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Gedunin Inhibits Oligomeric $A\beta_{1-42}$-Induced Microglia Activation Via Modulation of Nrf2-NF-κB Signaling

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder and the leading cause of dementia in aged populations worldwide. The deposition of toxic protein aggregates such as amyloid beta (Aβ) is a hallmark of AD, and there is growing awareness that a key driver of AD pathogenesis is the neuroinflammatory cascade triggered and sustained by these proteins. Consequently, interventions that suppress prolonged neuroinflammation represent viable therapeutic approaches for AD. In this context, we tested the natural product gedunin which is an anti-inflammatory molecule, found in the seeds of the neem tree (*Azadirachta indica*), whose mechanism of action remains to be fully elucidated. Using a mouse microglia cell line (IMG), we show that gedunin suppresses neuroinflammation arising from $A\beta_{1-42}$ oligomer exposure. Our results demonstrate that gedunin suppresses $A\beta_{1-42}$-induced NF-κB activation and its targets, including nitric oxide (NO) and IL-1β, known proinflammatory molecules. Further, we show that gedunin inhibits neuroinflammation by activating nuclear factor 2 erythroid–related factor 2 (Nrf2) and its downstream targets γ-glutamylcysteine synthetase, heme oxygenase 1, and NADPH quinone dehydrogenase 1, which are involved in quenching reactive oxygen and nitrogen species (NO) generated by NF-κB activation. Nrf2 activation appears essential for the anti-inflammatory effect because when silenced, the proinflammatory effects of $A\beta_{1-42}$ are enhanced and the protective effect of gedunin against NO production is reduced. Additionally, using human neuronal cells (SH-SY5Y), we show that gedunin prevents neurotoxicity secondary to Aβ-induced microglial activation. In conclusion, our findings highlight a potential therapeutic role of gedunin in neurodegenerative diseases.

Keywords Gedunin · Alzheimer's disease · Oligomeric amyloid beta · Microglial activation · Nrf2 · NF-κB · Neuroinflammation

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder worldwide. Over 5 million Americans are estimated to have AD, with the elderly population over the age of 65 being the primary group affected [1]. With disease progression, histopathological hallmarks of AD become more prominent within the brain. These hallmarks include extracellular amyloid plaques, formed by the deposition of amyloid beta 1–42 ($A\beta_{1-42}$) peptides outside of affected neurons, and intracellular neurofibrillary tangles (NFTs), stemming from the accumulation of hyperphosphorylated tau proteins within neurons. The accumulation of amyloid plaques and NFTs leads to neurodegeneration in the hippocampus and cortex, resulting in cognitive deficits including diminished memory, attention, and visuospatial skills [2]. This is accompanied by the activation of various glial populations within the brain, particularly the microglia [3].

Activation of microglial cells, the resident immune macrophage-like cells in the brain, is beneficial during acute infection or toxic insult through neuroprotective clearance of damaged cells [4]. However, in the presence of ongoing, progressive brain damage, they can become chronically activated, resulting in a sustained aberrant inflammatory response. In this case, microglial cells continue to secrete factors including proinflammatory cytokines and reactive oxygen and nitrogen

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species that are toxic to neurons [5] and, by doing so, may propagate progressive neurodegeneration. In this context, the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the "master switch" for microglial activation, is a compelling target for therapeutic intervention due to its key regulatory role in the production of inflammatory mediators that contribute to neurotoxicity.

Currently available drugs that suppress microglial NF-κB activation include nonsteroidal anti-inflammatory drugs (NSAIDs) and some anticancer drugs, which can have serious side effects with long-term usage [6]. In addition, preclinical drug discovery and development efforts of de novo synthesis of novel NF-κB inhibitors are ongoing [7], but remain many years from the potential introduction in clinical populations. In recognition that > 70% of approved drugs originate from nature [8], and herbal medicines are often low cost with fewer side effects [9], using an unbiased screening of > 3000 compounds from a natural product derivative library, our laboratory identified a number of compounds that substantially inhibit NF-κB activity in microglia. One of the most effective of these was gedunin, a component of neem plant extract [10], with an elucidated structure and availability in high purity from a standard commercial supplier.

