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# Investigating the role of ras-related c3 botulinum toxin substrate 1(rac1) in modulating tumor necrosis factor alpha (tnf-α) mediated inflammation in a streptozotocin-induced mouse model of Alzheimer's disease

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## Abstract

**Background and objective:** Alzheimer's disease (AD) is a slowly worsening brain disease linked to ongoing inflammation, with Tumor Necrosis Factor Alpha (TNF-α) playing a significant function in its progression. Ras-related C3 botulinum toxin Substrate 1(Rac1) regulates inflammatory signaling and may influence TNF-α expression in AD. The aim is to investigate the impacts of Rac1 inhibition on TNF-α expression and neuropathology in a streptozotocin (STZ)-induced AD mouse model.

**Methods:** There were three groups of male Swiss albino mice: Control (control), STZ (AD model), and NSC23366 + STZ (Rac1 inhibitor). The treatment group received 5 mg/kg NSC23366 before STZ administration. ELISA measured TNF-α, amyloid beta 42 (Aβ42) and tau levels in the brain and serum. Histopathological analysis assessed neuroinflammation and neuronal damage.

**Results:** Streptozotocin (STZ) significantly increased Aβ42, tau, and TNF-α levels ($P$ <0.05). NSC23366 treatment reduced these markers, indicating neuroprotection. In the STZ group, histological analysis showed severe neuroinflammation and neuronal damage. Conversely, NSC23366 reduced the quantity of inflammatory cells and scores of brain injury ($P$ <0.05).

**Conclusion:** Blocking Rac1 lowers neuroinflammation caused by TNF-α, lowers the damage caused by tau and amyloid plaques, and slows down neurodegeneration in AD. Targeting Rac1 may provide an achievable therapeutic strategy for the management of Alzheimer's condition.

**Keywords:** RAC1; Alzheimer’s disease (AD); TNF-α; Tau protein; Amyloid beta 42.

## Introduction

Alzheimer's disease (AD) is a neurological disorder that worsens over time, that profoundly affects public health, with its prevalence expected to double or triple by 2050.[1] The disease primarily affects long, thinly myelinated cortical projection neurons, leading to cognitive decline, memory loss, and impaired language and motor skills.[2]

Alois Alzheimer first described the pathology in 1906, identifying amyloid plaques and neurofibrillary tangles as key features, Alzheimer's disease presents in both early-stage (30–60 years) and late-onset (≥60 years) variants, with aging being the most significant risk factor.[3]

A key component of Alzheimer's disease progression is chronic neuroinflammation, which is characterized by the overgrowth of microglia and astrocytes and the secretion of inflammatory mediators that worsen neuronal dysfunction.[4]

Immune cells react with amyloid-beta plaques and increase phosphorylation tau, causing neuroinflammation and synaptic dysfunction.[2,5] TNF-α is a crucial inflammatory cytokine involved in neuroinflammation and neurodegeneration, TNF-α reacts with TNF receptors (TNFR1

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and TNFR2), triggering downstream pathways that regulate apoptosis, necroptosis, and inflammation.[5,6] Unbalanced TNF-α levels can weaken the blood-brain barrier and harm neurons.[7] Ras-related C3 botulinum toxin substrate 1 (Rac1), a tiny GTPase, is involved in neuroprotection and neurodegeneration by regulating cellular signaling, synaptic plasticity, and cytoskeletal dynamics.[8] Rac1 activation regulates inflammation by affecting TNF-α and reactive oxygen species (ROS) generation via NF-κB and NADPH oxidase pathways.[9] The inhibition of Rac1 has been linked to enhanced cognitive performance, indicating that it might serve as an agent of treatment for Alzheimer's disease.[10] This research aims to explore Rac1's role in influencing TNF-α expression and how it affects inflammation linked to AD.

## Methods

### Study Design

The present investigation is an experimental study and Alzheimer's Disease Induction. Inthis study, 15 male Swiss albino mice were allocated into three groups utilizing the basic random sampling method, with each group including five mice. Group one functioned as the negative group of controls, while group two served as the positive group of controls (STZ). Group three served as a treatment group (RAC1+STZ). The research was performed in the Animal House Unit/ College of Pharmacy/Hawler Medical University.

