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Comparative assessment of a novel neuroprotectant against citicoline in a thrombus-induced cerebral ischemia mouse model

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Stroke is one of the leading causes of mortality and morbidity worldwide. Among the two major forms of stroke (ischemic and hemorrhagic), ischemic stroke is the most prevalent and occurs when cerebral or cervical blood vessels are occluded. Ischemic occlusion can result from the formation of a thrombus within the cerebral or cervical vasculature (thrombosis), the migration of an embolus from a distant site, such as the heart, to the brain (embolism), or severe arterial stenosis in or leading to the brain. Despite substantial advancements in medical science over the past two decades, there remains a paucity of neuroprotective agents available for stroke management. Citicoline is an FDA-approved agent that has demonstrated promising efficacy in treating ischemic stroke. It functions as a neuroprotectant in ischemic stroke patients, facilitating their recovery. A novel spiro tricyclic compound [IM-1725-RS-109] or compound #3 has already exhibited significant anti-inflammatory, neurogenic, and neuritogenic properties in a preclinical BCCAo stroke model in mice. We compared our novel compound with citicoline in a thrombus-induced ischemic stroke model and found that our compound demonstrated superior anti-inflammatory and neurogenic effects. Behavioral assessments revealed that treated animals showed greater restoration of locomotor activity and motor coordination post-stroke than citicoline-treated animals. Hence, we propose that our novel spirotricyclic compound has the potential to emerge as a therapeutic agent for ischemic stroke in the near future.

Keywords: Neurodegeneration, Oxidative stress, Motor deficits, Reperfusion Injury, Anti-inflammatory, Neurogenesis

Introduction

Ischemic stroke is considered the most severe manifestation of cerebrovascular diseases and remains one of the leading causes of death (along with cardiovascular diseases) and disability, as well as the leading cause of hospitalisation worldwide[1.] Although advancements in therapeutics and a deeper understanding of stroke pathophysiology over the last three decades or so have reinforced the treatment strategies against ischemic stroke, which led to a significant decrease in door-to-needle time in acute ischemic stroke treatment, several shortcomings remain unresolved[2]. Some of the most common treatments of ischemic stroke available worldwide are reperfusion therapies that include the administration of clot-bursting agents like Tissue Plasminogen Activator(tPA) or mechanical thrombectomy. These procedures have definitely revolutionized stroke care, but the need for a neuroprotective agent that can be used globally to treat post-stroke neuronal damage still exists.

In recent years, Citicoline (cytidine-5′ -diphosphocholine, CDP-choline) has emerged as one of the most studied neuroprotective agents in stroke therapeutics. It serves as an intermediate in the synthesis of phosphatidylcholine, which is an important component of the cell membrane. The components of citicoline (Cytidine and Choline) can cross the blood-brain barrier (BBB) easily and facilitate the synthesis of CDP-choline[4]. Several studies conducted on focal and global cerebral ischemia models have shown that citicoline, alone or in combination with recombinant tPA (Alteplase), has significant therapeutic effects by reducing the infract size and improving neurological function5. In the acute phase of ischemic stroke, citicoline supports neural recovery by reducing glutamate synthesis,

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alleviating oxidative stress, and repairing the damaged BBB. In the chronic phase, citicoline has been shown to increase neurogenesis, angiogenesis, and neurotransmitter synthesis[4]. Although citicoline has a prolonged therapeutic window and is considered safe for long-term usage, many trials have shown that citicoline does not provide significant benefits in severe ischemic stroke, even in long-term administration[7]. This underscores the translational gap between citicoline's experimental effectiveness and its clinical efficacy. Failure of many such single-target neuroprotective agents to deliver significant results under clinical conditions has prompted a shift towards pleotropic compounds that can affect stroke recovery in multiple pathways. Our spirotrycyclic compound, patented under the name IM-1725-RS-109 (compound #3), showed significant neurogenic, anti-inflammatory, and neuritogenic properties in a global ischemia mouse model (BCCAo). For better readability, we will be referring to IM-1725-RS-109 as #3 from now on, including figures/graphs. This compound significantly reduced pro-inflammatory cytokines like IL-6, IL-1β, and promoted neural health by increasing levels of important growth factors such as brain-derived neurotrophic factor (BDNF)in the same model[8,19].

