Pomalidomide Reduces Ischemic Brain Injury in Rodents

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Abstract

Stroke is a leading cause of death and severe disability worldwide. After cerebral ischemia, inflammation plays a central role in the development of permanent neurological damage. Reactive oxygen species (ROS) are involved in the mechanism of post-ischemic inflammation. The activation of several inflammatory enzymes produces ROS, which subsequently suppress mitochondrial activity, leading to further tissue damage. Pomalidomide (POM) is a clinically available immunomodulatory and anti-inflammatory agent. Prior cellular studies demonstrate that POM can mitigate oxidative stress and lower levels of pro-inflammatory cytokines, particularly TNF-α, which plays a prominent role in ischemic stroke-induced brain damage and functional deficits. To evaluate the potential value of POM in cerebral ischemia, POM was initially administered to transgenic mice chronically over-expressing TNF-α surfactant protein (SP)-C promoter (SP-C/TNF-α mice) to assess whether systemically administered drug could lower systemic TNF-α level. POM significantly lowered serum levels of TNF-α and IL-5. Pharmacokinetic studies were then undertaken in mice to evaluate brain POM levels following systemic drug administration. POM possessed a brain/plasma concentration ratio of 0.71. Finally, rats were subjected to transient middle cerebral artery occlusion (MCAo) for 60 min, and subsequently treated with POM 30 min thereafter to evaluate action on cerebral ischemia. POM reduced the cerebral infarct volume in MCAo-challenged rats and improved motor activity, as evaluated by the elevated body swing test. POM's neuroprotective actions on ischemic injury represent a potential therapeutic approach for ischemic brain damage and related disorders, and warrant further evaluation.

Keywords

pomalidomide, thalidomide, stroke, cerebral ischemia, TNF-α, pulmonary fibrosis

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Introduction

Stroke is the second major cause of death and the leading cause of long-term neurological disability worldwide[1]. Ischemic stroke represents approximately 80–85% of all strokes and has been the target of most drug trials[2–4]. After cerebral ischemia, inflammation plays a key role in the development of permanent neurological damage[5], and its mitigation may provide a treatment strategy. Brain ischemia produces superoxide through xanthine oxidase and leakage from the mitochondrial electron transport chain[6]. Superoxide is the primary radical from which hydrogen peroxide (H_2O_2) is formed. In its turn, H_2O_2 is the source of the hydroxyl radical (OH), which readily crosses cell membrane and generates oxidative damage[7,8]. Such free radicals can induce a series of cellular effects that include enzymatic inactivation, protein denaturation, cytoskeletal and DNA injury, lipid peroxidation, and chemotaxis. Severe acute oxidative stress can provoke cell death via necrosis, whereas moderate oxidation gives rise to apoptosis[9–11]. In the latter case, mitochondrial function is impaired by free radical-mediated breakdown of the inner mitochondrial membrane and the oxidation of proteins that mediate electron transport, H^+ extrusion, and adenosine triphosphate (ATP) production. As a consequence, cytochrome c is released from the mitochondria, and, following its binding to apoptotic protease activating factor-1 (Apaf-1) and formation of an apoptosome with caspase-9, the ensuing active caspase-3 cleavage impacts the downstream protein poly (ADP-ribose) polymerase (PARP)[12]. Substrate cleavage causes DNA injury and subsequently leads to apoptotic cell death[8].

Pomalidomide (POM), clinically approved for treatment of multiple myeloma and increasingly used in the treatment of other cancers, is a powerful immunomodulatory agent that also possesses anti-angiogenic properties[13–15]. It is a third-generation analogue of thalidomide that is widely reported to be significantly more potent than its parent compound at lowering pro-inflammatory cytokine levels[13,16]. Additionally, as an immune modulator, POM is reported to have less unfavorable teratogenic and neurotoxic adverse actions in preclinical models[17,18]. Our recent studies have demonstrated that POM mitigates H_2O_2-induced oxidative stress injury in rat primary cortical neuronal cultures by inducing anti-oxidative and anti-apoptosis effects and thereby reduces neuronal cell death[19]. In the light of a recent study demonstrating that POM decreased neuronal cell loss, neuroinflammation, and behavioral impairments induced by traumatic brain injury in rat[20], we evaluated the ability of the agent to lower pro-inflammatory cytokine levels in vivo, to enter the brain, and to mitigate ischemic stroke. Specifically, in the current study, we first evaluated the ability of POM to effectively lower systemic TNF-α levels in a mouse model of moderate chronic TNF-α over-expression involving TNF-α generation from the lung. Pom achieved this is a well-tolerated manner. We next evaluated the brain uptake of POM in healthy mice following a well-tolerated systemic

dose. POM achieved a brain/plasma concentration ratio of 0.71. Finally, we evaluated the ability of a well-tolerated systemic dose of POM to mitigate ischemic stroke. POM reduced the size of the brain infarct following transient middle-cerebral artery occlusion (MCAo) in rats, which was associated with decreased functional damage. Our animal studies indicate that POM provides neuroprotective actions of potential in cerebral ischemia and reperfusion injury, and warrants long-term efficacy and safety evaluation as a new treatment strategy.

Materials and Methods

Pomalidomide Preparation

POM was prepared in a two-step process. Initially, 3-aminopiperidine-2,6-dione was condensed with 3-nitrophthalic anhydride in refluxing acetic acid. Successive precipitation with ice water yielded the corresponding nitrothalidomide as a grey-purple solid. Subsequent hydrogenation over a palladium catalyst generated POM as a yellow solid, which was then subjected to chemical characterization to confirm the structure and purity of the agent. A sample of POM was additionally obtained from Selleckchem (Houston, TX, USA). POM was prepared freshly for all studies and, following milling with glass beads, was administered as a suspension in a 1% carboxy methyl cellulose/saline solution (Fluka 21901, Muskegon, MI, USA). POM was administered to mice 0.1 ml/10 g body weight and rats 0.1 ml/ 100 g body weight, and 1% carboxy methyl cellulose/saline solution was administered as a similar volume to vehicle dosed animals.

