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Targeting NLRP3 signaling with a novel sulfonylurea compound for the treatment of vascular cognitive impairment and dementia



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Abstract

Background As a key inflammatory factor, the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome plays a crucial role in neuroinflammation and the progression of neurodegenerative diseases. Dysregulation of NLRP3 signaling can trigger various inflammatory responses in the brain, contributing to the development of neurodegenerative diseases such as ischemic stroke, vascular dementia (VaD), Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Therefore, the NLRP3 signaling pathway is a promising therapeutic target for the treatment of neurodegenerative diseases, including VaD.

Methods In this study, we investigated the therapeutic effects of a synthetic sulfonylurea NLRP3 inhibitor, AMS-17, in a VaD mouse model using bilateral common carotid artery stenosis (BCAS) and elucidated the underlying mechanisms. All mice were randomly divided into three groups: Sham, VaD + Vehicle, and VaD + AMS-17. Cognitive function was assessed using the Y-maze and Morris water maze (MWM) on the 50th day after BCAS. Brain sections and blood serum samples were collected for biomarker analysis and immunohistochemistry. Neurodegeneration, expressions of the molecules involved in the NLRP3 signaling pathways, tight junction proteins, and myelination were assessed using western blotting and immunofluorescence (IF). The levels of Interleukin-1 beta (IL-1 β), Tumor Necrosis Factor-alpha (TNF- α) and Interleukin-4 (IL-4) in the blood were measured using ELISA.

Results AMS-17 treatment improved cognitive function, enhanced blood-brain barrier (BBB) integrity, and promoted remyelination in VaD mice. Additionally, AMS-17 reduced neurodegeneration and decreased the expression of NLRP3 and its associated proteins, Apoptosis-associated speck-like protein (ASC), and cleaved caspase-1 in the brain. It also lowered pro-inflammatory TNF- α and IL-1 β levels, while increasing the anti-inflammatory IL-4 level in the blood.

Conclusions The findings of this study provide the first promising evidence for the use of AMS-17 in VaD treatment in mice. This study introduces AMS-17 as a novel chemical scaffold with NLRP3 inhibitory activity, which can be further developed for the treatment of VaD in humans.

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Introduction

Vascular dementia (VaD) is the second most common form of dementia after Alzheimer's disease among Alzheimer's disease and related dementias (ADRD). VaD is caused by cerebral vascular diseases, hypertension, chronic cerebral hypoperfusion, cerebral ischemia, and post-ischemic inflammation. It also has the highest mortality rate among all forms of dementia. A large cohort study conducted in the U.S. found that, on average, Alzheimer's patients live up to 7.1 years after diagnosis, whereas VaD patients only live an average of 3.9 years [1]. This lower survival rate in VaD may be due to co-existing vascular conditions, such as ischemic and hemorrhagic events. Cerebral ischemic injury leads to neuronal death through various mechanisms, with inflammation being a key contributor to neurodegeneration in VaD. The role of inflammation in VaD is becoming increasingly recognized, as numerous inflammatory cytokines are aberrantly elevated in the brain during VaD [2]. Therefore, targeting neuroinflammatory responses is essential for developing effective treatments for VaD. Despite significant efforts, a clinically effective drug for VaD treatment has yet to be identified. However, much effort has not yielded a clinically useful candidate drug for VaD treatment. Development of effective therapeutics for ADRD represents an urgent and unmet challenge.

The NLRP3-mediated microglial inflammation plays a crucial role in the pathophysiology of VaD. Elevated NLRP3 expression in microglia is implicated in brain injury, neurodegenerative, and neuroinflammatory disorders [3, 4]. NLRP3 activation stimulates caspase- [1] activity, leading to the maturation and release of proinflammatory cytokine IL-1 β [5, 6]. IL-1 β then triggers the expression of inducible nitric oxide synthase (iNOS), further promoting microglial activation, cell injury, and death. Increased amyloid- β (A β) level in VaD serves as danger-associated molecular patterns (DAMPs) that activate NLRP3. Hypoperfusion and the resulting hypoxia in VaD are also shown to promote A β production [2]. Chronic A β deposition in the brain, particularly in the hippocampus, leads to Toll-like receptor-4 (TLR4)mediated microglial activation, upregulating pro-inflammatory cytokines such as TNF-α, IL-1β, Interleukin-6 (IL-6), and Interleukin-18 (IL-18), and contributing to neuronal death [7-9]. Previous studies demonstrated the benefit of disrupting NLRP3 signaling in VaD; for example, Osthole, a natural product with NLRP3 inhibitory activity, was shown to reduce $A\beta$ deposition in a rodent model of VaD [10]. Additionally, MCC950, a small-molecule NLRP3 inhibitor, improved cognitive function and vascular integrity post-stroke [11]. Together, these studies highlight NLRP3 inhibition as a promising strategy for ADRD treatment.

Several compounds, such as isoliquiritigenin, resveratrol, and curcumin, have shown NLRP3 inhibitory activity [12–14]. However, these natural products are unsuitable for drug development due to stability issues [15]. To date, multiple BBB-penetrating small-molecule NLRP3 inhibitors, including RRx-001, Dapansutrile and ZYIL1 have successfully completed Phase II trial [16–19]. However, many of these candidates have drawbacks, such as renal failure and upper airway infections [19]. No NLRP3 inhibitor has yet been successfully applied for dementia treatment. Therefore, it is crucial to find a novel molecule for NLRP3 inhibition for developing effective treatment of ADRD. In the search for novel NLRP3 inhibitors, we synthesized a collection of sulfonylurea compounds designed to eliminate the instability seen in their natural counterparts while maintaining a similar three-dimensional structure (20). Our previous study showed that the newly developed sulfonylurea compound AMS-17 had anti-inflammatory effects by inhibiting expressions of NLRP3, its downstream components and cytokines, such as caspase-1, TNF- α , IL-1 β and iNOS in microglial cells [21]. In the present study, we further evaluated the efficacy of AMS-17 in improving cognitive function and its associated molecular changes in a mouse model of VaD to investigate the underlying mechanisms.