In our experiments, we employed a thrombus-induced focal ischemic stroke model in CD1 mice, as these strokes are more severe and better recapitulate the pathophysiology of human ischemic stroke. Since we used an $FeCl_3$-induced thrombotic stroke model in our study, we incorporated Tissue Plasminogen Activator (tPA) alongside our drugs as a combinatorial therapy, given that tPA remains the only FDA-approved thrombolytic agent for acute stroke management to date. We compared our novel compound IM-1725-RS-109 (#3) with citicoline (CT) to determine whether it demonstrates superior efficacy in promoting ischemic stroke recovery.

Materials and Methods

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

We employed LC-MS/MS to validate the blood-brain barrier (BBB) permeability of compound IM-1725-RS-109, as BBB penetration significantly enhances drug efficacy in neurodegenerative disorders. Mice were administered IM-1725-RS-109 (30 mg/kg body weight) intravenously (Iv). Brain and blood samples were collected and immediately homogenized at different time points (5 min, 15 min, and 30 min post-injection). Each sample was analyzed in duplicate. Brain samples (25 mg) and blood samples (25 µL) were treated with 400 µL of methanol, incubated in a thermomixer for 20 min, sonicated for 5 min, and then centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and injected into the LC-MS/MS system for analysis.

Cell Culture

Mouse Neuroblastoma cells (N2A) were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution, and maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Sulforhodamine B (SRB) Assay

This assay has been performed as per our previous protocols[9]. The cells were seeded at a density of 3000 cells/cm^2 per well. The two compounds were assessed at six different concentrations (0.01, 0.1, 1, 10, 100, and 1000µm) after 24 hours of incubation. 5% DMSO was used as the negative control for this assay. Three technical replicates were used in this study to improve accuracy and eliminate human errors.

Acetylcholine esterase (AchE) inhibition assay

Inhibition of acetylcholinesterase (AChE) is considered an important factor in improving neural health. AChE inhibition plays a critical role in ameliorating cognitive deficits associated with various neurodegenerative disorders, including stroke, dementia, and Alzheimer's disease. Galantamine, an FDA-approved drug, was used as a positive control for this experiment. All compounds were evaluated for AChE inhibitory activity at three different concentrations (1, 0.1, and 0.01 µM). The assay was performed following previously published protocols from our laboratory[10]. Three technical replicates were performed to ensure data accuracy.

Neuritogenic Assay and Imaging

This assay was performed according to our previously published protocols[9]. Neuro2A cells were seeded at a density of 8000 cells/cm² in six-well plates. After 24 hours, cells were subjected to serum deprivation for 6 hours, then exposed to two non-toxic concentrations (0.01 and 0.1 µM) of the compounds, and incubated. Post-incubation, cells were imaged using a MotiCam Pro 282A (Motic Microscopes)

microscope at 20× magnification at different time points (24 and 48 hours).

Animal Housing

Adult male CD1 mice were bred, housed, and procured from the institutional animal facility and used throughout the study. IICT Animal Ethics Committee approved (IAEC-IICT-084-2024) for all the experimental procedures. The experiments were conducted at the Indian Institute of Chemical Technology (IICT) in Hyderabad, India. Adult male mice aged between 6-8 months were utilized for the study. The animals were acclimatized to standard experimental conditions, maintained at 24°C with a 12-hour light-dark cycle, and provided ad libitum access to food and water. The animals were housed in individually ventilated cages (IVC).

Surgical Procedure and Experimental Design

Briefly, each mouse was anesthetized using a ketamine (100 mg/kg b.w.) and xylazine (10 mg/kg b.w.) cocktail administered via the intraperitoneal (i.p.) route. A small incision (approximately 1 cm) was made in the ventral region of the neck on the left side. The common carotid artery was carefully isolated from the trachea and vagus nerve. A strip of pre-soaked paper in PBS was carefully inserted beneath the artery to separate it from the adjacent musculature. A thin strip of Whatman filter paper soaked in 3% Ferric Chloride($FeCl_3$) solution was carefully positioned atop the artery, and gentle pressure was applied to ensure proper absorption of the solution. After 3 minutes, the strip was removed, and the adjacent area was thoroughly cleaned with PBS to prevent dissemination of the $FeCl_3$ solution. The incision was closed using silk sutures, and the animal was placed on a heating pad for recovery. Once the animal regained consciousness, it was transferred to the Individually Ventilated Cages(IVC). Laser Doppler perfusion imaging was performed before and after surgery.

