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ORIGINAL RESEARCH

Examination of Inter-α Inhibitor Proteins in Permanent and Transient Focal Ischemia

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BACKGROUND: Ischemic stroke is among the most prevalent diseases, with high death and morbidity. Numerous preclinical studies have reported efficacious interventions in rodent stroke models. However, reperfusion therapies remain the only clinically efficacious intervention to date. Rigor and reproducibility are now recognized as critical to bridge the preclinical–clinical disconnect. Inter-α inhibitor proteins (IαIPs) are a family of structurally related glycoproteins with 2 major forms (inter-α inhibitor and pre-α inhibitor) in blood. Purified human plasma–derived IαIP has beneficial effects in sepsis and hypoxic–ischemic brain injury. More recently, IαIP improved focal ischemic stroke outcomes in mouse models. Here, we tested IαIP efficacy in both transient and permanent stroke mouse models, mimicking previously published study designs and protocols to seek reproducibility.

METHODS AND RESULTS: Using healthy young male and female C57BL/6 mice, we induced transient or permanent endovascular filament middle cerebral artery occlusion (MCAO). Mice were divided into transient MCAO+vehicle, transient MCAO+IαIP (30 mg/kg), permanent MCAO+vehicle, and permanent MCAO+IαIP groups. IαIP or vehicle was administered intravenously at 6 and 18 hours after MCAO. End points were assessed at 2 days. Efficacy readouts included death, infarct volume and swelling, and 3 neurological tests. Contrary to the previous work, we did not find IαIP efficacious on any outcome readout in either transient MCAO or permanent MCAO.

CONCLUSIONS: Our data highlight the contribution of interlaboratory heterogeneity to study outcomes and suggest that interventions considered for clinical development should undergo rigorous testing in multiple single-laboratory studies before entering a multicenter preclinical trial.

Key Words: brain ischemia ■ MCAO ■ neuroprotection ■ stroke

Ischemic stroke is a devastating disease with high death and morbidity.[1] Intravenous thrombolysis, albeit successful in improving overall outcomes, has been limited by a narrow therapeutic window and incomplete reperfusion.[2,3] The recent success of endovascular thrombectomy in achieving early recanalization renewed interest in potential neuroprotective therapies in ischemic stroke.[4]

The inter-α inhibitor protein (IαIP) family is a group of structurally related endogenous serine protease inhibitors circulating at high concentrations in human plasma.[5,6] The major forms of IαIPs found in plasma are 250-kDa inter-α inhibitor (IαI), composed of 2 heavy chains (H1 and H2) and a single light chain, and 125-kDa pre-α inhibitor (PαI), composed of a single heavy chain (H3) and a single light chain. Both IαI and PαI are extracted and purified together from human plasma and have been collectively termed and referred to as IαIP.

IαIP is an important component of innate immunity. In acute inflammation, the circulating IαI component is downregulated and behaves as a negative acute-phase

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RESEARCH PERSPECTIVE

What Is New?

- Treatment with inter-α inhibitor proteins did not improve tissue and functional outcomes in transient or permanent focal cerebral ischemia mouse models, highlighting the variability among single-laboratory studies even when the experimental conditions were ostensibly similar.
- Sex and circadian stage at the time of stroke did not influence inter-α inhibitor protein efficacy.

What Question Should Be Addressed Next?

- In translational stroke research, efficacy should be tested in multiple independent laboratories before a candidate intervention moves on to multicenter preclinical trials as the next step toward clinical trials.
- Future studies will explore the relative efficacy and safety of inter-α inhibitor protein versus pre-α inhibitor and other potential determinants of variability among laboratories.