Materials and methods

Synthesis of AMS-17 and Preparation of AMS-17 solution AMS-17 was synthesized using a modification of our previously reported procedure for the synthesis of tertiary sulfonylurea compounds (Scheme 1) [22]. It began with the reaction of pyrimidin-5-amine with 3-chloropropyl isocyanate resulting in the formation of acyclic urea. The latter was subjected to deprotonation with excess sodium hydride to affect the intramolecular ring closure. Quenching the reaction with aryl sulfonyl chloride 5 afforded AMS-17 as a white solid. NMR (1 H and 13 C), FT-IR and ESI-MS mass spectra for the synthetic sample of AMS-17 were consistent with the proposed structure. For the preparation of the AMS-17 solution, 1 mg of AMS-17 powder was mixed with 1 mL of DMSO, yielding a stock concentration of 28.47 mM. This stock solution was subsequently diluted with 1X phosphatebuffered saline (PBS) to achieve the desired working concentrations.

Experimental animals and VaD establishment

The study was carried out in compliance with ethical guidelines set by the University of Houston's Institutional Animal and Use Committees (IACUC) and the National Research Council's *Guide for the Care and Use of Laboratory Animals*. The University of Houston is fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Briefly, 10-monthold male C57BL/6J mice were obtained from Charles River Laboratories, housed individually, and maintained on a 12-hour light cycle with food and water available ad

libitum at 23 °C. Mice were allowed to acclimate for at least one week before experimental procedures began. Mice were randomly assigned to three groups: Sham, VaD, and Treatment. The VaD group underwent bilateral common carotid artery stenosis (BCAS) surgery, involving the placement of microcoils around both common carotid arteries [23]. The treatment group underwent the same BCAS procedure and was administered either 20 mg/kg AMS-17 or Vehicle for five consecutive days. The sham group underwent carotid artery exposure without stenosis. Please refer to Supplementary Fig. 1 for an overview of the entire workflow.

Cerebral blood flow (CBF) quantification

Mice were subjected to sterile procedures. A cranial window was created approximately 1–1.5 mm anterior to the bregma and 0–3 mm lateral to the midline. A laser Doppler flowmetry probe (Moor Instruments, UK) was then secured at the center of this cranial window to measure CBF. Measurements of CBF were taken at intervals of 1, 3, 5, 7, and 60 days following the surgery.

Y-Maze

The Y-maze spontaneous alternation performance test was conducted on the 50th day after BCAS (Fig. 1A) to assess novel environment recognition. The Y-maze apparatus consists of three identical arms spaced 120 degrees apart, designated as the start, novel, and other arms. The test comprised two phases: a training phase and a testing phase. During the 8-minute training phase, animals were allowed to explore two arms (start and other arms), while the novel arm was blocked. After a 1-hour interval, animals were placed in the start arm and allowed to explore all three arms for 8 min during the test phase. The number of entries into the novel arm was recorded. To prevent olfactory cues, the arms were cleaned with 70% ethanol between tests.

Morris water maze

On the 53rd day after BCAS induction, mice underwent the Morris water maze test (Fig. 1A). This test consisted of a training session and a probe test session, during which the mice were placed in a circular swimming pool and allowed to explore for a maximum of 60 s. The test



Scheme 1 Chemical Synthesis of AMS-17



Fig. 1 AMS-17 improves CBF and memory function in VaD mice. (**A**). Temporal pattern in CBF for Sham, VaD + Vehicle and VaD + AMS-17 mice at different time points. (**B**-**D**) Changes in CBF following VaD at: (**B**) day 5, (**C**) day 7, and (**D**) day 60. (**E**) Number of times mice entered the novel arm during the testing phase in the Y-Maze. (**F**) Representative track map of mice during the testing phase of the Y-maze. (**G**) Latency to platform in MWM during training days for Sham, VaD + Vehicle and VaD + AMS-17 mice. (**H**). Number of times mice entered the platform quadrant during the probe trial. (**I**) Representative track map of mice on Day-5. (Mean \pm SD, **p<0.005, ***p<0.005, ****p<0.001, n = 6-8 animals per group)

was conducted four times a day for five consecutive days, with the probe test taking place on the sixth day. The movements of the mice were tracked using Ethovision software, and the water temperature was kept around 25 °C. A non-toxic white paint was added, and the mice were dried with a towel and any fecal matter was removed from the water after each session.

Samples collection

After the final behavior tests on day 60 post-BCAS, brain samples were collected. The left hemisphere was stored at -80 °C and cryosectioned for immunohistochemical analysis. The right hemisphere cortex was separated, solubilized in RIPA buffer, and homogenized with a Bead Blaster. Samples were centrifuged at 2000 g for 15 min at 4 °C, and supernatants were collected and stored at -20 °C for Bradford assay and western blot analysis. Blood samples were also collected in EDTA tubes and centrifuged at 2000 g and 4 °C for 10 min to obtain plasma for cytokine measurement.