Animals were divided into four groups: (i) vehicle group (receiving PBS via i.p. route), (ii) tPA group (receiving only tissue plasminogen activator via i.v. route post-stroke), (iii) tPA+IM-1725-RS-109 or tPA+#3 group (receiving our novel compound along with tPA), and (iv) tPA+CT group (receiving the FDA-approved drug citicoline along with tPA post-stroke). Dosages of citicoline and #3 (5 mg/kg b.w.) were determined based on our previous publications[8], and tPA dosage was 3 mg/kg b.w. After 2 days, animals were euthanized, and tissues were collected. Each group consisted of 10 animals (n=10; see supplementary section for additional details on experimental design, Supplementary Figure 1).

Justification of not using a Sham group

Since this study focuses on the comparative evaluation of two drugs, the FDA-approved drug citicoline and our compound #3 (patented under the name IM-1725-RS-109), we excluded a sham group from the primary analysis. Although sham surgery was performed and several parameters were assessed for stroke model accuracy, we decided to exclude these results from the main manuscript to avoid complications. Instead, we used a vehicle control group (receiving only PBS post-stroke) as the control and compared treatment group outcomes accordingly (see the supplementary section for a detailed explanation and Supplementary Figure 2).

Laser Doppler Perfusion Imaging (LDPI)

In this study, LDPI was employed to monitor changes in cerebral blood flow in the common carotid artery following thrombus induction. A Moor LDI2-IR system (Moor Instruments Ltd., Devon, UK) was used for this procedure. Following anesthesia, animals were secured in a supine position beneath the laser. LDPI measurements were acquired at two different time points: before thrombus (BT) and after thrombus (AT) induction. The region where the filter paper strip was placed was designated as the Region of Interest (ROI) to facilitate accurate quantification of blood flow (Fig 1).

Behavioural Tests

Neurological Deficit Scoring (NDS)

Neurological deficit scoring (NDS) was employed to assess the extent of impairment following the surgical procedure. Scoring was performed according to our previously published protocols (11) and based on post-surgery behavioral symptoms. A score of 0 indicates no visible impairment. A score of 0.5 is assigned if ptosis is observed in one eye. A score of 1 is assigned if both eyes exhibit ptosis, while a score of 1.5 is assigned if additional symptoms are observed along with ptosis, such as body bending or limb tremor. A score of 2 is assigned if more than one symptom is present in addition to ptosis. A score of 3 (the maximum) is assigned if the animal exhibits no resistance to lateral push, partial limb paralysis, and circling toward the paretic side.

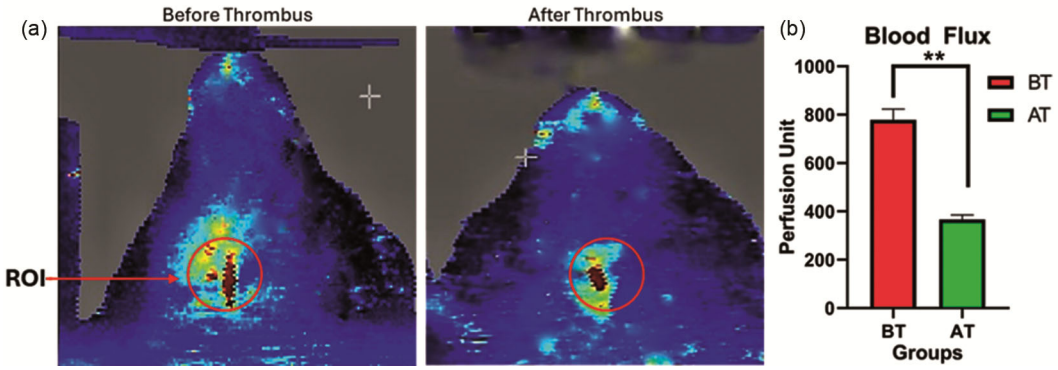


Fig. 1 — (a) shows the cerebral blood flow at two different time points, before thrombus (BT) and after thrombus (AT) induction, and (b) shows the reduction of blood flux post thrombus induction in the perfusion unit.

Open field test (OFT)

The open-field test was used to evaluate locomotor activity in animals post-surgery. Previously published protocols were followed[11]. The apparatus consisted of a 40 × 40 cm wooden arena. Each mouse was placed in one corner of the arena and allowed to move freely. Movement was recorded for 3 minutes using a video camera. Subsequently, video footage was analyzed using EthoVision XT 17 (Noldus Information Technology, Wageningen, The Netherlands). To eliminate olfactory cues, the arena was thoroughly cleaned with 70% ethanol between trials. Trials were conducted at 24 and 48 hours post-surgery.