Nonstandard Abbreviations and Acronyms

CCA	common carotid artery
IαI	inter-α inhibitor
IαIP	inter-α inhibitor protein
MCAO	middle cerebral artery occlusion
pMCAO	permanent middle cerebral artery occlusion
PαI	pre-α inhibitor
tMCAO	transient middle cerebral artery occlusion
TTC	2,3,5-triphenyl-tetrazolium chloride

protein, while PαI is upregulated and serves as a positive acute-phase protein.[7] The purified IαIP exerts anti-inflammatory effects by suppressing proinflammatory cytokines, inducing anti-inflammatory cytokines, and inhibiting complement activation.[5,8,9] Treatment with human IαIP attenuated lipopolysaccharide-induced blood–brain barrier disruption in mice[10,11] and hypoxic–ischemic brain injury from neonatal to adult rats.[12–19] Moreover, in both humans and mice, plasma IαIP levels are reduced 24 hours after stroke onset, suggesting that restoring IαIP levels via exogenous administration might be beneficial, especially given the gradual build-up of inflammation after ischemic injury.[20] This hypothesis was supported by a recent report showing that IαIP decreased infarct size and improved neurological deficits in permanent and transient focal ischemic stroke in mice.[21]

Independent confirmation of efficacy in multiple laboratories is increasingly recognized as an important translational step in the search for novel stroke therapies.[22–24] We undertook this study to reproduce previously published data[21] using a rigorous and unbiased study design in our laboratory. Our results underscore the importance of independent confirmation in translational stroke research.

METHODS

All data generated or analyzed during this study are the intellectual property of the authors. The data sets are available from the corresponding author on reasonable request for research purposes.

Experimental Animals

All experiments were reviewed and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee following the National Institutes of *Health Guide for Use and Care of Laboratory Animals*. Mice (C57BL/6J, male and female, 9–17 weeks old, 18–33 g) were purchased from Charles River Laboratories Inc (Wilmington, MA) or Jackson Laboratories (Boston, MA) and housed in standard facilities with a 12-hour light/12-hour dark schedule in a temperature- and humidity-controlled vivarium, with access to food and water ad libitum.

Study Design

A total of 102 mice were used to test IαIP on focal ischemic brain injury. Mice were randomly allocated into transient or permanent endovascular filament middle cerebral artery occlusion (MCAO) and 2 treatment arms (IαIP or vehicle). Given the possible effect of the circadian cycle on stroke outcomes and treatment efficacy,[25] the MCAO procedure was performed during the animal's active or inactive circadian stage in separate cohorts. The experimenters (T.I. and A.M.) were blinded to the allocation by 2 other investigators (T.Q. and Y.S.) who did not participate in surgery or data analysis. Exclusion criteria were technical error, inadvertent subarachnoid hemorrhage during filament insertion, or complete absence of ischemic injury. Mice that died before the day 2 end point were reported as part of the death and neurological deficit score analysis but excluded from the other outcome analyses. In secondary exploratory analyses, we imputed the infarct volume using the largest infarct volume in the data set and behavioral readouts using the worst possible score.

IαIP Preparation and Administration

IαIP, purified using a modified process compared with prior work, and vehicle controls were shipped from Takeda Pharmaceuticals (Lot FF_882_V002_04ASF; Lexington, MA) or ProThera Biologics, Inc. (Providence, RI) frozen in aliquots of 19.7 or 6.5 mg/mL in PBS. The modified chromatographic purification process improved the yield of IαIP with higher PαI content than the previous methods[21] (IαI:PαI 25:1 versus 2:1), measured using an established enzyme-linked immunosorbent assay with a specific monoclonal antibody against bikunin (MAb 69.26, ProThera Biologics, Inc., Providence, RI) and a rabbit polyclonal antibody against heavy chain 3 (anti-ITIH3) (Catalog No. HPA017373, Sigma-Aldrich, St. Louis, MO) that specifically detects PαI. Vials were stored at −80 or −20 °C and used within 3 hours of thawing on the day of the experiment. IαIP (30 mg/kg) or vehicle (PBS) was administered 6 and 18 hours after MCAO via the jugular vein under brief anesthesia.

