SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals, *a priori* sample size calculation, power analysis, blinding, and randomization

Fifty-seven aged male Sprague Dawley rats (18-20 months, Hilltop Laboratories, Scottdale, PA, USA) underwent 90 min of MCAO or sham operation. We performed an *a priori* sample size calculation using the G*Power v.3.1.3 software[1]. In order to calculate Cohen effect size (*d*), we compared two independent groups in a two-tailed unpaired *t*-test using α=0.05, and β (type II error) of 0.1 with a power of 90%. We utilized means and standard deviations from our preliminary studies in this stroke model in aged rats. The present study was powered with the expectation that we will detect a difference in infarct size of at least 25% between vehicle- and MRE-269-treated groups, which is a biologically meaningful effect[2]. For the difference to become statistically significant, we calculated a sample size of *n*=11 with an effect size of *d*=1.34. A final sample size of *n*=13 per group (vehicle- or MRE-269-treated) was utilized for the experiments described in this report after adjusting for a 15% attrition rate. Corrected sample size=calculated sample size/(1-[% attrition/100]). Five sham-operated control rats were also included for the molecular biology analyses performed with tissue collected after 18 h of ischemia. All rats were housed in individual cages in a controlled environment maintained under a 12-h light/dark cycle. Animals had free access to food and water, and they were acclimated to our animal facility for at least 7 days before any surgical procedure. All experimental animal procedures were performed in accordance with approved guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals, the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines), and the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida (protocol #201406503). Animals were randomly allocated to treatment groups using the GraphPad software randomization tool (http://www.graphpad.com/quickcalcs/randomize1.cfm). The investigators performing surgeries and euthanizing animals had no knowledge of the experimental group to which an animal belonged. An independent investigator administered either the vehicle or MRE-269 (coded vials) according to the randomization schedule to guarantee treatment allocation concealment. Similarly, investigators responsible for the assessment of outcomes (infarct size, behavior, and molecular biology analyses) were blinded to treatment groups (coded animals, samples, and MRI images).

Induction of transient MCAO in aged rats

Transient MCAO was induced using an intraluminal silicone-coated filament method as previously described by our group[3, 4]. Briefly, animals were subjected to anesthesia by 3% isoflurane and maintained by inhalation of 1.5-2% isoflurane in medical grade oxygen during surgery. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed via a midline vertical incision in the anterior neck. The CCA was ligated with a 4-0 suture at the proximal portion from the carotid bifurcation. Another 4-0 suture was loosely tied around the ICA and ECA bifurcation and micro-aneurysm clips were temporarily placed on the ICA and ECA. A small arteriotomy was made in the CCA approximately 2 mm proximal to the carotid bifurcation, and a 3-0 silicone-coated nylon filament was inserted via the incision and advanced gently in the ICA approximately 20-22 mm from the carotid bifurcation until a mild resistance was felt. The end of the occluding filament was coated with silicone rubber (6-7 mm coating length and 0.48 mm in diameter). The clips on the ICA and ECA were removed during the induction of focal cerebral ischemia, and rats were allowed to recover from

anesthesia. After 90 min ischemia, rats were re-anesthetized and the filament was gently retracted to allow reperfusion. The skin was closed and anesthesia was discontinued, the animals were allowed to recover in a temperature-controlled chamber. Buprenorphine hydrochloride (Buprenex; 0.05 mg/kg; s.c.) was given as an analgesic immediately before surgery. Sham-operated rats underwent the same surgical procedures except for the advancement of the silicone-coated filament into the ICA. Ischemia and reperfusion conditions were confirmed by regional cerebral blood flow (CBF) detected by a laser Doppler flowmeter (moorVMS-LDF, Moor Instruments, Delaware, DE, USA) positioned 1.0 mm posterior to bregma and 5.0 mm lateral to the midline before, during, and after withdrawal of the occluding filament. Only rats showing sustained CBF reduction to less than 55% of pre-ischemic baseline values as well as successful reperfusion (>90% of baseline) were included in the experiments. Animals showing complete loss of spontaneous activity for a prolonged time (>2 h), subarachnoid hemorrhage upon tissue harvesting, or lack of neurological deficits after 90 min of stroke were excluded from the analysis. Blood was collected from the femoral vein (~0.2 mL) to measure physiological parameters with an iSTAT clinical analyzer using CG8+ cartridges (Cat. No. 600-9001; Abbott, Princeton, NJ) 15 min before and after MCAO, as well as 15 min after initial dose of vehicle or MRE-269 injection.