Pole Test

This test was conducted to further evaluate the motor coordination of mice post-stroke and following treatment. Briefly, each mouse was placed atop a vertical pole (50 cm in length and 3 cm in diameter) and trained to descend the pole to reach its home cage. Trials were conducted at 24 and 48 hours post-surgery. To eliminate olfactory cues, the pole was thoroughly cleaned with 70% ethanol between trials.

Tissue collection and fixation

Mice were euthanized by cervical dislocation on day 3 (72 hours post-surgery). Specific brain regions, including the striatum and hippocampus, were carefully microdissected using a brain matrix [Stainless Steel Brain Matrix (#72-5032), 1 mm coronal sections, mouse; Harvard Apparatus, Holliston, MA, USA]. Collected tissues were immediately transferred to liquid nitrogen for snap-freezing and subsequently stored at -80°C.

RNA Isolation and qRT-PCR

Expression of different genes in the striatum and hippocampus was quantified by quantitative real-time PCR. The RNA was isolated using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. cDNA synthesis was done using the Thermo Fisher First-Strand cDNA synthesis kit as instructed by the company. qRT-PCR was performed using the SYBR Green PCR Master Mix Detection System (Applied Biosystems). The mRNA levels of different target genes were normalized against the fold change of RPL32 (housekeeping gene) (see supplementary section for forward and reverse primers).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism and Microsoft Excel. Two-way ANOVA followed by post hoc Bonferroni tests was used for in vitro and behavioral analyses. In the case of LDPI, the T-test has been used. The results were expressed as mean ± SEM with n=10-12 for the behavioral study and n=6-7 for qRT-PCR. One-way ANOVA was used. A p-value< 0.05 was considered significant.

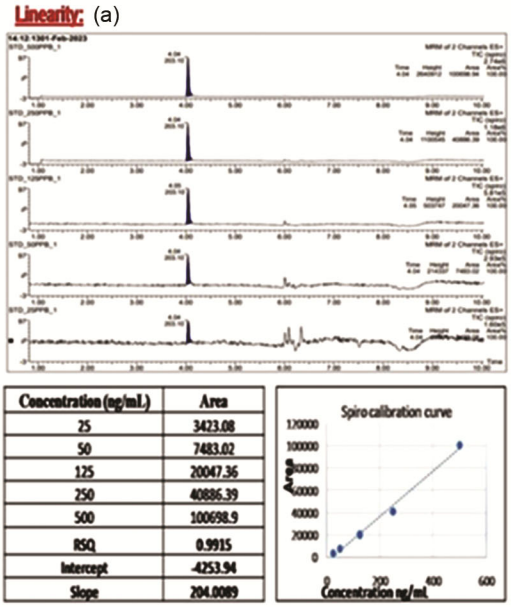
Results

IM-1725-RS-109 crosses the blood-brain barrier

LC-MS/MS data demonstrated that our compound successfully crossed the BBB. Mice were administered IM-1725-RS-109 and euthanized at various time points (5 min, 15 min, and 30 min). LC-MS/MS analysis revealed measurable concentrations (peaks) in both the 5 min and 30 min samples. These findings indicate that our compound crossed the BBB as early as 5 min after administration and remained at therapeutic levels for at least 30 min (Fig 2).

Acetylcholine esterase inhibition assay

Citicoline (CT) demonstrated significant inhibition at both concentrations compared to our compound (#3). These results suggest that our compound does



Recovery for brain sample: (b)

S.No	Preparations	Obtained Concentration (ng/mL)	Amount added (ng/mL)	% Recovery
1	Brain+ spike 25_PPB	31.68	25	126.72
	Brain+ spike 50_PPB	40.88	50	81.76
	Brain+ spike 125_PPB	101.48	125	81.18
	Brain+ spike 250_PPB	243.81	250	97.52

Recovery for blood sample:

S. No	Preparations	Obtained Concentration (ng/mL)	Amount added (ng/mL)	% Recovery
1	Serum+ spike 25_PPB	31.81	25	127.2
	Serum+ spike 50_PPB	41.71	50	83.4
	Serum+ spike 125_PPB	116.26	125	93.0
	Serum+ spike 250_PPB	321.59	250	128.6

Fig. 2 — (a) shows the MS peaks, and (b) shows the recovery percentage of the compound from blood and brain samples.