Immunofluorescence staining

Mouse brain tissues were fixed with 4% paraformaldehyde (PFA) in PBS at pH 7.4, cryosectioned into 8 μ m slices, and permeabilized with 0.1% Triton-X 100 for 30 min. Brain slices were blocked with 5% bovine serum albumin (BSA) for 1 h and incubated with primary antibodies (Supplementary Table 1) overnight at 4 °C. Secondary antibody incubation followed the next day for 1 h (Supplementary Table 3), after which slices were mounted with DAPI antifade media. TUNEL and Fluoro Jade C (FJC) staining were performed according to the instructions of manufacturer (TUNEL; Catalog #30063, Biotium, USA & Fluoro Jade C; Catalog TR-100-FJ, Biosensis, USA). Images were acquired using a Zeiss LSM 710 microscope.

Analysis and quantitation of images

Images were acquired at consistent exposure times and analyzed using automated ImageJ (NIH) software. Following TIFF export and 8-bit conversion, images underwent edge detection, sharpening, thresholding, and water shedding. Normalization was achieved using ImageJ's set scale function. Results presented as percentage of positive cells relative to total cells. Fiber length of myelinated axons was analyzed using the ImageJ plugin DiameterJ [24, 25]. First, images underwent segmentation by subtracting the background (Process > Subtract background) and applying a threshold to isolate MBP+staining from the background. Images were converted to 8-bit format, and noise/small particles were removed. Subsequently, DiameterJ was used to measure the fiber length of myelinated axons. For each mouse specimen, three cortical images from regions of interest (Fig. 2C) were acquired. All analyses were conducted in a blind fashion.

Western blotting

Protein concentrations were measured using a colorimetric Bradford assay kit (Biorad, USA), following the manufacturer's protocol. Cortical lysates were solubilized in sodium dodecyl sulfate (SDS) sample buffer at 40 µg per lane and subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 110 min. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% BSA for 1 h, and incubated with primary antibodies overnight at 4 °C (Supplementary Table 2). Membranes were incubated with an HRP-conjugated secondary antibody for 1 h (Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP, Invitrogen, CAT #31460) and visualized using Super Signal[™] West Pico PLUS Chemiluminescent Substrate (Cell Signaling, USA). Bands were imaged using a Bio-Rad Chemidoc Touch imaging system, and band density was quantified using Image Lab 6.1 software (Bio-Rad, USA). GAPDH and HSP-70 were used as internal controls for normalization, ensuring equal protein loading across lanes. Raw western blot bands are shown in the Supplementary file.

ELISA

Cytokine concentrations (TNF- α , IL-1, and IL-4), Liver enzymes (ALT, AST) and kidney function markers (BUN, Creatinine) in the blood were measured using ELISA colorimetric kits. Details of the kits with catalog number, limit and range of detection are provided in Supplementary Table S4. Briefly, the 96-well microplates were coated with a capture antibody overnight at 4 °C and washed the wells 4 times and then added a Diluent for 1 h and washed the wells again. The plate was then incubated with antibodies for 2 h, and washed 4 times afterwards, and incubated with streptavidin-HRP conjugate for 30 min. Finally, TMB solution was added for color development and the absorbance was measured at 450 nm.

Statistical analysis

Image analysis was performed using Fiji (ImageJ), an open-source image processing software (National Institutes of Health, Bethesda, MD, USA). Significant differences between and within multiple groups were examined using one-way and ANOVA test, followed by Tukey's multiple comparison test. Two-way ANOVA was employed to analyze escape latency times in the MWM test. The data from all groups met the assumptions of normality (Shapiro-Wilk test or D'Agostino-Pearson omnibus). Data are presented as means ± SD. To further quantify the magnitude of treatment effects, we calculated the generalized eta squared (η^2) values. This measure estimates the proportion of variance in the dependent variable explained by the independent variable. With the help of Prism software, η^2 was computed as the sum of squares (SS) for treatment effects divided by the sum of SS for treatment effects and SS for residual variation. η^2 values ≥ 0.14 were considered to represent large effects. A p-value of < 0.05 was considered significant. GraphPad Prism software was utilized for all statistical analyses.

Results

AMS-17 improves CBF and cognitive impairment in a VaD mice

CBF analysis revealed a steady decline in the VaD + Vehicle group, with a sharp drop on the day VaD was induced, reaching 47.33% of Sham levels (Fig. 1A). This decline persisted, with CBF remaining at 55.74% of Sham levels by day 5. In contrast, AMS-17 treatment led to significant CBF recovery, starting at day 5 post-VaD, with CBF reaching 72.06% of Sham levels (Fig. 1B; η^2 =0.9733). By day 7, VaD + AMS-17 cohort showed further improvement to 79.26% of Sham levels, compared to only 59.03% in the VaD + Vehicle group (Fig. 1C; η^2 =0.9844). Persistent CBF deficits were evident in the VaD + Vehicle group at 60 days (70.7% of Sham), whereas the AMS-17-treated mice achieved near-normalization on day 60 (93.21% of



Fig. 2 AMS-17 reduces neuron degeneration and fibrinogen deposition in the cortical regions in VaD mice. (**A**) Representative IF images of FJC-positive cells (green signals) indicating neuronal degeneration, stained with DAPI (blue signals). (**B**) Quantification of FJC-positive cells. (**C**) Region of interest (indicated by black squares) that was imaged in the cortical area (Created with BioRender.com). (**D**) Representative IF images for fibrinogen-positive cells, counterstained with CD31 and DAPI. (**E**) Quantification of merged fibrinogen and CD31-positive cells. Data are presented as mean \pm SD (n = 6) and analyzed by one-way ANOVA and Tukey's multiple comparison tests. ***p<0.0005, ****p<0.0001

Sham; Fig. 1D; η^2 =0.9434). These findings indicate that AMS-17 accelerated and enhanced CBF restoration in VaD mice, with recovery detectable as early as day 5.