Endovascular Filament MCAO

Mice were subjected to either permanent (pMCAO) or 90-minute transient MCAO (tMCAO) using a previously reported surgical approach.[24,26–28] Briefly, mice were anesthetized using isoflurane (2.5%–3.0% induction, 1.5%–2.0% maintenance in 70% N_2O/30% O_2), and rectal temperature was controlled by a servosystem (36.5±0.5 °C). Surgery was performed during the animals' active or inactive circadian phase using a normal or reverse lighting room. A laser Doppler flowmetry probe was attached over the right middle cerebral artery territory (2 mm posterior, 2 mm lateral from the bregma) to monitor cerebral blood flow. After a ventral midline incision, the right common carotid artery (CCA) bifurcation was exposed, taking care not to injure the surrounding nerves. The external carotid artery was ligated both proximally and distally. After the ligation of CCA, a silicone-coated nylon monofilament (602212PK10Re or 602312PK10, Doccol Corporation, Sharon, MA) was inserted into the external carotid artery between the ligations, proximal ligation was released, and the filament was advanced to the middle cerebral artery origin through the internal carotid artery until a major cerebral blood flow drop was observed.

In the pMCAO model, the CCA was opened after the filament insertion to avoid a high mortality rate, and mice were allowed to recover from anesthesia. In the tMCAO model, the CCA was kept ligated to maintain the cerebral blood flow reduction during the MCAO period, and mice were allowed to recover from anesthesia. After 80 minutes of occlusion, mice were reanesthetized and the filament was removed to restore cerebral blood flow at 90 minutes.

Immediately after surgery, mice received a 10-mL/kg intraperitoneal prewarmed saline supplement to prevent dehydration, moved into a warm recovery chamber until fully awake and then to their home cage. Approximately 48 hours later, the functional outcome was evaluated, and mice were euthanized to measure the infarct volume and swelling.

In the pMCAO cohort, 6 of 28 mice were excluded from the analysis because of subarachnoid hemorrhage (n=1) or surgical technical error (n=5). In the tMCAO cohort, 8 of 74 mice were excluded from the analysis because of either subarachnoid hemorrhage (n=2), no stroke (n=2), or drug dosing error (n=4).

Neurological Assessments

Three different tests were used to examine the behavioral outcomes.[29] *5-point neurological deficit score* (0=best to 5=worst) was recorded 6 hours after MCAO induction and then daily: 0, no deficit; 1, forelimb weakness and torso turning to the one side when held by the tail; 2, circling to 1 side; 3, persistently leaning to 1 side; 4, no spontaneous locomotor activity or barrel rolling seizures; and 5, death. The *corner test* was performed at baseline and 2 days after MCAO induction. Mice were placed in a 30° corner constructed using 2 tall panels forming a wedge, and their left or right turns were recorded. The test was repeated at least 10 times for each mouse. The ipsilesional turn rate was calculated by dividing right turns by the total turns for each time point and expressed as a change from the pre-MCAO baseline. The *horizontal wire test* was performed at baseline and 2 days after MCAO induction. Mice were placed on a 100-cm-length single wire stretched between 2 posts 50 cm above the ground. The investigator counted time until mice fell onto a cushioned surface below the grid. The maximum hanging time was capped at 90 seconds if the animal stayed on the grid. Performance was also scored (0–5): 0, fall before 30 seconds; 1, hang for at least 30 seconds; 2, mouse consistently brought all 4 limbs to midline to grip the wire; 3, mouse consistently brought all 4 limbs to midline and wrapped tail around wire; 4, mouse moved across the wire using 4 limbs; 5, mouse climbed down the pole and completed the test. The test was repeated 3 times, and the hanging time and score were averaged and expressed as a change from the pre-MCAO baseline.

Infarction and Swelling Assessment

Mice were euthanized by cervical dislocation under deep anesthesia (5% isoflurane). Brains were carefully removed and sectioned into 1 mm-thick coronal slices and immersed in 1% to 2% 2,3,5-triphenyl-tetrazolium chloride (TTC; Sigma-Aldrich, Inc., St. Louis, MO)/saline until intact tissue was sufficiently stained. After image acquisition, cortical and subcortical infarct areas were measured using ImageJ (National Institutes of Health,

Bethesda, MD) and integrated to calculate volumes. Hemispheric swelling was calculated as the ipsilateral hemisphere volume divided by the contralateral hemisphere volume and expressed as a percentage. Infarct volume was adjusted for swelling and expressed as indirect infarct volume.