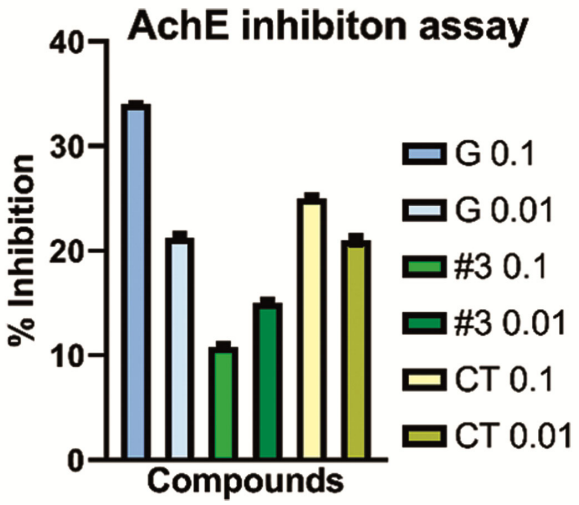


Fig. 3 — The figure shows the % inhibition of acetylcholine esterase in all the compounds; galantamine (G) is used as a positive control.

not exhibit significant acetylcholinesterase inhibitory activity (Fig 3).

In-vitro studies

To determine cell viability at different concentrations of the two compounds (citicoline and IM-1725-RS-109 or compound #3), we performed an SRB assay. This assay was conducted using N2A cell lines, and results demonstrated that both compounds exhibited minimal cytotoxicity at all tested concentrations at both 24 and 48 hours compared to the negative control (5% ethanol)(Fig 4). These findings suggest that the compounds are safe for therapeutic administration.

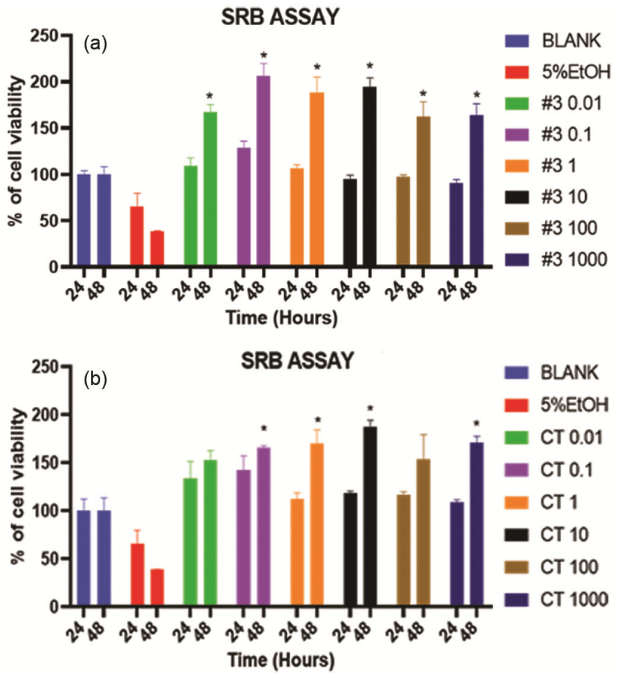


Fig. 4 — SRB cell viability assay of compound #3 (a) and Citicoline (b) at different concentrations and two different time points (24 and 48 hours).

Effect of compound #3 and Citicoline on neurite growth length

The neuritogenic assay, or neurite growth length assay, was performed to determine whether treatment affects axonal growth. Since axonal outgrowth is

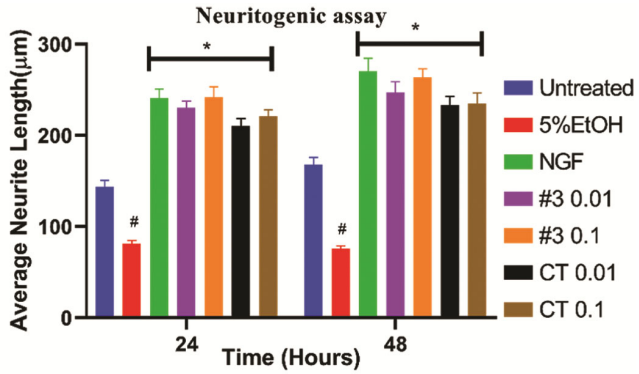


Fig. 5 — Average neurite length outgrowth at 24 and 48 h post-treatment with the two compounds at two different concentrations.

considered beneficial for neural health, this assay is critical for evaluating the neuritogenic potential of therapeutic compounds. Our compound demonstrated a significant increase in neurite length at both time points (24 and 48 hours) compared with the untreated and negative control groups, but did not differ significantly from citicoline (Fig 5). Notably, the average neurite length was comparable to that of nerve growth factor (NGF), which served as a positive control.