The Y-Maze test assesses spatial memory and learning. During the testing phase, the number of entries into the novel arm was recorded. The mice in VaD with Vehicle group showed impaired memory function, with an average of 1.83 entries into the novel arm. In contrast, mice treated with AMS-17 showed significant improvement in learning and memory with an average of 4.66 entries into the novel arm, which was closer to the performance of the sham mice (5.75) (Fig. 1E; η^2 =0.4789). These results indicate that AMS-17 treatment improves learning and memory functions in mice subjected to VaD.

Furthermore, the Morris water maze (MWM) was performed to evaluate cognitive functions. The results indicated that mice in the sham and AMS-17-treated VaD groups learned to reach the platform significantly faster than those in VaD with Vehicle group during the training phase (Fig. 1G; η^2 =0.64). The average escape latency in sham group decreased from 47.85 s on day 1 to 18.01 s on day 5. Similarly, in the AMS-17-treated VaD group, the average escape latency decreased from 48.02 s on day-1 to 15.09 s on day-5. However, the average escape latency of mice in VaD with Vehicle group did not change significantly during the 5-day training period, remaining at 45.16 s on day-5. A significant reduction in escape latency was observed in the sham group, indicating normal learning and memory functions, while the VaD group showed impaired learning and memory, as evidenced by a lack of significant decrease in escape latency. AMS-17 treatment significantly reduced escape latency in VaD mice, indicating a recovery of learning and memory functions (Fig. 1G). To further evaluate spatial memory, a 30-second spatial probe trial was conducted on day-6 following the training trial. The platform was removed from the pool, and the average frequency of mice entering the platform quadrant was recorded. The frequency of entering the platform quadrant (2.2) was significantly higher in the AMS-17-treated VaD group, closer to that of the sham mice (2.8), compared to the VaD with Vehicle group, which had a frequency of just 0.8 (Fig. 1H, η^2 =0.6088). The MWM results further indicate that AMS-17 treatment improves learning and memory functions in mice subject to VaD.

AMS-17 protects against neurodegeneration in VaD mice

We assessed the extent of neurodegeneration in the brains of VaD mice using Fluoro-Jade C (FJC) staining. The number of degenerating neurons (FJC-positive cells) in the brain cortex area was detected and compared across different groups. The results revealed a minimal percentage of FJC-positive cells in the cortical area of the brain in the sham group, at 1.3%. In contrast, this percentage increased to 26.94% in VaD with Vehicle group. Importantly, AMS-17 treatment significantly reduced the percentage of FJC-positive cells to 19.61% (Fig. 2A & B; η^2 =0.9569). The results of FJC staining demonstrate that AMS-17 treatment protects neurons from degeneration and exerts a neuroprotective effect in VaD conditions.

AMS-17 protects the BBB in VaD mice

BBB integrity was evaluated by measuring fibrinogen deposition, a marker of BBB leakage, in the brain using immunostaining. We co-stained fibrinogen with CD31, a marker for endothelial cells, which line the blood vessels. The results showed that only 4.01% of cells were fibrinogen-positive in sham mice (Fig. 2D & E; η^2 =0.8752). In VaD with Vehicle group, the percentage of fibrinogen-positive CD31 cells increased to 18.72%, but AMS-17 administration lowered this to 11.38% (Fig. 2D & E). Additionally, we assessed the tight junction proteins, Occludin and claudin-5. Our study revealed that VaD induction significantly decreased occluding-positive

CD31 cells from 19.42% (Sham) to 5.41% (VaD with Vehicle group). Importantly, AMS-17 treatment partially restored Occludin expression to 10.04% (Fig. 3A & B; η^2 =0.8959). Consistently, western blot analysis of cortical lysates from the same mice showed that AMS-17 increased Occludin expression from 0.22-fold in VaD with Vehicle group to 0.75-fold in the treatment group, relative to the sham group (Fig. 3C & D; η^2 =0.8393). A similar pattern was observed with claudin 5. As shown in Fig. 3E & F, the sham group had 25.42% claudin 5-positive CD31 cells, while in Vehicle-treated VaD group, the percentage significantly decreased to 10.16%. However, AMS-17 treatment increased claudin 5-positive CD31 cells to 15.44% (Fig. 3E & F; η^2 =0.8950). Western blot analysis further confirmed that claudin 5 expression decreased to 0.37-fold in VaD with Vehicle group compared to the sham group, but AMS-17 treatment restored claudin 5 expression to 0.59-fold (Fig. 3G & H; η^2 =0.8880). These findings indicate that AMS-17 reduced BBB leakage by promoting the expressions of tight junction, thereby helping maintain BBB integrity.

AMS-17 enhances remyelination in VaD mice

Demyelination is a key pathological feature in the brain of VaD and contributes to cognitive impairment. To study this, we assessed myelin basic protein (MBP) as a marker of myelin integrity and quantified fiber length to evaluate the complexity of myelination patterns in the mouse cortex, a sensitive indicator of myelination [25]. Our results show that, compared to the sham group (5965.24) μ M), the average fiber length in VaD mice treated with Vehicle was significantly reduced to 2385.7 µM (Fig. 4A & B η^2 =0.9725). In contrast, AMS-17 treatment substantially increased average fiber length in VaD mice to 4116.3 µM (Fig. 4A & B), indicating improved myelination in the brain. Next, we conducted IF analysis on the same cohort of brains to quantify oligodendrocyte progenitor cells (OPCs), identified by NG2 + labeling, across all mouse groups. Our study revealed that VaD induction significantly decreased NG-2 OPCs from 63.73% (Sham) to 16.01% (VaD with Vehicle group; Fig. 4C & D). Importantly, AMS-17 treatment partially restored NG-2 expression to 42.48% (Fig. 4C & D; η^2 =0.9705). All together, these findings indicate that AMS-17 has a potential role in suppressing demyelination in the VaD brain.