Animal Studies

Male C57Bl/6 mice (3 months of age and approximately 30 g) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) for pharmacokinetic studies to evaluate brain uptake of POM. Male transgenic mice overexpressing the proinflammatory cytokine TNF-α within lung type II alveolar epithelial cells under the control of the surfactant protein (SP)-C promoter (SP-C/TNF-α mice on a C57BL/6 background)[21] together with their wild-type (Wt) littermates were used to evaluate POM efficacy to lower inflammatory cytokine levels, and were generated in house from breeding pairs originally obtained from Dr. Li Zuo (Ohio State University). These mice were genotyped by PCR analysis of genomic DNA isolated from ear punches, as previously described[21]. Finally, male SD rats, weighing 250–300 g, were obtained from BioLASCO Taiwan for ischemic stroke studies. All animals were maintained under a 12/12-h day/night cycle, and were housed at an ambient temperature with free access to food and water at either the Animal Facility of the Intramural Research Program, National Institute on Aging, National Institutes of Health (NIH) (mice) or Experimental Animal Center of Taipei Medical University (rats). All animal study methods

were carried out in accordance with and complied to the NIH (DHEW publication 85–23, revised, 1996). Animal numbers for each assessment group and experimental measures were selected based upon our prior studies[19]. Their use in research was approved by the Animal Care and Use Committee of either the National Institute on Aging, Baltimore, MD, USA (protocol No. 331-TGB-2021 and 415-TGB-2021) or Taipei Medical University, Taipei, Taiwan (protocol No. LAC-1010147).

Evaluation of Pomalidomide to Lower TNF-α Levels in a Mouse Model of Moderate Chronic TNF-α Over-Expression

Transgenic (Tg) mice in which the 3'-untranslated region of the mouse tumor necrosis factor-α (TNF-α) gene was placed under the control of the transcriptional promoter of the SP-C gene within lung epithelial cells (i.e., Tg SP-C TNF-α mice) and Wt littermates that lacked the transgene were used to evaluate the ability of POM to lower elevated levels of TNF-α in vivo. These mice, originally developed by Miyazaki et al.,[21] in B6D2FI Tg founder mice, were backcrossed with C57BL16 mice to generate F1 hybrid Tg mice. Our studies involved male mice, 50–77 weeks of age, randomly separated into the following groups: (i) Wt + vehicle (Veh) 57–75 weeks old, (ii) Tg + Veh 50–77 weeks old, and (iii) Tg + POM 53–77 weeks old. The respective initial body weights (grams) were (i) Wt + Veh = 45.4 ± 1.9, $n = 10$; (ii) Tg+ Veh = 30.4 ± 1.0, $n = 9$; (iii) Tg + POM = 33.8 ± 3.0, $n = 9$. Mice were administered either POM 50 mg/kg once daily by intraperitoneal (i.p.) injection, or Veh once daily for 21 consecutive days, and their weight and physical appearance was noted as signs of wellbeing. Blood samples were obtained 24 h following the final dosing, placed on wet ice, and serum was removed and immediately frozen (–80°C) for later quantification of cytokines.

Cytokine analysis by multiplex ELISA. The Mesoscale Discovery (MSD) V-PLEX Proinflammatory Panel 1 was used to quantify mouse serum cytokine levels. The proteins of interest were: TNF-α, interleukin 1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, and interferon-γ (IFN-γ). The protocol used was that described by the manufacturer. Samples were briefly centrifuged (10,000 × g, 4°C, 60 s) to remove any particulate material, and then serum was diluted with the supplied assay diluent. A multiplex standard and the diluted serum samples were added to the V-PLEX ELISA plate, which was then incubated for 2 h. Following a series of washes, a multiplex detection antibody solution was added and the samples were incubated for a further 2 h. After an additional series of washes, the Read Buffer was added and the electrochemiluminescence (ECL) signal levels were measured (MESO QuickPlex SQ 120). The MSD Discovery Workbench software was used to determine the serum cytokine protein levels by comparing the mouse serum protein ECL signals to those of the appropriate protein standard curve. Protein concentrations were expressed as pg/ml.

Quantification of Pomalidomide Brain Uptake in Rodent

To evaluate the brain uptake of POM, its plasma and brain drug levels were quantified in C57Bl/6 male mice (approximately 30 g body weight and 3 months of age) following the administration of a 10 mg/kg dose by the i.p. route. Blood and brain samples (left cerebral hemisphere) were obtained at 0.5, 2, and 6 h, the blood was centrifuged (10,000 × g for 2 min at 4°C), and plasma and brain were immediately frozen (–80°C) for later analysis by LC-MS/MS. It has been established in the literature that the permeability of the blood–brain barrier is similar between mice and rats, with the quantified values of the drug transport measure—the permeability coefficient-surface area product (PS)—showing a correlation of 1:1 between the two species across a broad range of drugs[22].

Sample preparation for plasma. To each plasma sample volume of 50 μl, 1.0 ml of ethyl acetate was added and vortexed (10 min). Thereafter, the mixture was centrifuged (18,000 × g for 10 min) to separate the organic and aqueous phases. An 850 μl sample of the upper (organic) phase was collected, the organic solvent removed under vacuum in a centrifugal evaporator, and the dried residue reconstituted in 60 μl 50% (v/v) acetonitrile in water containing 50 ng/ml ethyl nicotinate (an internal standard for POM). Each sample then was vortexed (4 min), clarified by centrifugation (18,000 × g for 3 min), and transferred to HPLC vials for LC-MS/MS analysis.

Sample preparation for brain. Frozen brain samples were weighed, thawed to room temperature, and sonicated in phosphate-buffered saline (PBS: 0.4 ml per 100 mg brain tissue x 15 sec sonication). A 50 μl sample of this brain sonicate was then taken, to which 1.0 ml of ethyl acetate was added and vortexed (10 min). Samples were then treated as described for plasma.