We could not harvest the brain in 1 vehicle- and 1 IαIP-treated animal in the pMCAO and 1 vehicle- and 2 IαIP-treated animals in the tMCAO cohort fast enough for TTC staining after they died unexpectedly; therefore, these were excluded from all morphometric analyses. In the tMCAO cohort, we harvested 15 brains (n=9 vehicle, n=6 IαIP) quickly after the animals died prematurely and performed TTC staining. These proved to be of good quality and were included in the secondary analyses (Figure S1).

Data and Statistical Analysis

Data are presented as means±SD or SEM. Data were analyzed using Prism 8 (GraphPad Software, La Jolla, CA). Significant differences were determined using the log-rank *t* test for mortality analysis, Mann–Whitney *U* test for nonparametric comparisons, and repeated measures 2-way ANOVA followed by Šídák's multiple-comparisons test. $P<0.05$ was considered statistically significant. Primary outcome analyses were performed only using data from surviving animals. We also carried out 2 separate secondary analyses. First, we imputed the worst value of the entire study cohort for missing data for all readouts due to death before the 48-hour euthanasia end point. Second, we repeated the morphometric analyses by including the brains harvested quickly enough for reliable TTC staining after premature death.

RESULTS

In the tMCAO experiment, demographics and study performance indices did not differ between vehicle and IαIP treatment arms (Table 1). Compared with vehicle, IαIP treatment did not change survival, infarct areas and volume, swelling volume, or neurological deficits using neuroscore, corner test, and horizontal wire tests (Figure 1, Table 2). Subgroup analyses did not reveal an effect of the circadian stage at the time of MCAO on swelling-adjusted infarct volumes (73±35 mm^3 vehicle and 80±21 mm^3 IαIPs in the inactive stage, 70±31 mm^3 vehicle and 78±29 mm^3 IαIP in the active stage). Subgroup analyses based on sex yielded similar results (infarct volumes 82±28 mm^3 vehicle and 79±20 mm^3 IαIP in males, 61±32 mm^3 vehicle and 78±32 mm^3 IαIP in females). Secondary analyses, either by imputing for missing data due to death with the worst possible values or by including brains from the animals that died prematurely but harvested fast enough for reliable TTC staining (Figure S1), did not change our conclusions (Tables S1 and S2).

In the pMCAO experiment, demographics and study performance indices did not differ between vehicle and IαIP treatment arms (Table 1). Infarct areas were smaller in the IαIP arm ($P=0.031$), which appeared to be related to less hemispheric swelling ($P=0.085$) since swelling-corrected infarct volume did not differ between the study arms ($P=0.474$; Figure 2, Table 2). IαIP did not improve performance in neuroscore, corner, or horizontal wire tests. Secondary analyses, as described above, did not change our conclusions for the pMCAO experiment either (Tables S1 and S2).

DISCUSSION

IαIP is ubiquitously expressed in many cell types, including neurons and astrocytes in the human brain.[30] Previous reports suggested that exogenously administered IαIP might be efficacious in hypoxic or lipopolysaccharide-induced brain injury via an anti-inflammatory effect in various animal models.[9,10,13,15] A recent comprehensive study also suggested efficacy

Table 1. Study Cohort Characteristics and Performance Indices

Circadian stage	Transient MCAO (CCA closed during MCAO)						Permanent (CCA open)		
	Inactive			Active			Active		
Treatment arm	Vehicle	IαIP	*P* value	Vehicle	IαIP	*P* value	Vehicle	IαIP	*P* value
Enrollment sample size, n	12	11		26	25		14	14	
Exclusions	2	1		2	3		2	4	
Final sample size, n	10	10		24	22		12	10	
Sex, male/female (n)	6/4	6/4		11/13	10/12		12/0	10/0	
Age, wks	15.8±1.4	16.0±1.4	0.85	13.3±2.2	13.3±2.2	0.98	12.5±2.3	12.4±2.1	0.92
Weight, g	25.7±4.2	26.5±3.8	0.67	24.2±2.8	23.8±3.2	0.46	25.4±1.9	26.7±2.6	0.22
CBF after MCAO, %	14±3	15±7	0.68	14±7	18±10	0.25	20±10	18±10	0.72