### Animals

Hawler Medical University, Iraq, the College of Pharmacy, complies with ethical guidelines for animal care regulation and has received clearance from the Regional Animal Experimentation Ethical Committee (No. Sc. E. C. 11) on November 26, 2024. The criteria for inclusion only male mice weighing 25-30 g were included, whereas those above 30 g and female mice were excluded. Animals caged in a sterile environment with a 12-hour day/night cycle at 24°C and 55% ± 5% humidity. Food and drink are provided two times a day. Pure drinking water was made available and made available ad libitum via a designated nipple. Before the start of the trials, all subjects experienced a 7-day acclimatization phase to adapt to the experimental circumstances. Animals were categorized into three groups: the control group (receiving only saline injections), the vehicle group (receiving streptozotocin injections), and the treatment group was given pretreatment with NSC23366, followed by administration of streptozotocin (NSC23366 + STZ). Each group included five mice, housed in cages containing no more than five individuals per group, with environmental enrichment offered.

### Materials

Streptozotocin was purchased from (STZ; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). A citrate buffer can be used to make streptozotocin. This is done by mixing 6.07 grams of sodium citrate and 0.84 grams of citric acid in 200 milliliters of filtered water. HCl is then added to bring the pH of the solution down to 4.5.

NSC23766 (N6-[2-[[4-(Diethylamino) -1-methylbutyl] amino] -6-methyl- 4-pyrimidinyl] -2 methyl-4, 6-quinolinediamine trihydrochloride, Solarbio Science & Technology Co., Ltd. China. The GlucoNavii blood glucose meter.

### Animal Experiments

A fasting period of approximately 6–9 hours, during which they had unlimited access to water. Before administering injections, fasting blood glucose levels and body weights were recorded for each mouse. For the preparation of STZ, 11.25 mg of it was measured into a glass tube covered with aluminum foil and placed in a container filled with ice. Citrate buffer (pH 4.5) was added to the glass tube to dissolve the STZ.

In the control group, mice received an intraperitoneal injection of citrate buffer.[11] In the STZ group, a single dose of STZ was administered at 45 mg/kg body weight to induce diabetes mellitus in the

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experimental animals.[12] In the treatment group, five mg/kg of (NSC23766), was injected intraperitoneally, This dose was chosen based on previous research.[13] After a 30-minute interval, STZ was injected into the (NSC23766+ STZ) group. Following STZ administration, the animals exhibited signs of hypoglycemia. To alleviate this condition, a 5% (w/v) glucose solution was provided to the animals for one day.

Following the STZ injection, the mice's fasting blood sugar levels were assessed from the vein in the tail within one week to verify the induction of diabetes. More than 70% of the mice had blood glucose concentrations more than 200 mg/dL (11.1 mmol/L). These results demonstrate that the STZ and NSC23766+ STZ groups are in the early stages of diabetes. In diabetic mice, fasting blood glucose levels were defined as exceeding 11.1 mmol/L.[14]

After 21 days, all mice were anesthetized intraperitoneally with 25 mg/kg of xylazine and 75 mg/kg of ketamine. Blood was obtained from the vena cava, separated the serum via centrifugation, and stored in Eppendorf tubes at -20°C for subsequent ELISA testing. The brains of the mice were separated into two sections: first was immobilized in a 10% formalin solution for histopathological examination, and the second was placed in an Eppendorf tube, mixed with a few drops of phosphate buffer (pH 7.4), and homogenized with an Intelligent Ultrasonic Processor. After centrifuging the homogenate the supernatant was removed, moved to an Eppendorf tube, and preserved at -20°C for further ELISA analysis.

**Biochemical Determination**

Blood glucose levels of all experimental mice were monitored prior the commencement of the procedure. Until diabetes was definitively diagnosed, fasting blood sugar levels were routinely monitored, the diabetes status was established in mice when their fasting blood sugar levels reached 11.11 mmol/l or higher. Each experimental animal had 0.5-1 µl of blood taken from its tail veins. AGlucoNavii blood glucose meter was used for the purpose of monitoring the blood glucose levels.