HPLC-MS/MS analysis. Samples were subjected to HPLC (Waters 2795 Alliance Integrated System) and separated on a Kinetix C8 column (5 μm, 50 × 4.6 mm) column (20°C) in a mobile phase gradient that comprised of (a) 0.1% (v/v) formic acid in water, and (b) 0.1% (v/v) formic acid in acetonitrile. The gradient changed from 60%/40% at zero time to 5%/95% at 4 min, and back to 60%/40% at 5.1 min onward, with a constant flow rate of 0.3 ml/min. Retention times for POM and ethyl nicotinate were 2.7 and 3.1 min, respectively. MS conditions were evaluated on a Micromass Quattro Micro MS using electrospray ionization in the positive ion mode with multiple reaction monitoring (MRM). For POM, the MRM transition was 274.1 to 84.15

m/z, with a collision energy of 13 eV and cone voltage of 20. For ethyl nicotinate, the MRM transition was 152.1 to 124.1 m/z, with a collision energy of 25 eV and cone voltage of 25. The dwell time was set to 0.25 sec, the capillary voltage to 4000, the desolvation temperature to 350°C, and the source temperature to 115°C. Quantification of data was performed using the Quanlynx portion of Masslynx Software version 4.1.

Calibration curve standards. POM calibration standards (0, 10, 50, 100, 250, 500, 1000, 2500, and 5000 ng/ml) were prepared in blank mouse plasma from a stock solution of 2 mg/ml POM in DMSO. These were prepared fresh daily, and extraction was immediately undertaken to minimize potential POM degradation. These standards were analyzed in duplicate in each analytical run of the rodent POM samples. Mouse brain calibration standards were similarly prepared, using sonicated untreated C57Bl/6 mouse brains in PBS. Likewise, the calibration standards were prepared fresh daily, immediately extracted, and analyzed in duplicate in each analytical run. Each calibration standard curve was prepared by undertaking weighted (1/y) linear regression of the peak area ratio of POM to the internal standard, ethyl nicotinate. No POM signal was evident in samples prepared from blank plasma or brain samples that were not spiked with POM. The correlation coefficients for POM in plasma and brain were $r^2 = 0.994$ and 0.995 (10–5000 ng/ml, 50–5000 ng/g), respectively.

Lower limit of quantitative detection (LLOQD). The LLOQD was defined as the lowest concentration that could be measured with a back-calculated accuracy of at least 50% of the standards within 80–120% of nominal, and was thus set as the lowest concentration within the standard curve that was run concurrently with the samples derived from rodents. In the plasma and brain homogenate sample analyses the LLOQD for POM was 10 ng/ml for plasma and 50 ng/g for brain.

Rat Transient MCAo Model and Drug Treatment

The MCAo was performed as described previously[23,24]. Briefly, the rats were anesthetized with i.p. administered chloral hydrate (initially 400 mg/kg, i.p. followed with 100 mg/kg every hour). The bilateral common carotids were then ligated with non-traumatic arterial clips. A craniotomy of approximately 2×2 mm^2 was applied to the right squamosal bone. Cerebral ischemia was induced by ligation of the right MCA with a 10-O suture, thereby inducing MCAo using the intraluminal suture method for 60 min[23]. The ligature and clips were removed after 60 min ischemia to generate reperfusional injury. Core body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad throughout anesthesia. After recovery from the anesthesia, body temperature was maintained at 37°C using a temperature-controlled incubator. Immediately after the recovery from anesthesia, an elevated body swing test was used to evaluate the success of MCAo surgery. All animals used for this study demonstrated prominent motor bias contralateral to the lesion side, and animal numbers utilized were based on the variance of data and differences between treatment and control groups evident in both our prior studies and those of others[24–26].

The rats were randomly divided into two experimental groups: saline MCAo and POM MCAo. Following the period of 60-min ligation and 30-min reperfusion, the saline and POM groups received i.p. injections of saline or POM 20 mg/kg, respectively.

In relation to the anesthetic utilized in our study, chloral hydrate is a hypnotic anesthetic agent often used in laboratory rodents. Its advantages include: 1) rapid onset of action, 2) short duration of anesthesia, 3) a stable anesthetic plane, and 4) maintenance of body temperature. Its disadvantages include an association with adynamic ileus (loss of GI motility with consequent fluid sequestration and constipation) in laboratory rodents. In the NIH Animal Program's "Anesthesia Guidelines for Rodents", chloral hydrate is specifically listed as an acceptable anesthetic for laboratory rodent surgery, provided that the concentration of drug is kept at 4% or lower, that the users provide an acceptable scientific justification for its application in preference to other rodent anesthetics, and that the animal(s) be kept under observation for any signs of adynamic ileus such as bloating or constipation. Such observation was incorporated into our study.

Assessment of the Injured Cerebral Lesion Area and Neurological Deficit Scores

Infarct size was measured based on Shen et al.[24] at 24 h after MCAo, and was performed by an observer blinded to the treatment groups. Brains were quickly removed and immersed into cold saline for 5 min. Coronal slices were dissected into 2 mm from the frontal tips. Sections were immersed in 2% TTC at 37°C for 10 min and subsequently fixed in a 5% formaldehyde solution. The brain infarct volume was calculated as a percentage zone of the coronal section in the infarcted hemisphere[24,25]. Lateral movements and turning of the body were assessed using the body asymmetry test, which is a simple and easy behavioral assessment, as detailed by Borlangan and Sanberg[26]. Specifically, rats were lifted from their tails and maintained at 20 cm above the examination table. The frequency of initial turning of the upper body contralateral to the ischemic side was calculated in 20 subsequent trials. The maximum impairment in body swing in MCAo rats is 20 contralateral turns/20 trials, in which animals demonstrate asymmetric behavior ipsilateral to the MCAo[25,26]. An uninjured animal would show a value of 10 (i.e., an equal number of left and right turns).