Experimental design and drug administration

For the intravenous administration of vehicle or MRE-269, a Micro-Renathane catheter was inserted into the right femoral vein of ischemic rats at the time of MCAO surgery. The catheter was placed in the distal portion of the vein to minimize disruption of blood flow in the leg. This technique has been utilized in long-term behavioral studies in rodents after stroke[5] and results from our pilot studies indicate that distal catheterization of the femoral vein does not interfere with motor behavior in rats. MRE-269 (Cat No.10010412; Cayman Chemical, Ann Arbor, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) and then diluted in sterile saline. The final concentration of DMSO was 1%. For the infarct size and neurobehavioral tests experiments, twenty-six rats underwent transient MCAO and were randomly assigned to vehicle or treatment group with administration of 1% DMSO in saline (n=13) or MRE-269 (0.25 mg/kg, n=13) starting at 4.5 h post-MCAO. Additional doses were given every 12 h for the first 48 h, and then one injection daily for 7 days post-MCAO. The MRE-269 dose and treatment schedule was based on our preliminary findings in young rats (3-4 months) showing that MRE-269 (0.1-0.5 mg/kg, i.v.) given at 1.5 h after stroke onset produced a dose-dependent reduction in infarct volume as measured by TTC staining at 48 h (data not shown). The optimal dose of 0.25 mg/kg was therefore used in the present study in aged rats.

Rats were scanned by MRI at 48 h and 21 days post-MCAO and neurobehavioral tests were performed before ischemia and at 3, 7, 14 and 21 days after stroke. Five rats in the vehicle and 4 rats in the MRE-269 groups died during the 21 days post-MCAO period. For the biochemical experiments, 31 rats were randomly assigned to the following groups: sham-operation (n=5), vehicle (1% DMSO, n=13) or MRE-269 (0.25 mg/kg, n=13). Drug or vehicle were given intravenously starting at 4.5 h after stroke and an additional dose was given at 12 h after stroke onset. One animal in the vehicle group died shortly after MCAO. Animals were sacrificed at 18 h post-MCAO and perfused with ice-cold saline. Samples from the ipsilateral and contralateral cerebral cortices were obtained for RNA isolation, immunoblotting, and lipid peroxidation analyses.

MRI and image analysis

At 48 h and 21 days after MCAO, rats treated with vehicle or MRE-269 (0.25 mg/kg) were brought to the University of Florida's Advanced Magnetic Resonance Imaging and Spectroscopy Facility (AMRIS) for MRI measurements. During imaging sessions, rats were anesthetized under 1.5-2% isoflurane gas and placed supine on a custom-made bed and their heads accommodated inside a quadrature surface transmit/receive ^{1}H radiofrequency coil tuned to 200 MHz (airmri, LLC, Holden, MA). Diffusion and T2 weighted images were collected in a Magnex Scientific 4.7 Tesla MR scanner controlled by an Agilent VnmrJ 3.1 console. Respiratory rates and core body temperature were monitored and controlled continuously through the experiment (SA Instruments). For diffusion-weighted MRI, we collected eight interleaved coronal slices covering the entire rostral-caudal extent of the stroke lesion. The following parameters were optimized and used: conventional spin echo sequence with a repetition time (TR) = 2 sec, echo time (TE) = 37.5 ms, field of view (FOV) = 25.6^2 mm, slice thickness = 1.5mm, data matrix = 96 x 96 (for an in-plane resolution of 267 μm^2). A trace weighted sequence was used with one B = 0 and three orthogonal B images = 1270 s/mm^2, Δ = 17.5 ms, δ = 5.6 ms, and gradient (G) amplitude 18.61 Gauss/cm. Scan time was 13 minutes per rat. Region of interest (ROI) analysis for the stroke volume was used. Stroke volume was the primary measure for the present study. We quantified the apparent diffusion coefficient (ADC) for the side ipsilateral to the lesion and the contralateral side. The first image in the trace sequence was a T2 image that provided excellent contrast for delineating the outer borders of the lesion area across multiple slices. Manual ROI drawing for each subject at each time point was carried out using ITK-SNAP software. The T2 and diffusion weighted images were imported into NIH ImageJ software (http://rsbweb.nih.gov/ij) and ADC maps were constructed. ADC maps were set at a threshold between 0 and 200 s/mm^2 and ADC values extracted for the stroke lesion side and the contralateral side. Hyper-intense ischemic areas in the ADC and T2 weighted images were marked with a region of interest (ROI) tool in the ITK-SNAP software. The mean signal intensity of all pixels within the ROI was used to calculate infarct area. Stroke ROI volumes were converted to .vtk format and registered to an atlas of the rat brain in order to assess relative changes in lesion with and without MRE-269 treatment.