**Mouse ELISA**

Tau and Aβ42 levels were measured in brain tissue, while TNF-α levels were assessed in brain tissue and serum across all groups of animals. For the tests, Tumor Necrosis Factor α (TNF-α) ELISA Kit, (Cat. No SL0547Mo), the Mouse Tau Proteins (TAU) ELISA Kit (Cat. No. SL0868Mo), and the Mouse Amyloid Beta Peptide 1-42 (Aβ1-42) ELISA Kit (Cat. No. SL0040Mo) were used. at 450 nm Absorbance was measured under the manufacturer's guidelines, TNF-α levels were reported in ng/L, while Tau and Aβ42 levels were expressed in pg/mL.

**Histology**

The brain was preserved overnight in a 10% formaldehyde solution prior to dehydration and embedding in paraffin. To stain micrometer-sized fragments, hematoxylin and eosin were employed. A revised assessment method was employed to objectively assess brain damage using a blinded study design.[15] consisting of a mixture of inflammatory cells, edema, scattered pyknosis (degeneration), myeloid gliosis, necrosis (irreversible damage), and vascular congestion. The aforementioned criteria were evaluated and classified as mild, moderate, and severe, (0) normal histology; (1) mild involvement (<25%); (2) moderate involvement (25–50%); and (3) severe involvement (>50% of the affected tissues), The mean value was determined by analyzing five randomly selected regions of each tissue sample. The histopathology score was calculated by adding up all six criteria. This part was performed in the histology lab of the Par Hospital.

**Statistical Analysis**

The results are shown as means with their respective standard error of the mean (SEM) values. To assess the data, nonparametric tests (Mann-Whitney) were

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employed. For each group, the total number of mice is represented by "n," and a *P*-value below 0.05 was proposed as the threshold for statistical significance. All analyses were conducted using SPSS (IBM Corp., Armonk, N.Y., USA).

## Results

### Brain Amyloid Levels

Brain amyloid levels were assessed in the control, STZ, and NSC23766-treated groups. The results indicate a significant increase in amyloid accumulation following STZ administration, while NSC23766 treatment reduced amyloid levels, Control (58.3 ± 17.8), STZ (134.1±8) and NSC23766 (66.9 ± 14.3). The STZ group exhibited a significant increase in brain amyloid deposition, while NSC23766 treatment mitigated this effect, suggesting a neuroprotective role of RAC1 "Figure 1"

### Brain Tau Levels

Tau protein concentrations were significantly elevated in STZ-treated mice compared to Control. However, NSC23766 administration reduced tau levels in some instances. The mean values were, Control (305.8 ± 33.5), STZ (624.6 ± 250.2) and NSC23766 (317.6 ± 60.3). The STZ group showed a notable increase in tau protein aggregation, a hallmark of neurodegeneration. NSC23766 treatment resulted in a decrease, though not to Control levels, indicating partial mitigation "Figure 2".

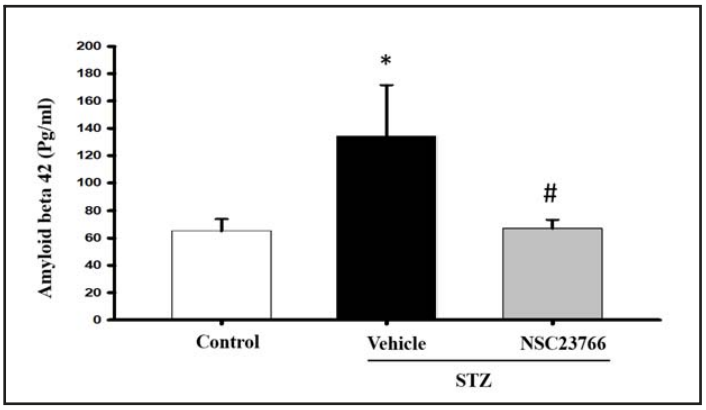


**Figure 1** The brains of animals with Alzheimer's disease were quantified using ELISA to measure the levels of Amyloid Beta 42 (Aβ42). The data are presented as the mean ± SEM (control = 5, vehicle = 5). In comparison to the control group, the vehicle-treated group exhibited a substantial increase in Aβ42 levels (*$P$ < 0.05 vs. control)