***In-vivo* studies**

All experiments were performed using an $FeCl_3$-induced thrombus stroke model in CD1 outbred mice. Cerebral ischemia or ischemic stroke often causes severe motor deficits, rendering survivors partially or completely paralyzed. Hence, we employed several behavioral tests of locomotor activity and motor function to determine whether our compound affects these parameters.

Neurodeficit score (NDS) analysis following thrombus induction

NDS analysis was performed at 24 and 48 hours post-surgery. The vehicle and tPA groups displayed significantly elevated scores on both days. The treatment groups demonstrated reduced NDS scores on both days. The group that received #3, along with tPA, showed a significantly lower score on day 1, suggesting that our compound is effective even at early time points (Fig 6).

Open field test (OFT)

This test was conducted to evaluate locomotor activity using two key parameters: distance traveled and velocity. (Fig 7a) represents the distance traveled, and (Fig 7b) represents the velocity. Results demonstrate that the treatment group receiving IM-1725-RS-109 (#3) along with tPA showed significant

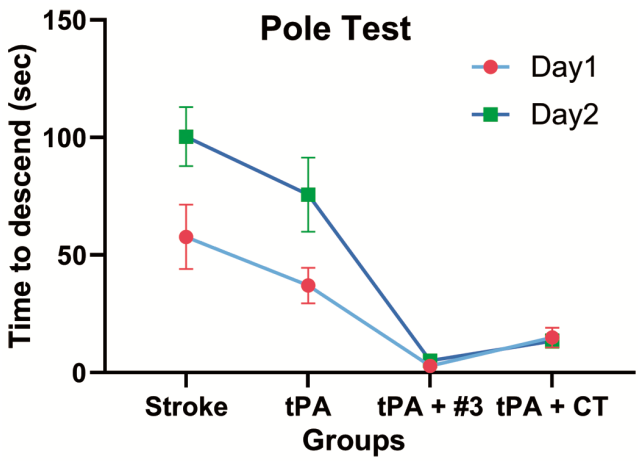


Fig. 6 — This figure represents the neurodeficit score across the four groups. The group receiving #3 showed significant improvement on day 1 compared with the group receiving CT.

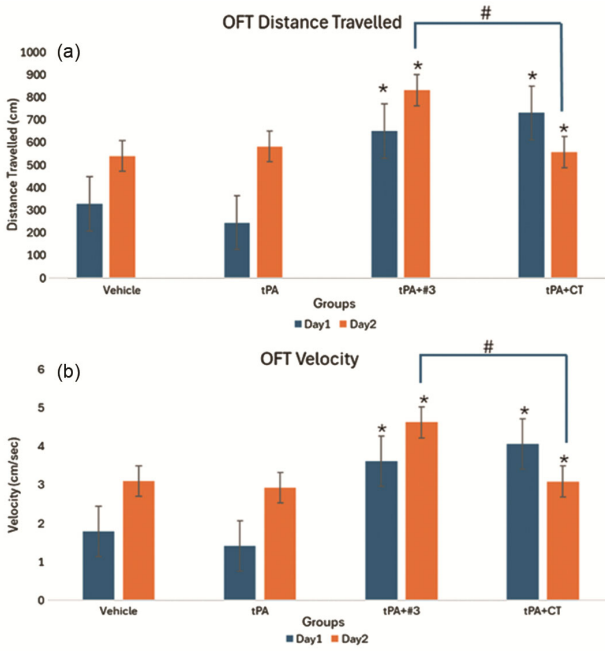


Fig. 7 — Open field test to assess locomotory activity (a) represents the distance travelled by the mouse in cm, and (b) represents the velocity of the mouse. The group receiving #3, along with tPA, showed a significant increase in distance traveled and velocity on day 2 compared to all the other groups.

improvement in locomotor activity on day 2 compared to the group receiving citicoline (CT) with tPA.