Behavioral tests

To examine the effects of MRE-269 on neurological outcomes in aged rats subjected to ischemic stroke, a battery of behavioral tests was performed pre-MCAO and at 3, 7, 14 and 21 days post-MCAO by an independent investigator blinded to the experimental groups. For the adhesive removal test, somatosensory deficits can be seen in the latency for the rat to notice the sticker and remove it from the paw as previously described[6] with some modifications. Briefly, adhesive tape (113.1 mm^2 round) was placed on the ventral surface of the contralateral forepaw. The observer recorded the latency for each rat to remove the adhesive tape with its mouth up to a cut-off time of 2 min. Before surgery, the animals were pre-trained for 2 days. Once the rats could remove the tape within 10 seconds, they were subjected to MCAO. Three trials per animal were performed and the average of the two best (smallest) latency values was used for analysis.

For the rotarod test, locomotor impairments were assessed with the accelerating rotarod as described previously[7] with minor modifications[8, 9]. Briefly, rats were placed on an accelerating rotarod (model ENV-575, MED Associates Inc., Fairfax, VT, USA) and the speed was slowly increased from 4 to 40 rpm during a 5-min period. Before surgery, the animals were pre-trained for 2 days. Latency to fall off the rotarod was recorded before ischemia and at 3, 7, 14 and 21 days after stroke. All animals were

required to stay on the accelerating rotarod for a minimum of 30 seconds. If they were unable to reach this criterion, the trial was repeated for a maximum of 5 times instead of 3 times. The average of the two best (largest) fall latency values was used for analysis and the motor test data were presented as percentage of mean latency compared with the internal baseline (before surgery).

RNA extraction and real-time PCR

As described previously by our group[3], total RNA from cortical tissue was extracted using the PureZOL™ RNA isolation kit (Cat. No. 738-6830; Bio-Rad, Hercules, CA) according to the manufacturer's protocol. cDNA was synthesized using iScript Reverse Transcription kit (Cat. No. 170-8841; Bio-Rad) and quantitative real-time PCR was performed with 20 ng of cDNA in a total volume of 10 µl using Sso Advanced Universal SYBR Green Supermix (Cat. No. 172-5272; Bio-Rad) according to the manufacturer's protocol. The following primers were used: *IL-1β*, GTGCTGTCTGACCCATGT (forward), TTGTCGTTGCTTGTCTCTCC (reverse); *TNF-α*, AGACCCTCACACTCAGATCA (forward), GTCTTTGAGATCCATGCCATTG (reverse); *IL-6*, CAGAGCAATACTGAAACCCTAGT (forward), CCTTCTGTGACTCTAACTTCTCC (reverse); *MCP-1*, ATCTCTCTTCCTCCACCACTA (forward), GAATGAGTAGCAGCAGGTGAG (reverse); *Ywhaz*, GAAGAGTCGTACAAAGACAGCA (forward), GCTTCTGCTTCGTCTCCTTG (reverse). PCR reactions were run in triplicate and cycle threshold (Ct) values were normalized to *Ywhaz* expression for each sample. Results are presented as normalized expression relative to sham-operated group.

Immunoblotting

Cortical tissue was homogenized in radioimmunoprecipitation (RIPA) lysis buffer containing protease and phosphatase inhibitors as detailed in our recent report[3]. Fifty micrograms of protein were incubated in non-reduced Laemmli's sample buffer for 5 min at 100°C to measure $gp91^{phox}$, while incubated at 60°C for 3 min to analyze 4-hydroxynonenal (4-HNE) as previously reported[10]. After the incubation step, samples were separated in 4-20% SDS-polyacrylamide gels, and then transferred onto nitrocellulose membranes. Membranes were then blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (TBS) before overnight incubation at 4°C with antibodies against either $gp91^{phox}$ (Cat. No. 611414, 1:1000; BD Biosciences, San Jose, CA), 4-HNE (Cat. No. ab48506, 1:200; Abcam, Cambridge, MA) or β-actin (Cat. No. A1978, 1:10,000; Sigma-Aldrich, Saint Louis, MO). Afterwards, membranes were washed and incubated for 1 hour with goat anti-mouse IRDye 800CW secondary antibody (1:30,000; Li-Cor, Lincoln, NE, USA) for detecting $gp91^{phox}$ or 4-HNE, and donkey anti-mouse IRDye 680LT (1:40,000; Li-Cor) for β-actin. Immunoreactive bands were visualized and densitometrically analyzed using Odyssey infrared scanner and Image Studio 2.0 software (Li-Cor).