AMS-17 suppresses expressions of NLRP3 and its associated proteins in VaD mice

To elucidate the underlying mechanism of AMS-17 in VaD treatment, we investigated its effect on the NLRP3 pathway. We hypothesized that AMS-17 inhibits NLRP3 and its associated proteins, ASC and cleaved caspase-1 expressions. To test this, we measured the protein levels



Fig. 3 AMS-17 increases tight junction protein expressions in the cortical regions of the brain in VaD mice. Representative IF images of (**A**) Occludin and (**E**) Caludin 5 positive cells, both counterstained with CD31 and DAPI. Quantification of (**B**) Occludin, (**F**) Claudin 5 -positive cells from IF. Representatives immunoblot for (**C**) Occludin and (**G**) Caludin 5 from cortical lysates of mice. (**D**) Quantification of (**D**) Occludin and (**H**) Caludin 5 from blots. Data are presented as mean \pm SD (n=6) and analyzed by one-way ANOVA and Tukey's multiple comparison tests. *p<0.05, **p<0.005, ***p<0.0001

of NLRP3, ASC, pro-caspase-1 and cleaved caspase-1 through western blotting of cortical brain homogenates and immunostaining of frozen brain sections collected on day 60 after VaD induction. As expected, NLRP3 was expressed in microglia, as indicated by double IF staining of NLRP3 with CD68. IF analysis of mouse brain cortex revealed that only 1.5% of microglia (CD68-positive cells) expressed NLRP3 in sham mice (Fig.5A & B; η^2 =0.9654). In the Vehicle-treated VaD group, the percentage of microglia expressing NLRP3 increased to 47.21%, but treatment of AMS-17 lowered this to 31.58% (Fig. 5A & B). We also examined ASC and cleaved caspase 1 expression. VaD induction increased the expression of ASC-positive CD68 cells from 1.3% (Sham) to 14.91% (VaD with Vehicle), and AMS-17 reduced the expression of ASC-positive CD68 cells to 7.91% (Fig. 5E & F; η^2 =0.9695). Western blot analysis of mouse cortical lysates revealed that AMS-17 treatment significantly reduced the average expression of NLRP3 from 4.38-fold to 2.43-fold (Fig. 5C & D; η^2 =0.9573), ASC from 4.35fold to 2.09-fold (Fig. 5G & H; η^2 =0.9276), and cleaved caspase-1 from 1.46-fold to 1.23-fold (Fig. 6C & D; η^2 =0.7666) in the AMS-17 treated VaD group compared to the VaD with Vehicle group. Interestingly, we observed NLRP3 expression in neurons, in line with prior research (26). Only 2.51% of neurons (Neu-N-positive cells) expressed NLRP3 in sham mice. But the percentage of

NLRP3-positive neurons increased to 33.75% in VaD with Vehicle group, but the AMS-17 treatment reduced it to 24.91%. (Fig. 6A & B; η^2 =0.9649). We also investigated the molecular mechanism of AMS-17 by measuring the phosphorylation levels of MST1 in the cortex of mice using IF and western blotting. We co-stained P-MST1 with NeuN. Our staining results showed that the percentage of P-MST1-positive neurons increased to 49.23% in Vehicle-treated VaD group, while only 4.2% of neurons were P-MST1-positive in the sham group. AMS-17 treatment significantly reduced the percentage of P-MST1-positive neurons in VaD mice to 36.7% (Fig. 6E & F; η^2 =0.9690). Western blot data further confirmed these changes in P-MST1. Compared to the sham group, the expression of P-MST1 was 3.55-fold higher in the VaD with Vehicle group. Interestingly, AMS-17 treatment reduced the P-MST1 level to 1.72-fold (Fig. 6G & H; η^2 =0.9384). These findings indicate that AMS-17 treatment inhibits NLRP3 pathway activation and neuroinflammation in VaD, while also modulating the Hippo-MST1 signaling pathway to exert neuroprotective effects.

AMS-17 treatment modulates inflammatory and antiinflammatory cytokine level in VaD mice

We have assessed the levels of various cytokines, IL-1 β , TNF- α (both downstream products of NLRP3 activation and key drivers of inflammation), IL-4 (a key