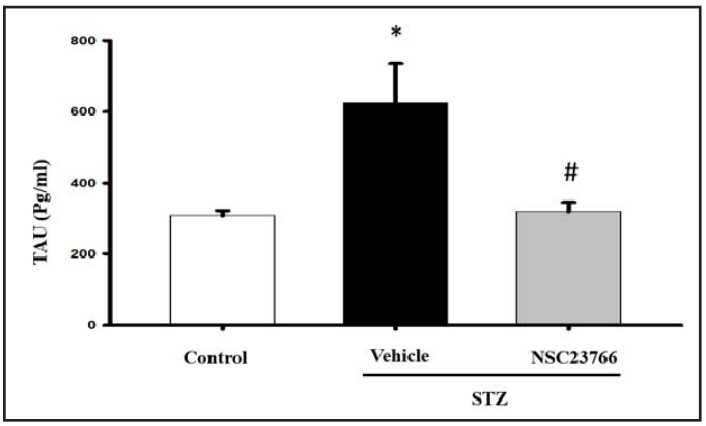


**Figure 2** The concentrations of TAU protein in the brains of animals with Alzheimer's disease were determined using ELISA. The data are presented as the mean ± SEM (control = 5, vehicle = 5). The vehicle-treated group demonstrated a statistically significant elevation in TAU levels compared to the control group (*$P$ < 0.05 vs. control)

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**Brain TNF-α Levels**

TNF-α, another major pro-inflammatory cytokine, was significantly elevated in the STZ group, Control (350.0±22.5), STZ (700.0±55.0), NSC23766 (368.0±30.4).STZ -induced neuroinflammation was evident through elevated TNF-α levels. However, NSC23766 treatment helped reduce TNF-α levels, further indicating its neuroprotective potential "Figure 3".

**Serum TNF-α Levels**

Systemic inflammation was further confirmed by measuring serum TNF-α levels, Control (250.0 ± 15.6), STZ (750.0±78.4), NSC23766 (315.0 ± 24.1). STZ treatment led to a significant increase in TNF-α, supporting its role in neuroinflammation. NSC23766 administration substantially reduced levels of TNF-α, highlighting its anti-inflammatory effects.STZ administration significantly increased brain amyloid and tau levels, as well as inflammatory cytokines TNF-α ($P$ <0.05). NSC23766 treatment effectively reduced these markers, demonstrating potential neuroprotective and anti-inflammatory effects. The differences in brain amyloid ($P$ <0.05) and tau ($P$ <0.05) between groups were statistically significant, confirming STZ's neurotoxic effects and RAC1 inhibitor's protective role "Figure 4".

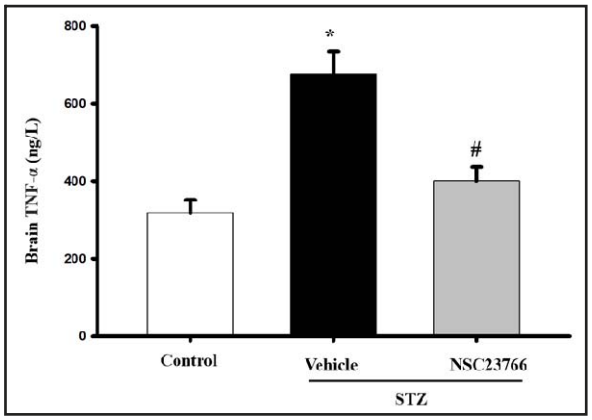


**Figure 3** ELISA was conducted to measure TNF-α concentrations in the brains of mice afflicted with Alzheimer's disease. Data are presented as mean ± SEM (control = 5, vehicle = 5). It was found that the group that was given vehicles had significantly higher levels of TNF-α than the control and NSC23766 + STZ groups (*$P$ = 0.05 vs. Control),* indicates significant

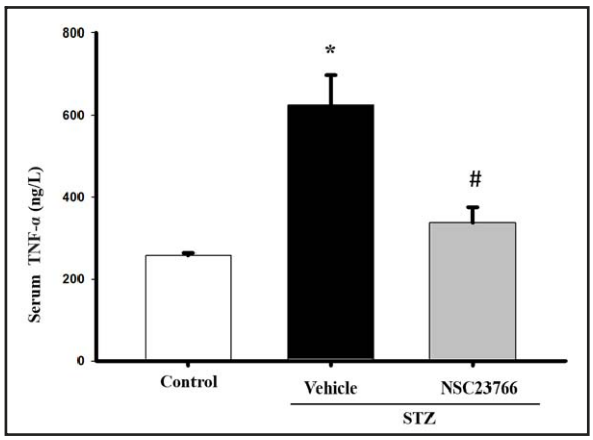


**Figure 4** The levels of TNF-α in the serum of Alzheimer's disease mice were measured using an ELISA. The mean ± SEM is used to express the data, with control = 5 and vehicle = 5. There was more tumor necrosis factor-alpha in the group that got the vehicle treatment compared to the control and NSC23766 + STZ groups (*$P$ < 0.05 vs. control)