Statistical Evaluation

All cytokine measurements underwent assessments for intragroup statistical outliers by use of the Grubb's Test, and

if any were identified they were excluded from the analysis. One Tg SP-C TNF-α mouse displaying an eye infection and overt health issues prior to initiation of the study was initially included but ultimately euthanized during the study, and thus was removed from the analysis. All data are expressed as mean $\pm$ SEM, where n refers to the numbers of samples (animals). Statistical analysis was performed by GraphPad Instat3. The treatment groups were compared using either the Dunnett or Bonferroni Multiple comparisons one-way analysis of variance (ANOVA) followed by *t*-tests, as appropriate. Where sample variances were different between the groups a Kruskal-Wallis ANOVA was performed. Differences were considered statistically significant at $P < 0.05$. GraphPad Prism 7 was used to generate summary graphs of the cytokine data.

Results

Effects of Systemic POM Treatment on Elevated Systemic TNF-α Expression in Vivo

To determine the ability of POM to lower elevated levels of TNF-α in vivo, we evaluated the actions of systemically administered POM in Tg SP-C TNF-α mice and their Wt littermates, as the former chronically and moderately over-express TNF-α from alveolar epithelium[21]. In the light of prior preclinical studies in rodents demonstrating that no neurological or respiratory effects were observed after a single dose of up to 2000 mg/kg (reaching an estimated maximum plasma drug concentration (Cmax) 94 times the clinical Cmax[13]), an initial dose of 50 mg/kg POM was evaluated in Tg SP-C TNF-α mice. This daily dose proved to be well tolerated, as evaluated by body weight (Fig. 1), which remained unchanged in comparison to both starting weight and the weight of vehicle administered Tg SP-C TNF-α mice. The physical appearance of POM treated Tg SP-C TNF-α mice was no different from those administered vehicle, and both groups of animal were smaller and weighed less than their Wt littermates (Fig. 1B and C versus A).

Quantification of a pro- and anti-inflammatory cytokine panel is shown in Fig. 2 (same mice as in Fig. 1), in relation to changes evident in Tg SP-C TNF-α vehicle mice versus their Wt littermates. As expected in a genetic mouse model of chronic moderate TNF-α over-expression in alveolar epithelial cells[21], as compared with Wt littermates serum levels of TNF-α were found significantly elevated in Tg SP-C TNF-α vehicle mice (Fig. 2A). Specifically, a concentration increase from 47.5 $\pm$ 4.0 pg/ml to 153.4 $\pm$ 40.2 pg/ml (3.2-fold) was evident. Notably, this TNF-α over-expression was substantially mitigated (by 76.3%) by POM (50 mg/kg x 21 days) administration to Tg SP-C TNF-α POM mice, whose levels were not significantly different (1.5-fold) from Wt littermates (Fig. 2A). To evaluate selectivity associated with the TNF-α transgene over-expression and actions of POM in Tg SP-C TNF-α mice, serum levels of

IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, and IFN-ɣ were additionally quantified (Fig. 2B–J). With the exception of IL-5, which was significantly elevated (2-fold (Fig. 2B) in Tg SP-C TNF-α vehicle, but not POM mice, versus Wt littermates, none were significantly different between the three groups of mice (Fig. 2C–J).

Plasma and Brain Levels of POM Following Systemic Administration

Time-dependent plasma and brain concentrations of POM were evaluated following a 10 mg/kg i.p. dose in Wt mice to evaluate brain uptake of drug into healthy brain (Fig. 3). Following systemic administration, plasma POM levels declined from a peak level of 2210 $\pm$ 160 ng/ml at 0.5 h to 107 $\pm$ 14.3 ng/ml at 6 h, whereas brain levels fell from a peak of 1997 $\pm$ 161 ng/g to 69.5 $\pm$ 8.6 ng/g over the same period. The brain/plasma ratio varied from 0.81 $\pm$ 0.03 at 0.5 h, to 0.70 $\pm$ 0.02 at 2 h to 0.58 $\pm$ 0.02 at 6 h; providing a mean brain/plasma ratio value over time of 0.71 $\pm$ 0.03.

Effects of Systemic POM Treatment on Brain Damage and Neurological Deficits in Vivo

Rats were subjected to MCAo for 60 min followed by reperfusion, and were subsequently treated with 20 mg/kg i.p. of POM. Based on TTC staining, the total infarct volumes were greatly reduced in the POM treated rats compared with the vehicle group. (Fig. 4A and B, infract volumes: $n = 5$, $P = 0.0131$). The elevated body swing test was applied to evaluate the physiological significance of the infarct, and revealed a reduced asymmetry in stroke animals that were treated with POM (Fig. 4C, body swing test: $n = 5$, $P = 0.0138$).