Fig. 4 AMS-17 enhances myelination in VaD mice. (A) Representative IF images of MBP (myelin marker) with NeuN and DAPI. (B) Quantification of fiber length. (C) Representative IF images of NG2-positive cells (green signals) indicating OPC marker, stained with DAPI (blue signals). (D) Quantification of NG2-positive cells. Data are presented as mean \pm SD (n=6) and analyzed by one-way ANOVA and Tukey's multiple comparison tests. ****p<0.0001

anti-inflammatory cytokine) in the serum samples of mice using ELISA. Our findings revealed that TNF- α level was significantly elevated in the Vehicle-treated VaD group (138.39 pg/ml), showing a 6.6-fold increase compared to the sham group (20.94 pg/ml). AMS-17 treatment reduced TNF- α levels to 60.58 pg/ml, representing a 1.8-fold decrease compared to the Vehicle-treated VaD group, indicating significant attenuation of TNF- α expression (Fig. 7A; η^2 =0.9213). We also found that AMS-17 significantly reduced systemic IL-1 β levels from 107.48 pg/ml in the Vehicle-treated VaD group to 59.57 pg/ml in the AMS-17-treated group, representing a 1.8-fold reduction (Fig. 7B; η^2 =0.9874). In addition to

evaluating pro-inflammatory cytokines, we examined the levels of the anti-inflammatory cytokine IL-4 in the serum to better understand the immune response. Our results showed that AMS-17 treatment significantly increased IL-4 levels in VaD mice, boosting IL-4 level by 2.1-fold, from 27.86 pg/ml in the Vehicle-treated VaD group to 59.71 pg/ml in the AMS-17-treated group (Fig. 7C; η^2 =0.9868). Collectively, these data show that AMS-17 not only reduces pro-inflammatory cytokines but also promotes the production of the anti-inflammatory cytokine IL-4, exerting anti-inflammatory effects.



Fig. 5 AMS-17 inhibits microglial NLRP3 and ASC expression in the cortical regions of the brain in VaD mice. Representative IF images of (**A**) NLRP3 and (**E**) ASC positive cells, both counterstained with CD68 and DAPI. (**B**) Quantification of (**B**) NLRP3 and (**F**) ASC -positive cells from IF. Representatives immunoblot for (**C**) NLRP3 and (**G**) ASC from cortical lysates of mice. Quantification of (**D**) NLRP3 and (**H**) ASC expressions from blots. Data are presented as mean \pm SD (n = 6) and analyzed by one-way ANOVA and Tukey's multiple comparison tests. ****p<0.0001



Fig. 6 AMS-17 inhibits neuronal NLRP3, Cleaved caspase-1 and P-MST1 expression in the cortical regions of the brain in VaD mice. Representative IF images for (A) NLRP3 and (E) P-MST1 positive cells counterstained with NeuN and DAPI. Quantification of (B) NLRP3 and (F) P-MST1 -positive cells costained with NeuN. Representatives immunoblot for (C) Pro-caspase-1, Cleaved caspase-1 and (G) P-MST1 from cortical lysates of mice. Quantification of (D) Cleaved caspase-1 and (H) P-MST1 expressions from western blot. Data are presented as mean \pm SD (n=6) and analyzed by one-way ANOVA and Tukey's multiple comparison tests. (***p<0.0005, ****p<0.0001)



Fig. 7 Effects of AMS-17 treatment on cytokine production and liver & kidney function. (A-C) Serum cytokine levels: (A) TNF-a, (B) IL-1β, and (C) IL-4. (D) Body weight measurements. (E-H) Kidney and liver function assessments: (E) BUN, (F) creatinine, (G) ALT, and (H) AST. Data are presented as mean ± SD (n=6) and analyzed by one-way ANOVA and Tukey's multiple comparison tests., ns = not significant, ****p<0.0001

AMS-17 treatment shows no adverse effects on liver or kidney function

Given the widespread expression of the NLRP3 inflammasome and its components across multiple organs, evaluating the systemic impact of our NLRP3 inhibitor, AMS-17 treatment on major organs is critical. As an initial evaluation, body weights of mice across all experimental groups were monitored at 1, 3-, 5-, 7-, and 60-day post-treatment. No statistically significant differences in body weight were observed between groups at any time point (Fig. 7D; η^2 =0.32). To assess kidney-specific effects of AMS-17, blood urea nitrogen (BUN) (Fig. 7E, η^2 =0.0003678) and creatinine (Fig. 7F, η^2 =0.003407) levels were analyzed, both of which showed no significant alterations across all the groups. Liver function was evaluated by measuring serum alanine aminotransferase (ALT) (Fig. 7G, η^2 =0.2252) and aspartate aminotransferase (AST) (Fig. 7H, η^2 =0.005847) levels, which similarly exhibited no significant changes in AMS-17-treated mice compared to controls. Collectively, these data indicate that in vivo administration of AMS-17 at the tested dose and duration did not induce detectable abnormalities in kidney or liver function, nor did it affect systemic physiological parameters such as body weight.

Discussion

Targeting NLRP3 signaling pathway to inhibit brain inflammation could be effective for treating ADRD and neurodegenerative diseases Although some NLRP3 inhibitors have been tested in clinical trials for inflammatory diseases, none have been successful in treating ADRD. AMS-17, a novel synthetic sulfonylurea compound, was developed using pharmacophore modeling and computational chemistry, structurally derived from isoliquiritigenin [20]. Our previous studies showed that AMS-17 reduced NLRP3 along with pro-inflammatory markers (caspase-1, IL-1 β , and TNF- α) in N9 microglia cells and inhibited LPS-induced microglial activation in mouse brains [21]. Based on these findings, we proposed that AMS-17 has therapeutic effects in VaD by inhibiting the NLRP3 pathway.