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**Modifications in Brain Histology in an Alzheimer's Disease Mouse Model**

Histological analysis showed that the brains of Alzheimer's disease (AD) mice that had been given STZ had major pathological changes. These changes included the infiltration of mixed inflammatory cells (Arrow 1 in the vehicle group), edema and scattered pyknosis, myeloid gliosis and necrosis, and vascular congestion. Notably, irreversible degeneration (Arrow 2 in the vehicle group) was observed, as illustrated in Figure 2B. The induction of AD via streptozotocin (STZ) injection resulted in a moderate degree of brain injury (25–50%), with a significantly higher histological injury score in the AD group (1.13 ± 0.08) compared to the control group (0.12 ± 0.01) (*P* <0.05). But giving NSC23766 worked to lessen the damage to the histology, lowering the score to 0.32 ± 0.01, (*P* <0.05) "Figure 5. These results suggest that NSC23766 protects neurons by easing the damage caused by STZ and lowering the changes that come with Alzheimer's disease.

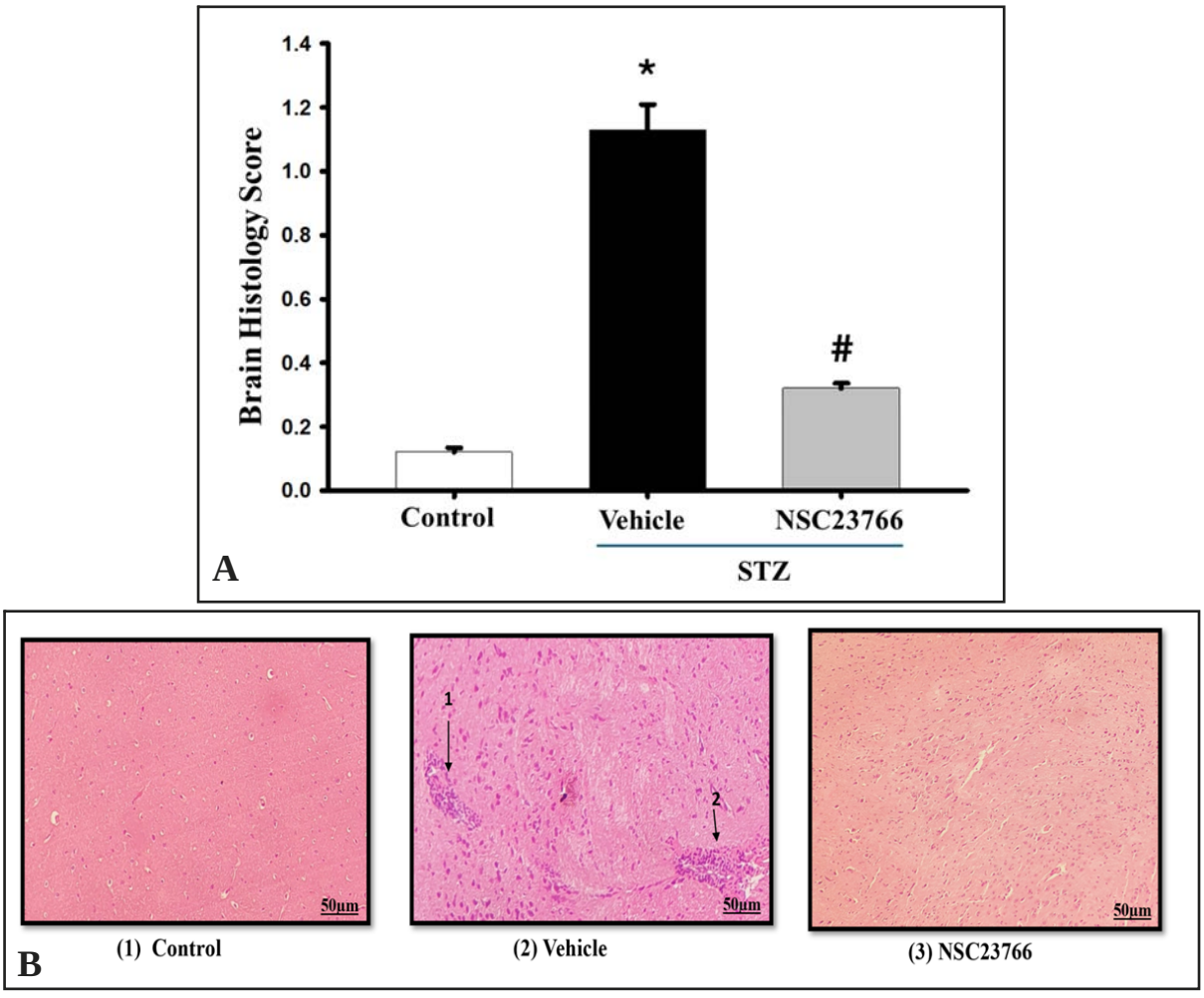


**Figure 5** In Alzheimer's disease, RAC 1 affects neuronal degeneration. (A) is brain histology scores. (B) Shown here are some representative H&E brain slices. Prior to streptozotocin-induced diabetes mellitus induction, mice were given either STZ (vehicle) or a RAC 1 inhibitor (NSC23766), Negative controls were performed using control animals. **P* <0.05 when compared to Control; #*P* <0.05 when compared to NSC23766 + STZ. The data are shown as mean ± SEM, and the sample size is 5.

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## Discussion

This study highlights the significant role that RAC1 plays in the neuroinflammation that is produced by TNF-α. In an animal model of Alzheimer's disease caused by streptozotocin (STZ), the results show that NSC23766 greatly lowers neuroinflammation, amyloid buildup, and tau clumping by stopping RAC1. Furthermore, the levels of TNF-α, a crucial cytokine in neuroinflammation, were significantly elevated in the STZ group, thereby confirming its role in the pathology of Alzheimer's disease (AD) "Figure 3&4". The findings of this study are in agreement with previous studies that have shown that TNF-α has a role in the breakdown of the blood-brain barrier, synaptic dysfunction, and neuronal apoptosis.[5,7] Nevertheless, the administration of NSC23766 resulted in a significant decrease in the levels of TNF-α, indicating that the inhibition of RAC1 may effectively control inflammatory pathways in order to limit neuroinflammatory responses.[10]