Determination of 8-*iso*-prostaglandin $F_{2\alpha}$

To determine the effects of delayed MRE-269 treatment on lipid peroxidation, 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$), a highly sensitive biomarker of oxidative stress, was measured using a commercially available ELISA kit (Cat. No. 516351, Cayman Chemical, Ann Arbor, MI) following the manufacturer's protocol. Briefly, the 8-*iso*-$PGF_{2\alpha}$ from brain cortical tissue was hydrolyzed with 15% (w/v) KOH before purification using an 8-isoprostane affinity sorbent protocol. Each sample was assayed in duplicate, and total 8-*iso*-$PGF_{2\alpha}$ in samples was determined based on a standard curve and expressed in nanogram per gram of tissue.

Immunoglobulin G (IgG) ELISA

To study the blood brain-barrier (BBB) permeability, the extravasation of IgG from the blood into the brain parenchyma was measured by a commercial IgG ELISA kit (Cat. No. E-25G; Portland, OR) according to the manufacturer's instructions. A total of 50 µg of protein extracted from ipsilateral and contralateral cerebral cortices of rat brain were used for the IgG measurement. All samples were assayed in duplicate and optical absorbance at 450 nm was measured with a Synergy™ HT Multi-Mode Plate Reader (Biotek Instruments, Winooski, VT). A standard curve was constructed (0-400 ng/ml) and the concentrations of IgG in brain tissue were determined from the standard curve.

Fluorometric immunocapture assay of matrix metallopeptidase (MMP)-9 enzymatic activity

Enzymatic activity of matrix metallopeptidase (MMP)-9 in cortical tissue was measured using a fluorescence resonance energy transfer (FRET) peptide immunoassay as described in our previous studies[3, 11]. Briefly, 96-well plates were coated with 5 µg/ml protein A/G for 2 hours at room temperature before the addition of the MMP-9 antibody (Cat. No. sc-6841R; Santa Cruz Biotechnology, Dallas, TX) for another 2 hours incubation. A total of 50 µg protein extracted from rat brain cerebral cortex was added to each well and incubated overnight at 4°C with gentle shaking. After incubation, wells were washed with TCNB buffer (50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35) and 1 µM of 520 MMP FRET substrate III (Cat. No. 60570-01; AnaSpec, San Jose, CA) was added. Plates were incubated for 48 h at 37°C, then relative fluorescence units (RFUs) were read and monitored at excitation/emission wavelengths of 485/528 nm in a Synergy™ HT Multi-Mode Plate Fluorescence Reader (Biotek Instruments, Winooski, VT). The average value from one paired substrate control wells was used to subtract baseline fluorescence from sample wells.

Supplemental References

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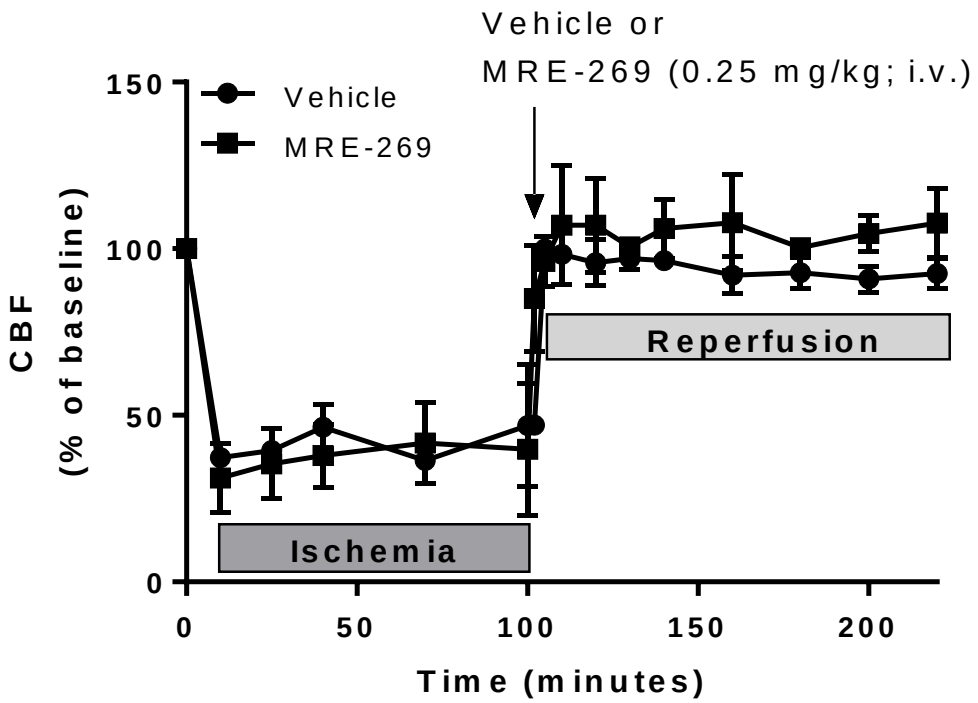
Supplemental Table I. Physiological parameters in aged rats treated with vehicle- or MRE-269