In this study, we first investigated CBF change in VaD mice induced by BCAS surgery. Consistent with previous reports [27], we observed reduced CBF in the VaD + Vehicle group. Furthermore, we found that AMS-17 treatment led to significant CBF improvement as early as day 5 (Fig. 1B). This enhanced perfusion recovery likely contributes to the observed reductions in cognitive impairment, neurodegeneration, and neuroinflammation. Recent mechanistic insights implicate that NLRP3-IL-1 β signaling pathway exacerbate vascular dysfunction by destabilizing endothelial NO synthase, thereby impairing vasodilatory capacity [28-30]. Prior investigations from our group revealed that AMS-17 suppresses inducible NO synthase expression providing further evidence regarding this aspect [21]. Therefore, inhibition of NLRP3 by AMS-17 improves CBF recovery after BCAS may be due to the inhibition of NLRP3/IL-1β-driven endothelial NO synthase dysregulation and restoration of vascular tone. Next, we investigated if AMS-17 has therapeutic effects on cognitive functions in VaD mice, as described previously [23]. The BCAS model, which induces chronic ischemia through bilateral common carotid artery, mimics VaD and impairs spatial memory and learning abilities, as evidenced by Y-Maze and MWM tests. However, AMS-17 treatment (20 mg/kg for 5 consecutive days) significantly improved cognitive functions in VaD mice (Fig. 1). Since cognitive improvement is associated with reduced neuronal death and degeneration, we further investigated histological and molecular evidence to elucidate the mechanism of action of AMS-17. As expected, FJC staining revealed that AMS-17 reduced the number of degenerating neurons in the cortex of VaD mice. In addition, AMS-17 treatment reduced BBB leakage and maintained BBB integrity by promoting the expression of tight junction proteins. AMS-17 also enhanced myelin repair in VaD mice, as shown by MBP and NG-2 expression recovery. Having demonstrated therapeutic effects at the behavioral and histological levels, we further explored the molecular pathways involved in AMS-17's protective effects. We observed upregulation of NLRP3 and its associated proteins, ASC and cleaved caspase-1, in the cortex of VaD mice. AMS-17 treatment effectively inhibited the expression of these proteins. Although the initial understanding of the roles of NLRP3 signaling is in immune response cell, microglia, recent studies have expanded the knowledge of NLRP3 in neurons and other cell types in the brain suggesting a more widespread and complex role for NLRP3 in the brain [31]. Peng's group showed that NLRP3 can spread from microglia to nearby neurons and endothelial cells, perpetuating inflammation and damage. Our findings confirm that NLRP3 is expressed in both microglia and neurons, and that AMS-17 decreases the expression of NLRP3, ASC, and cleaved caspase-1. Our results align with previous studies using the same VaD model, which showed activation of NLRP3, caspase-1, and IL-1 β in the brain during VaD [32, 33].

Preclinical studies have robustly demonstrated that IL-1 β and IL-18 are key drivers of the inflammatory response that occurs in the context of VaD [32, 33]. We hypothesized that the anti-inflammatory effect of AMS-17 is not only reflected in the brain but also should be shown in peripheral tissue. Indeed, pro-inflammatory cytokines TNF- α and IL-1 β were elevated in the blood of VaD mice, while anti-inflammatory cytokine IL-4 was significantly reduced compared to normal mice. AMS-17 modulated cytokine levels in blood showing as a reduction of pro-inflammatory TNF- α and IL-1 β level, while an increase of anti-inflammatory IL-4 level in blood. These results suggest a potential disruption in the balance between pro- and anti-inflammatory responses in VaD. Remarkably, AMS-17 nullified these detrimental changes in pro- and anti-inflammatory cytokines and resumed the balance of pro- and anti-inflammatory cytokines resulting in protective effects on the brain.

The activation of NLRP3 leads to microglial activation, leukocyte recruitment, and BBB disruption [34, 35]. Consistent with previous findings [36, 37], we observed BBB disruption characterized by decreased expression of tight junction proteins Occludin and claudin 5 and increased fibrinogen expression in VaD mice. Furthermore, recent studies have revealed that fibrinogen plays a crucial role in activating the microglial NLRP3 inflammasome [38]. The implications of this mechanistic study suggest that fibrinogen-driven NLRP3 activation contributes to BBB dysfunction. Recent studies have demonstrated that IL-1β, a key downstream effector of the NLRP3 signaling pathway and plays a crucial role in disrupting the integrity of the BBB [39] highlighting the importance of NLRP3 pathway in regulating BBB function. Suppressing NLRP3 activity by AMS-17 used in the present study has shown its protective effects on BBB integrity in VaD mice. In addition, accumulating evidence indicate that NLRP3 inflammasome plays important roles in demyelination after injury [40, 41]. IL-1 β has been shown to hinder relocation of oligodendrocyte and increase myelin repair in VaD mice [42]. On the contrary, reducing IL-1 β secretion has been found to repair myelin formation remyelination in VaD mice model [32]. Pharmacological inhibition of NLRP3 and caspase-1/IL-1β production has been shown to prevent myelin loss [43–45]. Intriguingly, our findings revealed aberrant myelination and decreased OPCs in the VaD brains indicating a disruption in normal brain function, and inhibition of NLRP3 by AMS-17 restored myelination and OPCs. The Hippo pathway plays a crucial role in neurodegenerative processes, particularly

MST1 signaling, which is implicated in microglial activation following ischemic injury [46]. While the Hippo pathway regulates NLRP3 activation in liver ischemia/ reperfusion injury [47], its relationship with NLRP3 in the brain remains unexplored. Increase of NLRP3 activity and P-MST1 were observed in inflammatory and apoptotic conditions and lead to cell death [48, 49]. In the present study, our findings reveal there are increased levels of NLRP3 and P-MST1 expressions in cortical tissue, while MST1 levels remained unchanged in Vehicle treated VaD mice brain. AMS-17 treatment inhibits P-MST1 to protect neurons and BBB from injury. The results also imply potential crosstalk between NLRP3 and Hippo-MST1 pathway in VaD, highlighting the intricate mechanisms of this disease.