Data are mean±SD. CBF indicates cerebral blood flow; CCA, common carotid artery, IαIP, inter-α inhibitor protein; and MCAO, middle cerebral artery occlusion.

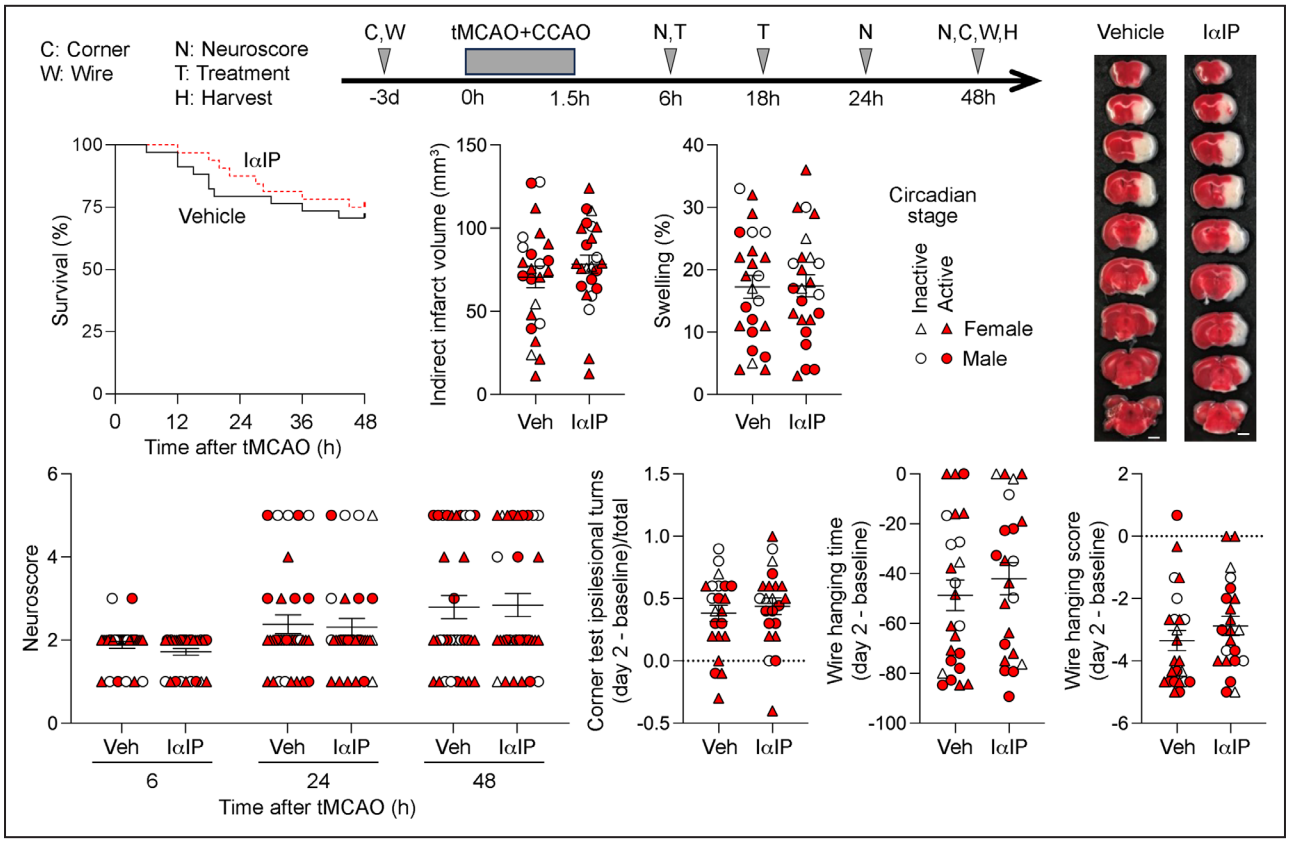

Figure 1. Effects of IαIP in transient MCAO.
The experimental protocol and timeline are shown. IαIP or vehicle was administered at 6 and 18 h after 90-min transient MCAO. Survival, tissue outcomes, and neurological outcomes are shown in each panel for IαIP and vehicle. Representative TTC-stained brains are also shown. Individual data points and mean±SEM are shown. Data are not imputed for death except neuroscore. The scale bar shows 2 mm. CCAO indicates common carotid artery occlusion; IαIP, inter-α inhibitor proteins; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyl-tetrazolium chloride; and Veh, vehicle.

on tissue and behavioral outcomes in mouse models of transient and permanent focal cerebral ischemia.[21] Here, we attempted to replicate selected experiments from the latter study using nearly identical experimental protocols, therapeutic paradigms, and sufficiently large sample sizes but did not find efficacy on any outcome measures.