Gedunin has been reported to have anti-malarial, insecticidal, and anticancer properties [11], in addition to neuroprotective effects. While its mechanisms of action remain to be fully characterized, they appear pleiotropic. With regard to neuroprotection, gedunin analogs have been reported to produce neuroprotection via induction of brain-derived neurotrophic factor [12]. Gedunin itself has been shown to have neuroprotective effects against the toll-like receptor (TLR)–mediated inflammation through the regulation of proinflammatory inflammasome activation and cytokine production [13]. Furthermore, another study determined gedunin to induce astrocyte-dependent neuroprotective effects through the activation of nuclear factor 2 erythroid–related factor 2 (Nrf2) [14]. These latter observations are particularly noteworthy because Nrf2 is a transcription factor that regulates the transcription of antioxidant genes when oxidative stress occurs [15]. Moreover, pharmacological and genetic studies increasingly demonstrate crosstalk between Nrf2 and NF-κB pathways, indicating that the activation of Nrf2 can suppress NF-κB pathways [16].

In the current study, we examined the effects of the natural product gedunin on oligomeric $A\beta_{1–42}$-induced microglial activation and subsequent inflammation. Oligomeric $A\beta_{1–42}$ is known to stimulate the activation of proinflammatory pathways in glial cells [17]. For this study, we selected a model system, immortalized microglia (IMG) which display the same features of primary microglia, including robust responses to inflammatory signals and stimuli such as lipopolysaccharide (LPS) and Aβ peptide, and the secretion of inflammatory markers [18]. We tested the ability of gedunin to modulate the proinflammatory NF-κB pathway by measuring markers of cytokines and reactive nitrogen species, after microglial activation by oligomeric $A\beta_{1–42}$. We also evaluated the potential role of gedunin as an Nrf2 activator and as a mechanism for inhibiting NF-κB pathways and resulting neuroinflammation following oligomeric $A\beta_{1–42}$-induced microglial activation. Finally, we determined whether gedunin could protect against the secondary neurotoxicity elicited by $A\beta_{1–42}$-induced microglial activation. Together, the data demonstrate the ability of gedunin to mitigate damaging neuroinflammatory responses which are triggered by oligomeric $A\beta_{1–42}$-induced microglial activation through the modulation of Nrf2-NF-κB signaling.

Materials and Methods

Cell Culture and Treatment

The immortalized microglia (IMG) cell line used in this study was purchased from Kerafast (Boston, MA, USA). Originally, these cells were prepared from primary mouse microglia, isolated from the brains of 8-week-old adult mice, which were immortalized via retroviral infection of v-Raf/v-myc to create IMG microglial cells. IMG cells display the same features of primary microglia, including robust responses to inflammatory signals and stimuli such as LPS and Aβ peptides, and the secretion of inflammatory markers [18]. IMG cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Thermo Scientific, Waltham, MA, USA) containing 10% fetal bovine serum, 1% penicillin-streptomycin (Thermo Scientific, Waltham, MA, USA) and grown in an environment of 37 °C with 5% CO_2 and 95% O_2. Dissociation of adherent cells from confluent IMG cultures was done via trypsinization using 0.25% trypsin (CorningTM, Corning, NY, USA). Varying concentrations of gedunin were used to pre-treat IMG cells, and $A\beta_{1–42}$ oligomers at 1 μM were used to co-treat IMG cells and stimulate microglial activation.

Human SH-SY5Y neuroblastoma cells stably transfected with an expression construct encoding the longest human tau isoform (htau40) were obtained from Prof. Goetz laboratory, Queensland Brain Institute, St Lucia, Australia [19]. The cells were cultured in DMEM/F12 media supplemented with 10% fetal bovine serum and antibiotics.