Discussion

The pathophysiology of stroke is complex, and the main pathogenic mechanisms of ischemia include oxidative damage, excitotoxic neurotransmitters, inflammatory pathways, ionic imbalance, and apoptosis[27–31]. The terminal results of acute ischemic cascades are neuronal death along with an irreversible loss of neuronal function. Clinically relevant neuroprotective pharmacological approaches to preserve damaged brain regions are lacking, and their development represents an important area of current medical need. In the light of prior studies demonstrating the ability of POM to mitigate H_2O_2-induced oxidative stress injury[19] as well as glutamate excitotoxicity[20] in rat primary cortical neuronal cultures and to lower microglial activation and their TNF-α generation thereby reducing neuronal cell death[20], we evaluated the ability of POM to mitigate ischemic stroke in vivo. Our current study demonstrates the ability of POM to (i) effectively lower elevated TNF-α levels following systemic administration in mice, (ii) enter the brain, and (iii) reduce infarct volume measured by TTC staining and mitigate motor
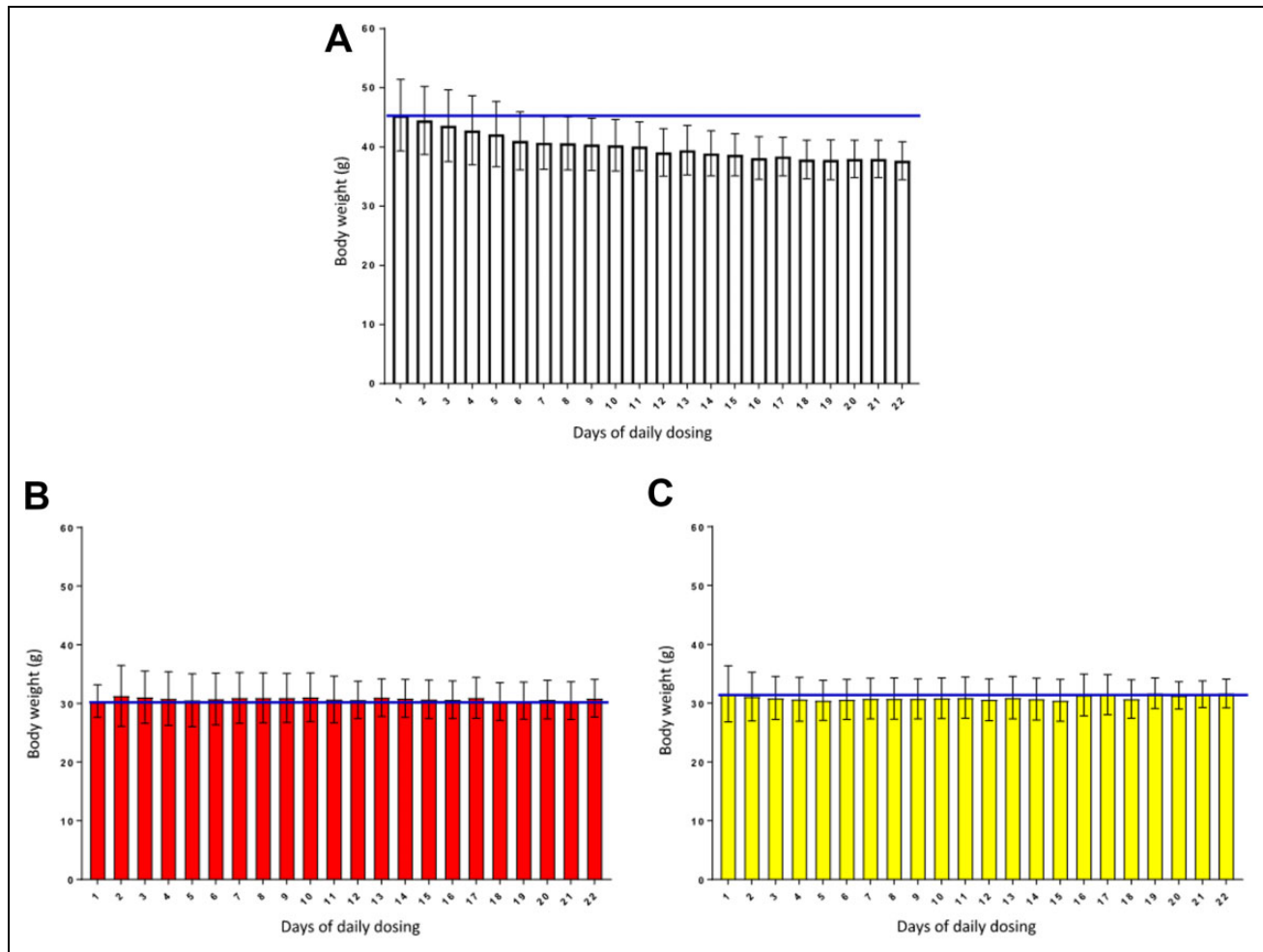
Fig. 1. Pomalidomide (POM) is well tolerated in mice in daily doses of 50 mg/kg, as evaluated by the maintenance of body weight: TNF-α over-expressing transgenic (Tg) mice in which the 3'-untranslated region of the mouse TNF-α gene was placed under the control of the surfactant protein (SP)-C promoter in alveolar epithelial cells (SP-C TNF-α mice) were dosed once daily with either vehicle (Veh) or POM (50 mg/kg) for 21 days by the i.p. route, and their body weights compared with Veh-dosed wild type (Wt) littermates. (A) Wt littermates administered Veh, (B) Tg SP-C TNF-α mice administered Veh, and (C) Tg SP-C TNF-α administered POM. The respective initial body weights (grams) of the groups were (A) Wt mice + Veh = 45.4 $\pm$ 1.9, $n = 10$; (B) Tg SP-C TNF-α mice + Veh = 30.4 $\pm$ 1.0, $n = 9$; (C) Tg SP-C TNF-α mice + POM = 33.8 $\pm$ 3.0, $n = 9$. Mean $\pm$ SEM values. No statistically significant decline in body weight was determined within each group ($P > 0.05$).

impairment following classical MCAo in rat. These results support the further evaluation of POM as a drug to mitigate neurodegenerative disorders underpinned by oxidative stress, neuroinflammation and glutamate excitotoxicity. It should be noted that, in our publications and those of others, TTC staining is a very sensitive technique to evaluate infarct size for up to at least 72 h after stroke in rats produced by the MCAo technique used here. One does not see significant problems with inflammatory cell infiltrate at later times.

To our knowledge, there are no prior reports in the literature evaluating POM as a neuroprotective agent in animal models of cerebral ischemia. Whereas we are similarly unaware of any literature studies of POM's close analogue lenalidomide, there are several publications in relation to thalidomide in ischemic stroke. Specifically, thalidomide