A critical component in pharmacological development involves rigorous evaluation of therapeutic safety, particularly regarding adverse effects. NLRP3-targeting agents, while promising, carry inherent risks such as renal toxicity and increased susceptibility to infections. In this investigation, AMS-17 was administered IP at a dose of 20 mg·kg⁻¹ body weight once daily over five consecutive days. Biochemical analyses revealed no statistically significant alterations in hepatic or renal functional markers, confirming the absence of detectable organ damage at this dosage. These collective findings indicate that short-term AMS-17 treatment at the tested concentration effectively mitigates VaD-associated neuropathology in murine models without compromising systemic organ integrity.

The VaD model used in this study previously revealed sex-dependent cerebrovascular dynamics, with male and female mice exhibiting distinct responses to chronic cerebral hypoperfusion [50]. Notably, male mice demonstrated prolonged CBF deficits compared to females [50], a pattern congruent with epidemiological data showing elevated vascular risk including susceptibility to VaD and major cardiac events in human males [51–53]. By focusing on males in the present study, we aimed to minimize confounding variables and prioritize mechanistic clarity.

Future studies should address how sex-specific disparities in CBF regulation within the current VaD model influence the progression of cognitive deficits, given their translational relevance to VaD pathogenesis. Moreover, the safety and efficacy of prolonged use or higher doses require further exploration to fully characterize the compound's therapeutic window. Subsequent studies should also prioritize evaluating AMS-17 in female mice to address sex-specific differences in VaD progression and therapeutic response, while comprehensive pharmacokinetic profiling including dose rationale (20 mg·kg⁻¹), plasma concentration kinetics (half-life, Cmax, AUC), and administration route optimization will clarify its bioavailability. Critical assessment of BBB penetrability, using methods such as in situ perfusion and brain-toplasma LC-MS ratios, will determine whether AMS-17 crosses intact or pathologically altered BBBs, directly informing its neurotherapeutic potential.

Conclusion

In conclusion, AMS-17 was able to improve cognitive functions, reduce neuronal degeneration and death, mitigate damage, and promote myelin repair in a VaD mouse model. The therapeutic effects of AMS-17 are associated with inhibition of NLRP3 and Hippo pathways, as well as suppression of inflammation. The molecular mechanism of action involves AMS-17 inhibiting the NLRP3 and Hippo pathways, which suppresses inflammation and apoptosis induced by chronic ischemic injury. Further studies are needed to investigate the safety and pharmacokinetic profiles of AMS-17 in vivo and to elucidate its mechanism of action in greater detail. This study provides evidence supporting the potential therapeutic application of NLRP3 inhibitors for VaD, with AMS-17 emerging as a promising therapeutic candidate for its treatment.

Abbreviations

AAALAC	American Association for the Accreditation of
	Laboratory Animai Care
	Alzheimer's disease and related demontion
AURU	Alzheimer's disease and related dementias
ALS	Amyotrophic lateral scierosis
ALI	Alanine aminotransferase
ANOVA	Analysis of Variance
ASC	Apoptosis-associated speck-like protein
AST	Aspartate aminotransferase
Αβ	Amyloid-β
BBB	Blood-brain barrier
BCAS	Bilateral common carotid artery stenosis
BSA	Bovine serum albumin
BUN	Blood Urea Nitrogen
CBF	Cerebral Blood Flow
CD31	Cluster of differentiation 31
CD68	Cluster of Differentiation 68
CNS	Central nervous system
DAMPs	Danger-associated molecular patterns
DAPI	4'.6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
FLISA	Enzyme-linked immunosorbent assay
ESI-MS	Electrospray ionization mass spectrometry
FIC	Eluro-lade C
FT-IR	Fourier transform infrared spectroscopy
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HRP	Horseradish peroxidase
HSP-90	Heat Shock Protein 90
	Institutional Animal Care and Use Committees
IF	Immunofluorescence
и IaG (Н±1)	Immunorlabulin G (beavy chains + light chains)
IGG (IT + L) II_1R	Interleukin-1 beta
ПЛ	Interleukin 4
IL-4 II 6	Interleukin-4
IL-0 II 19	Interleukin-0
	Interieukiii-io Inducible pitric evide supthace
	Muclinetian (Muclin Pasis Protoin
	Myelination/Myelin basic Protein
MBP	Myelin Basic Protein
	Manageralian Charila 20 lika kinasa 1
	iviammalian Sterile 20-like kinase l
MWWM	Morris water maze
NeuN	Neuronal nuclear protein

NLRP3	Nucleotide-binding oligomerization domain (NOD)-like
NMR (1H and 13C)	Nuclear magnetic resonance (Proton and Carbon)
OPC	Oligodendrocyte Progenitor Cells
P-MST1	Phosphorylated Mammalian Sterile 20-like kinase 1
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation Assay
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TLR4	Toll-like receptor-4
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-a	Tumor Necrosis Factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end
	labeling
US	The United States
V	Voltage
VaD	Vascular dementia
VAD	Vascular contributions to cognitive impairment and
	dementia
YAP	Yes-Associated Protein
YAP-TAZ	Yes-Associated Protein-transcriptional co-activator with PDZ binding motif

Supplementary Information

The online version contains supplementary material available at https://doi.or g/10.1186/s12987-025-00665-6.

Supplementary Material 1

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Author contributions

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Data availability

No datasets were generated or analysed during the current study.

Declaration

Compliance with ethical standards

All animal procedures were approved by the Institutional Animal Care and Use Committees at University of Houston, and they complied with pertinent NIH guidelines for care and use of animals.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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