From the outset, we designed our study to replicate the previous report (Table S3).[21] The 2 studies used the same species (C57) and strain of mice from the same

Table 2. Outcome Indices

	Transient MCAO (CCA closed during MCAO)			Permanent (CCA open)		
	Vehicle	IαIP	*P* value	Vehicle	IαIP	*P* value
Death, n (%)	10/34 (29)	8/32 (25)	0.62	1/12 (8)	1/10 (10)	0.89
Day 2 weight loss, %	−19±1% (n=25)	−18±1% (n=25)	0.49	−18±2% (n=11)	−15±1 (n=9)	0.25
Swelling-adjusted infarct volume, mm³	71±6 (n=24)	78±5 (n=24)	0.36	71±9 (n=10)	62±8 (n=9)	0.47
Swelling, %	17±2 (n=24)	17±2 (n=24)	0.95	15±3 (n=10)	7±3 (n=9)	0.08
Day 2 Neuroscore	2.8±0.3 (n=34)	2.8±0.3 (n=32)	0.87	2.3±0.3 (n=12)	2.4±0.3 (n=10)	0.93
Corner test, Δ right turn	0.38±0.07 (n=22)	0.44±0.07 (n=22)	0.55	0.47±0.06 (n=11)	0.46±0.13 (n=9)	0.92
Wire test, Δ time, sec	−49±6 (n=24)	−42±6 (n=22)	0.46	−56±9 (n=11)	−50±9 (n=9)	0.62
Wire test, Δ score	−3.3±0.3 (n=24)	−2.9±0.3 (n=22)	0.30	−4.2±0.2 (n=11)	−3.9±0.2 (n=9)	0.31

Data are mean±standard error and sample size. CCA indicates common carotid artery; IαIP, inter-α inhibitor protein, and MCAO, middle cerebral artery occlusion.

