle cerebral artery occlusion (MCAO) analysis. (A) Peri-ischemic laser Doppler flow as percent of pre-ischemic baseline, (B) ipsilateral hemisphere and contralateral hemispheric volumes 8 weeks after MCAO, (C) adhesive tape-removal test, (D) corner test, (E) grid-walk test, and (F) cylinder test. \* $P < 0.05$  versus vehicle, † $P < 0.05$  versus BB3 6 hours, # $P = 0.067$  versus vehicle, ‡ $P < 0.05$  versus contralateral. Corner test showed a strong trend for improved function at 56 days in BB3 10-minute group compared with vehicle ( $P = 0.067$ ).

Phosphorylated c-Met was similarly, but not significantly increased 2.5-fold in the contralateral hemisphere (data not shown).

For synaptophysin, a main effect was present for treatment group ( $F = 6.44$ ,  $P = 0.01$ ) in the ipsilateral cortex. *Post hoc* comparison found an increase in synaptophysin in rats treated with BB3 for 28 days versus vehicle ( $P = 0.01$ , Figure 5). There was no effect of BB3 treatment in the contralateral cortex ( $P = 0.81$ ).

## DISCUSSION

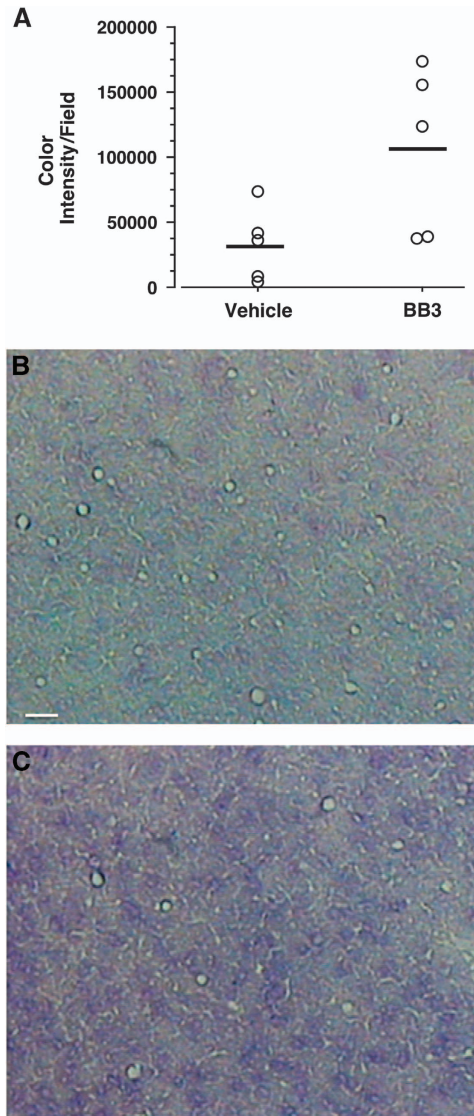
Results from this study, designed to conform to STAIR and Good Laboratory Practice guidelines,<sup>23,24</sup> suggest that the HGF pathway can potentially be harnessed for neurologic benefit in ischemic stroke with use of a small molecule. The extent to which STAIR and Good Laboratory Practice guidelines have been met is detailed in the Supplementary Table. With BB3 approaching end-of-Phase II studies in renal recipients and awarded both Fast-Track and Orphan Status by the United States Food and Drug Administration for that indication, from a clinical developmental perspective this therapeutic also holds promise for stroke survivors.

We have herein demonstrated that BB3 (1) crosses the BBB, (2) exerts dose-dependent effects within a dose range consistent with Investigational New Drug-enabling toxicology analysis, (3) exerts functional efficacy in male rats subjected to sustained post-ischemic pericranial thermoregulation for both tMCAO and pMCAO and long-term recovery (4 weeks), (4) exerts functional efficacy in female rats, (5) promotes functional efficacy in aged rats subjected to pMCAO and long-term recovery in the absence of

blood pressure effects, and (7) has a therapeutic window of at least 6 hours in rats and at least 10 minutes in mice. The enhanced c-Met phosphorylation and increased synaptophysin expression observed in the ischemic cortex of the BB3-treated cohort are consistent with expected functions of an HGF-like compound.