Pole test

Motor coordination in mice following treatment was further validated using the pole test, which measured the time each animal took to descend the pole and reach its home cage. Results demonstrate

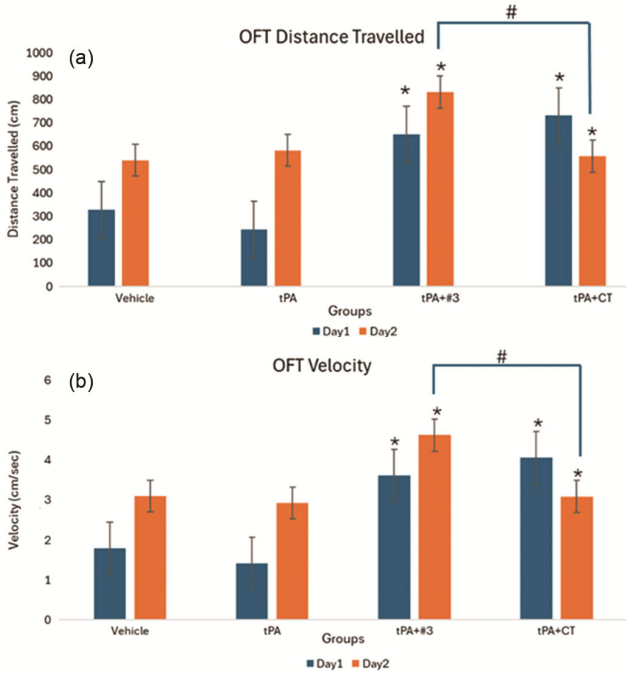


Fig. 8 — Analysis of behavioral functional outcome post stroke using the pole test across all 4 groups. The tPA + #3 group took the least amount of time to descend the pole compared to the other groups.

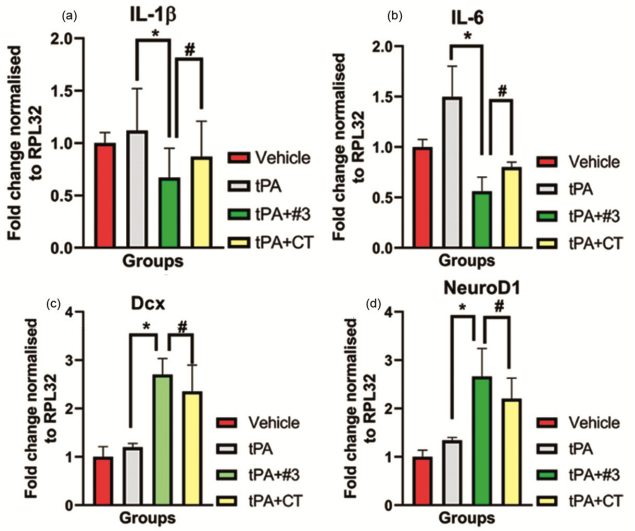


Fig. 9 — Figure shows the gene expression of anti-inflammatory markers IL-1β (a) and IL-6 (b), and neurogenic markers Dcx (c) and NeuroD1 (d). The fold change is normalized to that of RPL32.

that the treatment group receiving #3, along with tPA, required significantly less time to descend the pole compared to the tPA and vehicle groups. These findings indicate that our spirocyclic compound significantly improved motor coordination in mice post-stroke (Fig 8).

qRT-PCR Analysis of striatum and hippocampus post stroke showed significant downregulation in the expression of pro-inflammatory markers in the striatum and upregulation of neurogenic markers in the hippocampus.

It is well established that the striatum and hippocampus are the two most severely affected regions following ischemic stroke. In our study, we microdissected these two brain regions as described in the Materials and Methods section. Based on our previous studies demonstrating that our novel compound exhibits significant anti-inflammatory and neurogenic activity, we examined several pro-inflammatory and damage markers (Fig 9a & 9b) in the striatum and neurogenic markers (Fig 9c & 9d) in the hippocampus. Results demonstrate that our compound IM-1725-RS-109 exhibited significantly greater neurogenic and anti-inflammatory activity compared with the groups receiving only tPA or tPA + CT. These results give us an insight that reduction of pro-inflammatory activity might be linked to better locomotor activity in our treatment groups, especially in the group treated with #3