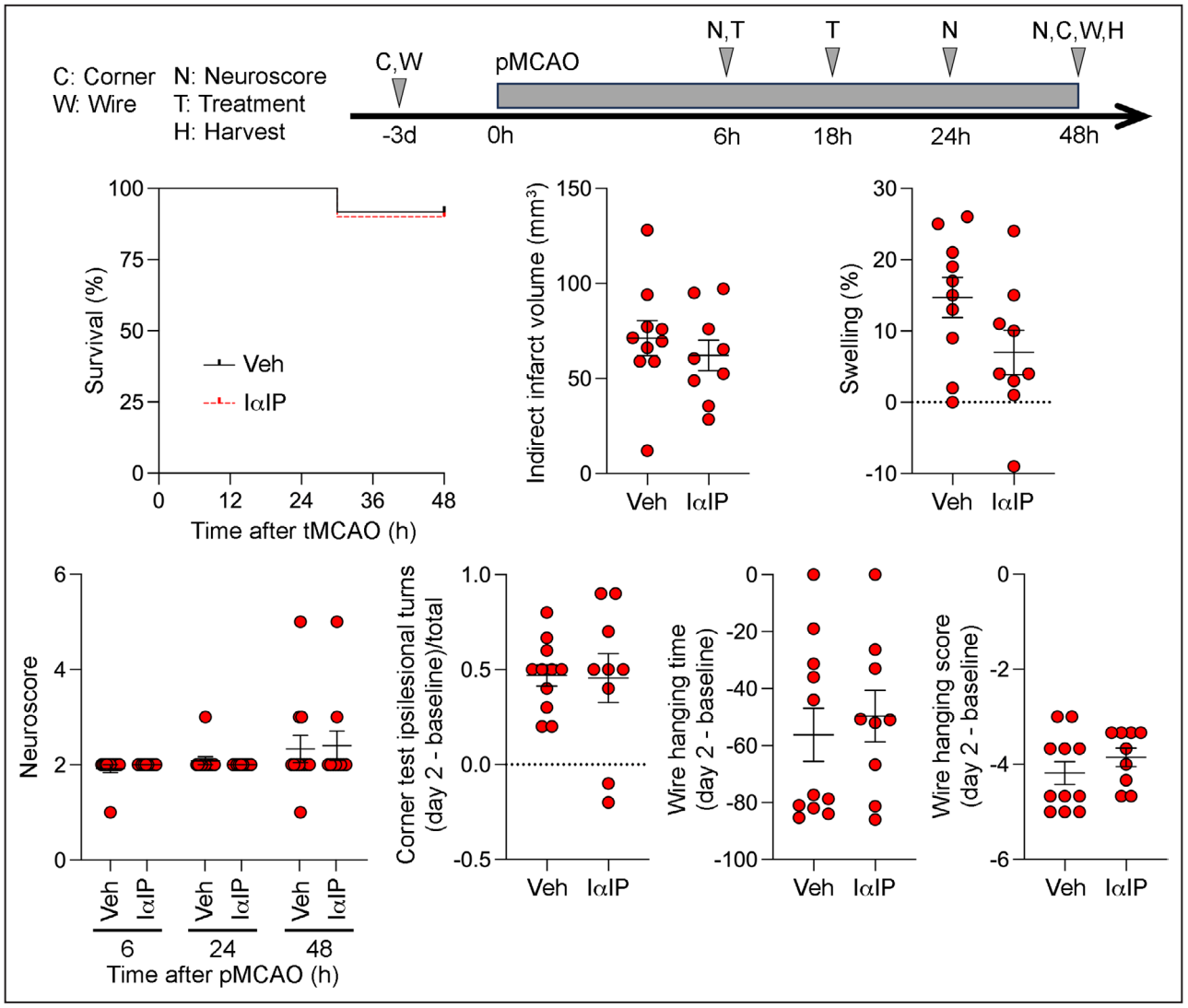

Figure 2. Effects on IαIP in permanent MCAO.
The experimental protocol and timeline are shown above. IαIP or vehicle was administered at 6 and 18 h after permanent MCAO. Survival, tissue outcomes, and neurological outcomes are shown in each panel for IαIP and vehicle. Representative TTC-stained brains are also shown. Individual data points and means±SEM are shown. Data are not imputed for death except neuroscore. The scale bar shows 2 mm. IαIP indicates inter-α inhibitor protein; pMCAO, permanent middle cerebral artery occlusion; TTC, 2,3,5-triphenyl-tetrazolium chloride; and Veh, vehicle.