In the context of acute brain injury, HGF's neurotrophic properties may be an advantage to both post-ischemic resident and stem cell-derived tissue.<sup>16,34,35</sup> Of particular interest was the report of Shang *et al*<sup>17</sup> in which recombinant human HGF was applied topically to the cortex immediately after MCAO. Hepatocyte growth factor decreased infarct size, whereas neurogenesis, angiogenesis, and synaptogenesis were increased at 14 days after ischemia, in contrast to glial cell line-derived neurotrophic factor, which decreased infarct size only. Neurologic function, the most clinically relevant end point, was not measured. Doepfner *et al*<sup>16</sup> subjected mice to 3 days of post-tMCAO intrastriatal HGF beginning immediately after reperfusion onset. Motor coordination was assessed over 28 days recovery. Hepatocyte growth factor ameliorated post-MCAO motor deficits and this was associated with post-ischemic neurogenesis and inhibition of matrix metalloproteinases 2 and 9. Of note, both infarct size and Evan's blue extravasation were decreased at 24 hours after MCAO and infarct size remained decreased on day 7 with an intermediate, but not low HGF dose. The metalloproteinase inhibitor BB-1101 emulated the effect of HGF on infarct size implicating this pathway in the efficacious response.

Findings from the current experiment using a small molecule HGF-like compound can be contrasted with these findings.



**Figure 4.** Phosphorylated c-Met. After temporary middle cerebral artery occlusion, rats ( $n=5$  per group) were randomly assigned to receive either BB3 (6 mg/kg) or vehicle at 2 and 23 hours after reperfusion. Brains were harvested 1 hour after the second dose and phosphorylated c-Met was measured in the ischemic penumbra. **(A)** BB3-treated rats had greater phosphorylated c-Met immunoreactivity. Open circles represent individual animal values. Horizontal bars = mean values. **(B)** Representative vehicle-treated animal. **(C)** Representative BB3-treated animal.  $P < 0.05$ . Scale bar = 50  $\mu\text{m}$ .

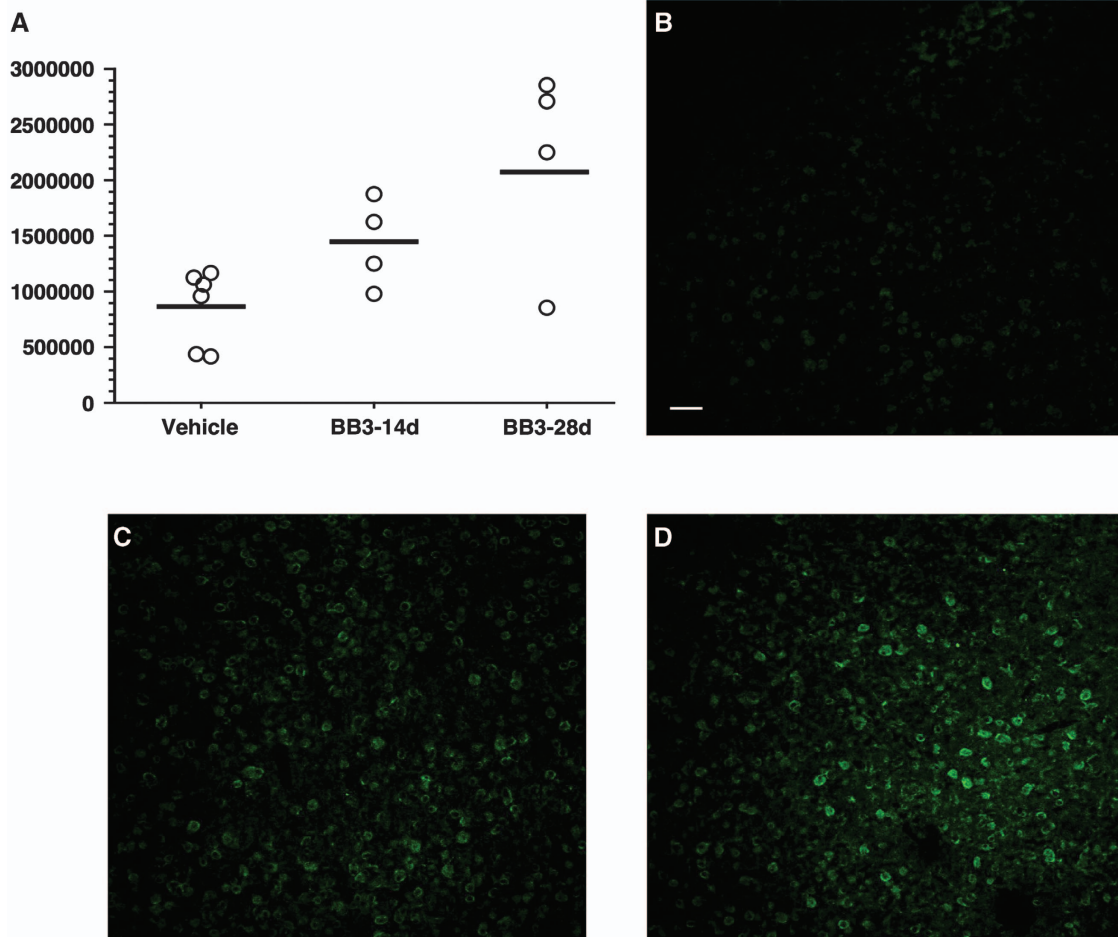
Evidence varies with respect to HGF BBB penetration. Pan *et al*<sup>36</sup> reported substantial blood to brain transfer of intravenously injected radiolabeled-HGF in mice and implicated a transporter system. In contrast, Kern *et al*<sup>13</sup> found no correlation between serum and cerebrospinal fluid HGF concentrations in patients with bacterial meningitis. Radiolabeled-BB3 was found to have rapid and substantial BBB penetration. This suggests a therapeutic advantage over HGF protein, as all preclinical stroke studies, to date, have used direct HGF microinjection or viral transfection into the brain.