	Vehicle	MRE-269 (0.25 mg/kg; i.v.)
15 min before MCAO		
pH	7.33±0.01	7.30±0.04
PCO_2 (mmHg)	54.62±2.12	59.36±6.38
PO_2 (mmHg)	291.60±29.74	274.60±14.94
HCO_3^- (mmol/L)	28.84±0.51	28.92±0.43
Na^+ (mmol/L)	135.00±0.45	135.00±1.45
K^+ (mmol/L)	3.90±0.09	3.68±0.17
Ca^{2+} (mmol/L)	1.28±0.05	1.22±0.04
Glucose (mg/dL)	392.80±26.85	395.80±41.42
Hematocrit (%)	42.20±1.07	39.00±1.92
Hemoglobin (g/dL)	14.36±0.36	13.28±0.65
Temperature (°C)	36.63±0.06	36.52±0.09
15 min after MCAO		
pH	7.39±0.06	7.33±0.02
PCO_2 (mmHg)	46.98±9.45	56.93±3.30
PO_2 (mmHg)	240.25±44.40	229.25±13.60
HCO_3^- (mmol/L)	26.85±2.47	30.03±0.21
Na^+ (mmol/L)	135.50±0.87	135.25±1.31
K^+ (mmol/L)	4.18±0.14	4.18±0.21
Ca^{2+} (mmol/L)	1.27±0.07	1.25±0.03
Glucose (mg/dL)	349.50±33.00	382.25±49.06
Hematocrit (%)	41.50±1.07	39.00±2.04
Hemoglobin (g/dL)	14.13±0.55	13.25±0.70
Temperature (°C)	37.09±0.52	36.77±0.17
CBF (% of Pre-MCAO baseline)	**41.74±3.31**	**35.78±3.91**
15 min after treatment		
pH	7.38±0.01	7.38±0.02
PCO_2 (mmHg)	51.68±1.81	55.43±3.79
PO_2 (mmHg)	368.20±36.84	396.67±48.64
HCO_3^- (mmol/L)	30.74±1.05	30.74±1.05
Na^+ (mmol/L)	137.60±0.40	138.80±0.58
K^+ (mmol/L)	3.96±0.14	4.16±0.16
Ca^{2+} (mmol/L)	1.21±0.05	1.23±0.05
Glucose (mg/dL)	325.40±25.06	275.00±31.47
Hematocrit (%)	39.40±0.75	38.00±2.49
Hemoglobin (g/dL)	13.40±0.26	12.92±0.82
Temperature (°C)	36.91±0.25	36.42±0.40
CBF (% of Pre-treatment baseline)	**93.23±2.74**	**92.70±1.80**

Values are mean ± SEM. MCAO, middle cerebral artery occlusion; CBF, cerebral blood flow.



Supplemental Figure I. Temporal profile of regional cerebral blood flow (CBF) in rats subjected to 90 min of transient MCAO followed by 120 min of reperfusion. Regional CBF reduced to about 40% of baseline immediately after MCAO occlusion and restored to baseline after reperfusion in both vehicle- (n=5) and MRE-269-treated (n=5) groups. A single bolus injection of MRE-269 (0.25 mg/kg) at the start of reperfusion via the right femoral vein has no significant effect on regional CBF compared to vehicle-treated animals. Data are expressed as mean ± SD.

Table II. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☑ An overall study timeline is provided.
Inclusion and exclusion criteria	☑ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☑ Type and methods of randomization have been described. ☑ Methods used for allocation concealment have been reported.
Blinding	☑ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☑ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☑ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☑ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☑ Statistical methods used have been reported. ☑ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. ☑ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☑ Statements on funding and conflicts of interests have been provided.