NSC23766 not only reduced TNF-α but also decreased levels of Aβ42 and tau, which are two hallmark proteins associated with Alzheimer's disease. This aligns with research indicating that RAC1 plays a role in amyloidogenic processing and tau hyperphosphorylation via cytoskeletal and oxidative stress mechanisms.[11] The decrease in Aβ42 and tau indicates that RAC1 inhibition could slow down disease progression by reducing protein aggregation “Figure 1&2".

An additional confirmation of the neuroprotective effects of RAC1 inhibition was provided by histological examination. "Figure 5" shows that mice that were treated with STZ had substantial neuronal injury, which included mixed inflammatory cell infiltration, edema, and vascular congestion. All of these results are consistent with prior observations that neuroinflammation makes neuronal damage worse in Alzheimer's disease.[2,4] NSC23766 showed a considerable reduction in histopathological damage, which suggests that it has the ability to protect against neurodegeneration associated with Alzheimer's disease.

While these results are encouraging, there are some limitations to consider. As the research was mainly concerned with biochemical and histological indicators, it did not include any evaluation of cognitive function. Additionally, behavioral evaluations should be included into future research in order to figure out whether or not inhibition of RAC1 results in cognitive enhancement. Moreover, there is a need for more research on the long-term effects of inhibiting RAC1 on neurodegeneration.

## Conclusion

This study underscores the role of RAC1 in regulating TNF-A-mediated neuroinflammation in AD. Targeting RAC1 with NSC23766 significantly reduced inflammatory and neurodegenerative markers, proposing its potential as a target for therapy. Future research should explore RAC1 inhibitors for clinical applications in AD management.

## Competing interests

The authors declare that they have no competing interests.

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