Discussion

According to a recent survey by the World Health Organization (WHO), stroke is the second leading cause of death and the third leading cause of disability worldwide. Among the two types (ischemic and hemorrhagic), ischemic stroke accounts for approximately 75% of all cases[11]. In recent years, numerous preclinical stroke models have emerged that have significantly advanced stroke research and therapeutic strategies. To date, first-line antiplatelet agents and oral purinergic receptor $P2Y_{12}$ ($P2Y_{12}$) inhibitors represent the most common treatments for stroke patients, along with thrombolytic and surgical intervention techniques; however, these approaches have certain limitations. Antiplatelet agents such as aspirin often affect hemostasis and cause bleeding complications. Some patients exhibit intolerance to antiplatelet therapy due to various genetic factors and experience severe complications, including aspirin-induced respiratory difficulties, drug hypersensitivity, and gastrointestinal disturbances[12]. Ischemia-induced damage often triggers severe neuroinflammation by recruiting various pro-inflammatory cells, including macrophages and microglia. These cells, in turn, release pro-inflammatory cytokines that cause severe neuronal damage and blood-brain barrier disruption[13].

Numerous studies have demonstrated that anti-inflammatory compounds capable of suppressing cytokine storms exhibit remarkable therapeutic efficacy in stroke treatment, particularly in preclinical rodent models[14].

In this study, citicoline was selected as an active comparator since it is an FDA-approved drug and has demonstrated beneficial effects in stroke patients across numerous clinical and preclinical studies. Citicoline in stroke patients has been shown to reduce infarct size, promote neuroplasticity, and improve cognitive deficits[15]. Citicoline has also been shown to exhibit anti-inflammatory properties by downregulating NF-κB, a master regulator of various pro-inflammatory cytokines, including TNF-α and IL-1β[16]. However, some research groups did not find any significant difference in infarct size reduction between the citicoline and placebo groups following 6 weeks of citicoline administration[17]. Another study published in the Journal of Neuroscience Research demonstrated that treatment with CDP-choline following ischemia/reperfusion significantly reduced neuroinflammation and BBB leakage by preventing the release of free fatty acids (FFAs) such as arachidonic acid[18].

Although citicoline demonstrated positive results in various clinical and preclinical trials, several research groups did not find significant differences between placebo and citicoline-treated groups. Our novel spirocyclic compound (compound #3) demonstrated significant efficacy in ameliorating post-stroke complications in preclinical rodent models of BCCAo[19]. Even compared to citicoline, our compound demonstrated remarkable potential to reduce inflammation and cellular damage while improving motor coordination and locomotor activity in a thrombus-induced ischemic model. Several limitations must be considered when extrapolating these results. First, the thrombus-induced model was performed in only one strain (CD1), and one sex (male). But neuroprotective efficacy can vary across ischemia paradigms, comorbidities, and biological sex. Second, dosing and timing were optimized for the experimental agent and may not directly correspond to human pharmacokinetics or clinical treatment windows typically associated with thrombolysis or thrombectomy. Third, the inability to conduct clinical trials or to use clinical samples from stroke patients is a limitation of this study. Additionally, the inability to validate gene expression changes at the protein level through Western blot analysis is also considered another limitation. Nevertheless, it is worth noting that our compound demonstrated significant efficacy in ameliorating stroke symptoms across multiple preclinical models.

Conclusion

In this study, the novel neuroprotectant IM-1725-RS-109 demonstrated robust efficacy in a thrombus-induced ischemic stroke model. Behavioral assessments revealed that our novel compound exhibited marked improvement in motor coordination and locomotor activity, particularly on day 2 post-stroke, indicating its potency in accelerating functional recovery. qRT-PCR analysis further demonstrated that IM-1725-RS-109 significantly downregulated pro-inflammatory genes (IL-6 and IL-1β) while upregulating neurogenic markers (Dcx and NeuroD1). Notably, these effects were more pronounced than those observed in citicoline-treated animals. Collectively, these findings suggest that our novel compound (IM-1725-RS-109) may be a promising candidate among stroke therapeutics.

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Author Contributions

S.G. and S.C. planned and designed the biological experiments. S.C. and S.P. contributed reagents, materials, analytical tools, and software. J.O. and S.P. synthesized the novel compound. S.G., S.K.P., R.K., S.W.P., and S.C. performed biological experiments. S.C. supervised all biological experiments. S.G. analyzed the results and wrote the original draft. S.C. and S.G. edited the original draft to generate the final manuscript. All authors reviewed and approved the final manuscript.

Competing Interests

The authors declare no competing interests.

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