(20 mg/kg i.p., but not either 10 or 50 mg/kg) when administered three times 10 min before, immediately before, and 1 h after MCAo, reduced neuronal damage (infarct volume) at 24 h and 72 h, and functional damage (neurological deficits) at 72 h after permanent MCAo occlusion in mice[32]. This same 20 mg/kg thalidomide dose provided neuroprotective effects against histological injury in both the core and penumbra of the stroke, as assessed by quantifying TUNEL-positive cells. Additionally thalidomide reduced oxidative stress evaluated by 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of DNA damage[32]. In line with this, our prior studies in a mouse MCAo/reperfusion model demonstrated that a single 50 mg/kg thalidomide dose pre-administered 1 h before the procedure reduced infarct volume and neurological impairment[33]. Likewise, in a rat
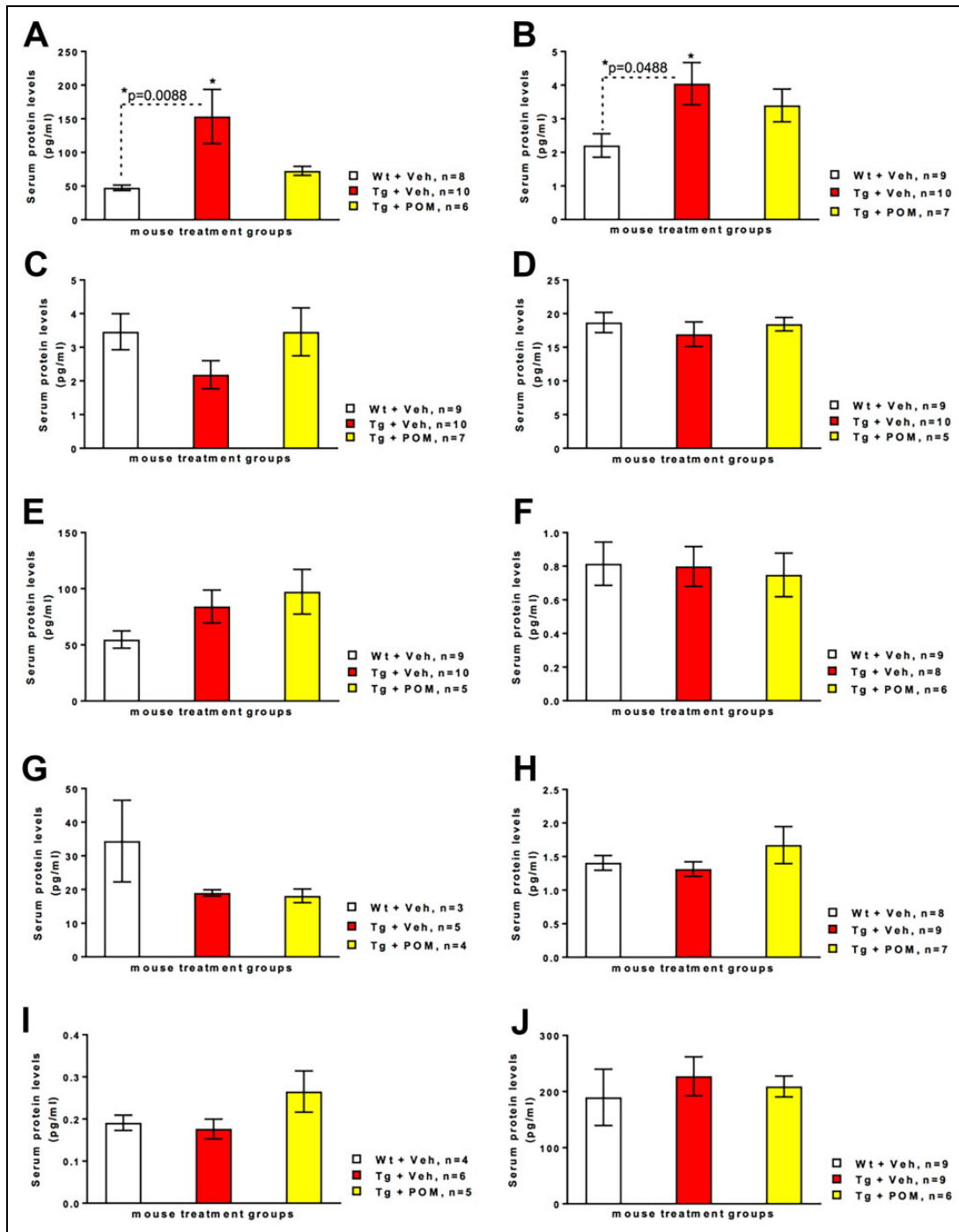
Fig. 2. POM (50 mg/kg for 21 days) lowers serum TNF-α and IL-5 levels in TNF-α chronically over-expressing Tg SP-C TNF-α mice. Serum levels of a broad panel of pro- and anti-inflammatory cytokines were quantified following 21 consecutive days of POM (50 mg/kg i.p.) dosing to Tg SP-C TNF-α mice and similar animals administered Veh. Cytokine levels in these two groups of animals were then compared with Wt mice administered Veh. Serum concentrations of (A) TNF-α and (B) IL-5 were significantly elevated in Tg SP-C TNF-α mice administered Veh (i.e., Tg + Veh mice) but not in those dosed with POM (Tg + POM mice), as compared with wild type (Wt + Veh) littermates (for the Tg + Veh mice: TNF-α *P = 0.0088—Kruskal-Wallis Test (nonparametric ANOVA); for IL-5 *P = 0.0488—one-way analysis of variance (ANOVA) vs. Wt + Veh mice. There was no significant difference between Tg + POM and Wt + Veh mice for either TNF-α or IL-5 (P > 0.05). IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, KC/GRO, and IFN-ɣ were additionally quantified, and were found to be unaltered in Tg SP-C TNF-α mice (whether administered either Veh or POM) as compared with Wt littermates (C–J). In relation to Tg + Veh vs. Wt + Veh mice. (C) Interferon-ɣ (P = 0.1584, ANOVA); (D) IL-10 (P = 0.7066, ANOVA); (E) IL-6 (P = 0.1261, ANOVA); (F) IL-1β (P = 0.9361, ANOVA); (G) IL-12 p 70 (P = 0.7066, ANOVA); (H) IL-2 (P = 0.4919, Kruskal-Wallis Test (nonparametric ANOVA); (I) IL-4 (P = 0.1703, ANOVA); (J) KC/GRO (P = 0.6339, Kruskal-Wallis Test (nonparametric ANOVA)). Values are mean ± SEM (n = 3 to 10 per group, as noted within the figure).
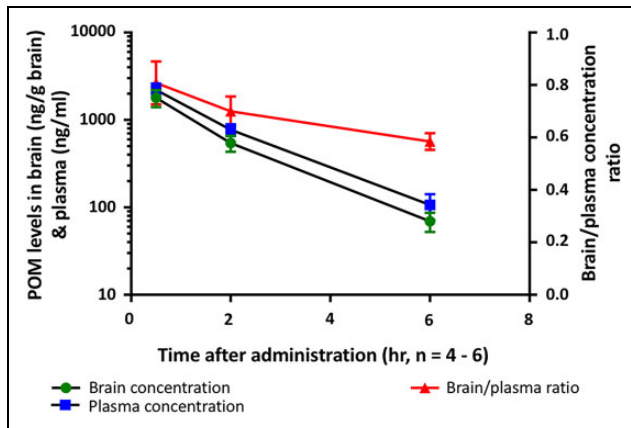
Fig. 3. POM enters the brain following systemic administration to rodents: Time-dependent plasma and brain concentrations of POM were quantified in mice using HPLC-MS/MS analysis following a 10 mg/kg i.p. dose in separate animals at 0.5 ($n = 6$), 2 ($n = 6$) and 6 h ($n = 4$) after administration. Plasma and brain POM levels (blue and green, respectively: left *y*-axis) declined in parallel to provide a mean POM brain/plasma ratio across time of 0.71 $\pm$ 0.03 (red: right *y*-axis).