Of note, our study found little benefit from BB3 on cerebral infarct size, which corresponded with a lack of BB3 effect on acute brain water content. This is in contrast to studies using HGF. Thus, BB3 appears provide functional benefit through a sustained

neurorestorative effect, consistent with the observed increase in synaptophysin expression. It is plausible that BB3 lacks some properties associated with HGF. Alternatively, the preponderance of our work was performed with a 6-hour delay to treatment onset and long-term outcome analysis, in contrast to HGF studies that used either pretreatment or immediate post-reperfusion treatment paradigms for infarct volume analysis.<sup>16,34,37</sup> A delay to treatment onset of 6 hours would presumably occur after considerable irreversible ischemic injury is present, but before completion of full infarct evolution.<sup>38</sup> Although this may explain the lack of a substantive effect of BB3 on infarct size in the rat experiments, we would have expected an effect on infarct size in the mouse study where treatment onset began 10 minutes after reperfusion. Doepfner *et al*<sup>16</sup> reported that HGF effects on post-MCAO infarct size were dose-dependent. BB3 may offer greater benefit at doses higher than those investigated herein. However, to the extent rat studies are predictive, this would present major clinical challenge. Higher BB3 doses examined in Experiment 2 (Figure 1) caused major and sustained hypothermia, which currently is not indicated for ischemic stroke and would be dose-limiting. Our study may also have been underpowered to detect a BB3 effect on rat infarct size if an effect was present, given the small magnitude of difference between treated and untreated animals. Sample sizes were calculated to detect a 37% decrease in infarct size on the basis of our previous experience with this model.<sup>27</sup> Cerebral infarct volume was numerically decreased in BB3-treated animals in all the experiments except male rats in Experiment 6 and mice in Experiment 8, but the effect size was substantially less than the study was powered to detect. Finally, all of our measures of infarct size occurred at 2 to 8 weeks after ischemia. It is plausible that BB3 delayed infarct maturation, albeit final infarct size became similar over time. Hence, we cannot rule out a direct effect of BB3 on infarct size as a potential contributor to the consistent improvement in neurologic function observed throughout the series of experiments.

BB3 consistently improved functional performance independent of sex, age, rodent species, or temporary versus permanent MCAO and studies were conducted in three independent laboratories. Improved functional performance was present in all rat studies. The scoring system we used includes the major elements of the classic Bederson and Garcia scoring systems.<sup>39,40</sup> We believe the scoring system is valid in that it has repeatedly correlated with cerebral infarct size.<sup>26,27</sup> Despite the potential maximal neurologic deficit score of 48 with this system, rats with scores in excess of 15 are typically moribund with major gross neurologic deficit. As a result, insult severity (i.e., ischemia duration) was titrated to produce the maximal deficit consistent with long-term recovery under laboratory husbandry conditions. BB3 also showed benefit in two of four functional measures in mice, with a strong trend for benefit in a third. The implications of the functional effect size in rodents for clinical translation are unknown. No molecule has successfully translated from bench to bedside that we can use as a standard to define a preclinical effect size sufficient to predict that a clinical effect could be detected. It is possible that the BB3 effect size is not sufficient; yet, given the robustness of the effect observed across models and laboratories, we believe that continued BB3 development is warranted. This is particularly true because the compound exists as a pharmaceutical grade substance, has completed clinical phase I testing and is in international multicenter phase II trials for a renal indication.