commercial vendor (Charles River Laboratories). Both studies used males, and the age and baseline body weight were comparable. Both studies used isoflurane anesthesia during MCAO and thermostatically maintained the rectal temperature while under anesthesia. Both studies used permanent and 90-minute transient MCAO. Both studies induced transient MCAO in animals' inactive circadian stage. Both studies allowed the animals to recover from anesthesia during the occlusion period. Both studies kept the CCA ligated after filament insertion in tMCAO and opened the CCA after filament insertion in pMCAO. Both studies used laser Doppler flowmetry to monitor successful filament insertion. Treatment paradigms were nearly identical. We tested 30 mg/kg IαIP administered via the jugular vein 6 and 18 hours after MCAO, whereas the previous study administered the treatments intraperitoneally or via the tail vein. Infarct volume was measured on TTC-stained coronal slices at 2 days in both studies, although the number and thickness of the slices differed (9 sections, 1-mm thickness in our study versus 5 sections, 2-mm thickness in previous study). Infarct volumes and neurological deficit scores were comparable between the two studies, suggesting similar severity of outcomes, and neither study employed imputation for missing data due to death in their primary analyses. Although we experienced a higher mortality rate in the tMCAO cohort, secondary analyses, either

by imputation or inclusion of TTC infarct volumes from dead mice, did not change our conclusions (Tables S1 and S2). Therefore, mortality bias was unlikely to have affected our conclusions. Exclusion reasons differed only slightly (technical or drug-dosing error and subarachnoid hemorrhage in our study versus subarachnoid hemorrhage and excessive weight loss in the previous study), and subtle differences in TTC slice preparation were not plausible explanations for the discrepant findings. Altogether, side-by-side comparisons show that the 2 studies were more similar than not.

One notable difference was the IαIP purification process. IαIP is a highly complex biologic with unique molecular structures that include a glycosaminoglycan chain connecting multiple heavy and light chains, making it challenging to produce these moieties recombinantly. Human plasma, with relatively high IαIP content (300–800 mg/L), is an excellent natural source for IαIP. The modified chromatographic purification of IαIPs from fresh frozen plasma or plasma intermediates resulted in higher recovery of the 250 kDa IαI and especially the 125 kDa PαI, altering the IαI:PαI ratio. Nevertheless, both studies used the same IαIP dose and administration frequency, and it is unclear whether the IαIP purification process or differences in IαI:PαI content might account for the differences in outcome compared with previous work.[21]

Importantly, we did not repeat the entire range of experiments in which IαIP showed efficacy in the previous report[21] but selected the 90-minute transient and permanent occlusions and administered the first dose of the treatment 6 hours after MCAO onset, that is, worst-case scenarios. Therefore, we cannot rule out the possibility that IαIP is efficacious when tested in shorter ischemic durations or administered earlier after MCAO onset. Another important point to be underscored is that each independent laboratory has an established procedural protocol that yields a certain range of outcomes after filament MCAO. These protocols often differ among the labs. When a procedural protocol used by 1 lab is adopted verbatim by another lab, experiments often yield outcomes that are very different from the original lab. Indeed, our adoption of the previously published protocol as faithfully as possible (ie, 90 minutes of tandem MCAO+CCA occlusion) likely yielded a higher mortality rate in our hands, at least in tMCAO. Therefore, our results highlight the heterogeneity of outcomes among independent laboratories, even when similar experimental protocols are used. Intangible factors (eg, surgeon, housing environment) likely play a role in divergent outcomes.

In conclusion, our data did not confirm the previously reported efficacy of IαIP, underscoring how laboratory-specific intangibles can radically alter the observed efficacy.[28] Whether an investigational therapy should be advanced to clinical testing if the efficacy is observed only under a given set of laboratory conditions is an important debate. Future studies must define the specific roles of IαI and PαI in neuroinflammation and test their relative efficacies in experimental models of ischemic stroke.

ARTICLE INFORMATION

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Drs Imai and Ayata designed and coordinated the research. Dr Lim provided purified IαIP. Drs Imai and de Morais performed the experiments and analyzed the data. T. Qin and Dr Sasaki supported the experiments. Drs Imai and Ayata wrote the manuscript text and prepared the figures. All authors edited the final manuscript.

Disclosures

Dr Lim is a cofounder of the company ProThera Biologics and holds a significant financial interest in the company. The remaining authors have no disclosures to report.

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Supplemental Material

Tables S1–S3
Figure S1

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