MCAo/reperfusion model three thalidomide (20 mg/kg) administrations initiated before and during reperfusion lowered stroke volume, neurological loss and markers of apoptosis, lipoperoxidation, and pro-inflammatory cytokines, as well as augmented the levels of anti-apoptotic and anti-oxidant markers[34]. Notably, however, the administration of thalidomide "after" permanent MCAo or MCAo/reperfusion had no significant protective actions[32,33]. In other studies involving neuronal ischemia, oxidative stress, and apoptosis, post-treatment with thalidomide (20 mg/kg, i.p.) did not mitigate ischemic injury of spinal cord in rabbits, whereas pre-treatment significantly ameliorated the injury[35].

Clearly, the post-administration of a drug following an ischemic event is of greater clinical translational relevance than is pre-administration. Our present study demonstrates that POM (20 mg/kg i.p) given after 1.5 h MCAo/reperfusion injury reduces brain infarction and mitigates neurological damage in rats, as evaluated by the elevated body swing test[25,26], which has been reported as a valuable and sensitive test for the evaluation of motor asymmetry in animal models of PD, TBI, and ischemic stroke involving MCAo in rats and
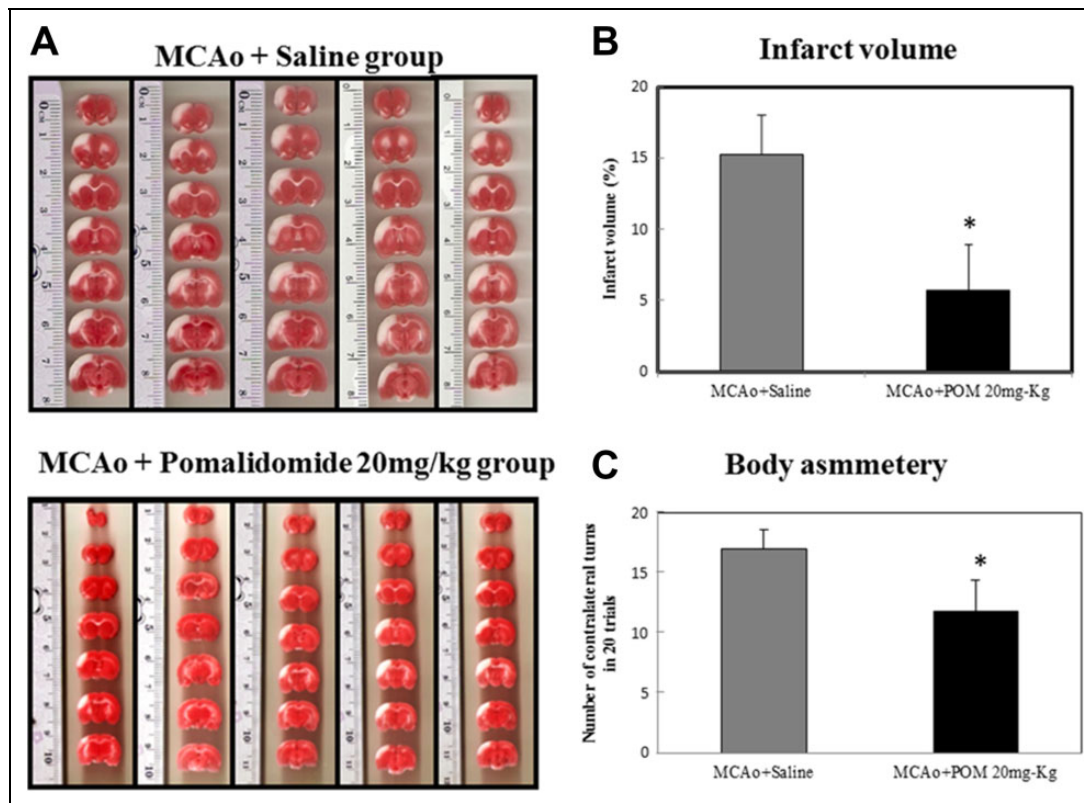

Fig. 4. POM post-treatment lowers cerebral infarct volume and reduces motor asymmetry in rats when administered 30 min following 60 min middle cerebral artery occlusion (MCAo) and reperfusion. (A) Representative coronal brain sections from vehicle-treated or POM (20 mg/kg)-treated rat groups stained with 2% TTC solution at 24 h after MCAo/reperfusion. (B) Quantitative analyses of infarct volumes. (C) Body asymmetry assessment evaluated by the elevated body swing test at 24 h after 60 min MCAo/reperfusion, comparing without and with POM treatment (mean $\pm$ standard error of the mean, $n = 5$) *$P < 0.05$ versus vehicle group. Notably, the maximum impairment in body swing in MCAo rats is 20 contralateral turns/20 trials, in which animals demonstrate asymmetric behavior ipsilateral to the MCAo[22,23]. An uninjured animal would show a value of 10 (i.e., an equal number of left and right turns).