Although the work to date is comprehensive and rigorous, one weakness may be the absence of sham-operated rats (and mice), which could provide a reference for functional changes attributable to anesthesia, surgery, and/or aging over the 4- to 8-week recovery intervals. Shams were not included to conserve statistical power and time, but this may limit interpretation. In contrast, preischemic neurologic function, as determined by our standardized scoring system, found negligible deficits in aged rats despite



**Figure 5.** Ipsilateral synaptophysin immunofluorescence (y-axis) in rats subjected to temporary MCAO and treated with either vehicle or BB3 for 14 or 28 days. Rats were allowed to survive 28 days after ischemia. A main effect was present for treatment group ( $P=0.01$ ). *Post hoc* comparison found a significant increase in synaptophysin in 28 days BB3-treated rats versus vehicle. (A) Open circles represent individual animal values. Horizontal bars = mean values. (B) Representative vehicle-treated rat. (C) Representative rat treated with BB3 for 14 days. (D) Representative rat treated with BB3 for 28 days. Scale bar = 50  $\mu\text{m}$ . MCAO, middle cerebral artery occlusion.

obvious senescence. Hence, it is unlikely that sham-operated controls would present persistent neurologic deficits over the extended recovery periods used. Additional functional testing, including assessment of cognitive factors, may provide a more sensitive measure of efficacy.

This constellation of data is consistent with potential clinical efficacy. However, data remain lacking for (1) efficacy in the context of comorbidities common to stroke patients, (2) interactions with drugs commonly encountered in stroke patients, (3) and efficacy by a route of administration consistent with clinical use (e.g., intravenous) with minimum and maximum tolerated doses defined. Further, a treatment duration-response experiment has not been performed to determine the minimal treatment duration necessary to obtain maximal therapeutic benefit. Consequently, it remains premature to recommend advancement of BB3 to clinical trial status in ischemic stroke until this additional research is completed.

In conclusion, a series of experiments was performed using the STAIR and Good Laboratory Practice guidelines to determine preclinical efficacy of the HGF-like small molecule BB3 in focal ischemic stroke. Using rigorous pericranial temperature control during and for 20 hours after ischemia, rats treated with BB3 for 14 days beginning 6 hours after tMCAO reperfusion (or pMCAO onset) exhibited improved neurologic function after a 28-day recovery interval without a detectable effect on cerebral infarct

size. BB3 efficacy was independent of laboratory, sex, age, or MCAO model and persisted with sustained strict post-ischemic pericranial temperature control. Mice appeared to have a narrower therapeutic window with efficacy noted with treatment onset at 10 minutes, but not 6 hours after MCAO reperfusion. On the basis of the large body of positive data presented, BB3 warrants further investigation as a neurorestorative agent after ischemic stroke.

#### DISCLOSURE/CONFLICT OF INTEREST

DES is the principal investigator on NIH NINDS 2R44NS045373, 5R44NS045373 and participates in the stock option plan of Angion Biomedica Corporation. IDG is an officer and director of Angion Biomedica Corporation and principal stockholder. BD, J-SL, BS, and KJ participated in the stock option plan of Angion Biomedica Corporation. CA received subcontract funding to support this research through NIH NINDS 5R44NS045373. DSW received subcontract funding to support this research through NIH NINDS 5R44NS045373. The remaining authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

REC, HSh, DES, IDG, CA, and DSW were involved in the experimental design, data analysis, data interpretation, revising manuscript for critical intellectual content and approval of submitted version of manuscript. MI, TS, YZ, H5a, TQ, DvB, FH, BD, J-SL, KJ, MP, and RDP were involved in data acquisition and analysis, revising manuscript for critical intellectual content and approval of submitted version of manuscript.

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