mice[36–39]. FDA has accepted EBST as the primary behavioral outcome for many IND applications that led to approval of clinical trials of cell-based regenerative medicine for stroke, including those by Athersys, Sanbio, NeuralStem, Celgene, Layton Bioscience, etc. Also notable is the activity of POM to lower chronically elevated TNF-α overexpression in serum in a well-characterized mouse model of pulmonary fibrosis[21], for which more effective treatment strategies are similarly being sought. Likewise, the levels of IL-5, a proinflammatory cytokine known to be involved in asthma and to augment the progression of pulmonary fibrosis[40,41], were lowered by POM in our study. In this regard, prior investigations in mouse models of pulmonary fibrosis have highlighted the ability of thalidomide to dramatically attenuate pulmonary fibrosis, oxidative stress, and inflammation in mouse lungs[42,43], and provide anti-inflammatory and antioxidant actions in human lung fibroblast cultures[43]. Thalidomide has been reported to mitigate cigarette extract-induced, as well as paraquat-induced, pulmonary damage in mice[44,45], and was identified as a promising treatment for intractable cough in humans with idiopathic pulmonary fibrosis[46,47]. In this light, our POM data in Tg SP-C TNF-α pulmonary fibrosis mice warrants follow up.

POM is a third-generation derivative of thalidomide that combines more potent immunomodulatory actions with tolerability in preclinical and clinical studies[14–16,48,49]. It is likely that this improved potency of POM, in relation to thalidomide, underpins the activity evident following POM's post-injury administration in the current study as the, likewise, more potent thalidomide analogue, 3,6'-dithiothalidomide, proved similarly able to mitigate neuronal damage, reduce infarct size and improve neurological function when administered following ischemia/reperfusion injury in mice[33]. 3,6'-Dithiothalidomide provided anti-inflammation and anti-oxidant actions, and reduced disruption of the blood–brain barrier and, thereby, ischemia-induced edema[33]. It is very likely that POM mitigates ischemic stroke-induced cerebral damage in the present study by combining anti-inflammatory (lowering TNF-α and IL-5) and anti-oxidant actions demonstrated here and elsewhere[19,20], in a manner similar to 3,6'-dithiothalidomide[33]. Importantly, POM, unlike 3,6'-dithiothalidomide, is an FDA-approved clinically available drug whose actions, if cross-validated and extended in future studies, could potentially be applied to, and rapidly evaluated in, humans. In the light of the cerebral hypoperfusion that occurs within the ischemic and post-ischemic brain, and associated drug delivery problems of potential stroke therapeutics[30,50], candidate drugs should possess brain entry across an uncompromised blood–brain barrier to support rapid and adequate delivery throughout both compromised and uncompromised brain[51]. Our study demonstrates that POM enters the brain rapidly following its systemic dosing and has a brain/plasma ratio of 0.71, as evaluated between 0.5 and 6 h, which is in accord with a report indicating that POM concentrations in brain are

roughly 70% of those in plasma[52], as well as data made available by the manufacturer (Celgene) indicating that POM distributes to brain with a brain to plasma/blood ratio of 0.39 to 0.49[13]. Thalidomide, likewise, has been noted to readily enter brain, but, in contrast, lenalidomide brain entry is reported to be relatively poor[53] (0.9% to 2.3% of plasma levels). Although the structures and lipophilicities of POM, thalidomide, and lenalidomide are similar, lenalidomide has been reported to be a substrate of the efflux transporter, P-glycoprotein[54], which mediates xenobiotic removal from the central nervous system as a part of the blood–brain barrier.

The POM doses evaluated in our study are in line with those reported in the literature[13]. Additionally, our 10, 20, and 50 mg/kg doses can be considered well tolerated in the light of preclinical toxicological studies demonstrating that POM lacked adverse effects for doses in rats up to 5000 mg/kg/day in a 7-day study, 2000 mg/kg/day in a 28-day study, 1500 mg/kg/day in a 90-day study, and 1000 mg/kg/day in a 6-month study[13]. Drawing knowledge from the broad POM dose range evaluated in prior preclinical rodent studies[13], we selected a POM dose of 50 mg/kg in our initial tolerability and systemic cytokine studies as this dose had previously been selected in mouse efficacy studies during the initial development of POM[13,55]. This dose was lowered by 5-fold (to POM 10 mg/kg) in our mouse pharmacokinetic study, as POM concentrations achieved in human plasma are substantially less than those achievable in rodents administered POM 50 mg/kg. Importantly, in our rat cerebral ischemia study, mitigation was achieved with a single POM 20 mg/kg dose (notably, this POM 20 mg/kg dose is equivalent to the POM 10 mg/kg that we evaluated in mice, following normalization to body surface area between species, according to U.S. Department of Health and Human Services Food and Drug Administration guidelines[56]). Toxicological studies of POM across species indicate that a single dose is far better tolerated than repeat daily dosing[13], potentially permitting the use of a far higher dose than that suggested for routine clinical use of POM for multiple myeloma (4 mg daily on 21 of a 28-day cycle)[13]. In this regard, future studies are warranted to define the time-dependent window of opportunity and optimal dose of POM to reduce ischemic brain injury, particularly in light of our prior study demonstrating efficacy of a 0.5 mg/kg single i.v. dose of POM in mitigating moderate TBI[20].

In summary, cerebral ischemia and reperfusion injury encompass several molecular mechanisms which are seen across neurodegenerative disorders as well as systemic diseases[5–8]. POM is shown herein to reduce brain infarct volume as well as motor asymmetry following MCAo-induced ischemia/reperfusion injury. POM and analogues hence warrant further evaluation as potential therapeutics for ischemic stroke and other neurological and systemic disorders involving oxidative stress and neuroinflammation, particularly since POM is clinically available, and consequently can be rapidly repurposed for disorders where current treatment options are lacking.

Author Contributions

Ethical Approval

Statement of Human and Animal Rights

Statement of Informed Consent

Data Availability

Declaration of Conflicting Interests

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