Preparation of Gedunin and $A\beta_{1–42}$ Oligomers

Gedunin was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) with a purity of > 98%. Gedunin (10 mg) was dissolved in dimethyl sulfoxide (DMSO) to create a working stock concentration of 10 mM which was finally dissolved in media within a range of 1–10 μM. Initial characterizations demonstrated that gedunin could compromise cell

viability on its own in IMG microglial cells ($F[5, 18] = 6.18$, $P < 0.005$). Post hoc Dunnett's tests revealed statistically significant cytotoxicity at a concentration of 10 μM ($q = 4.59$, $P < 0.001$). Therefore, in the studies reported herein, gedunin was used at a maximum concentration of 7.5 μM, which by itself was not cytotoxic (Fig. S1A).

An Aβ aggregation kit was purchased from rPeptide (Watkinsville, GA, USA). Aβ oligomers were generated by incubating $A\beta_{1–42}$ peptides at 37 °C for 3 h according to the kit protocol. To determine proper oligomer formation, aliquots of the solution were mixed with thioflavin every 15 min for fluorescence readings at an excitation wavelength of 440 nm and an emission wavelength of 485 nm. The final stock concentration of the $A\beta_{1–42}$ oligomers was 100 μM.

Cell Viability Assay

Cell viability was determined using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. IMG microglial cells were seeded at a density of 10,000 cells in 100 μL cell culture medium per well in a 96-well plate. Cells were incubated for 24 h to settle before treatment with gedunin (0–10 μM) for the next 24 h. After 24 h of gedunin treatment, 10 μL MTT 5 mg/mL (MilliporeSigma, St. Louis, MO, USA) was added to the culture media in each well and incubated at 37 °C for 2–4 h. Media was aspirated without disrupting the formed formazan crystals at the bottom of each well. The formazan crystals were dissolved in 200 μL of DMSO, and the optical density (OD) was measured at 570 nm using a SpectraMAX 340PC microplate reader (Molecular Devices, San Jose, CA, USA).

Measurement of Nitric Oxide Levels

The levels of nitric oxide (NO) were measured using the Griess Reagent Assay (MilliporeSigma, St. Louis, MO, USA). IMG microglial cells were treated with gedunin (0–7.5 μM) for 2 h and followed by exposure to $A\beta_{1–42}$ oligomers (1 μM) for 24 h in the presence of gedunin. After 24 h, culture media (100 μL) was collected and used for measuring NO after addition of the Griess reagent (100 μL). The OD was measured at 540 nm using a SpectraMAX 340PC microplate reader.

Enzyme-Linked Immunosorbent Assay for IL-1β

IMG microglial cells were pre-treated with gedunin (0–7.5 μM) for 2 h, then followed by exposure to $A\beta_{1–42}$ oligomers (1 μM) for 24 h along with gedunin. After 24 h, culture media from the cells were collected, and the concentration of IL-1β was measured using an enzyme-linked immunosorbent assay (ELISA) kit for IL-1β and the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA). The OD was measured at 450 nm using a SpectraMAX 340PC microplate reader.

Quantitative Polymerase Chain Reaction

Total RNA was prepared from IMG microglial cells using Trizol reagent (Thermo Scientific, Waltham, MA, USA) as described previously [20]. The quality and concentration of isolated RNA were determined using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). A cDNA synthesis kit (Bioline USA, Taunton, MA, USA) was used to synthesize cDNA from 2.5 μg of isolated RNA. Prepared cDNA was used for the quantitative polymerase chain reaction (qPCR) analysis using SYBR Green PCR Master Mix reagent and gene-specific primers from Roche Diagnostics (Indianapolis, IN, USA). Relative mRNA levels were estimated by the comparative analysis of Ct values (ΔΔCt method) and normalized to values measured for actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) within the same samples. The primer (Eurofins Genomics, Louisville, KY, USA) sequences used were as follows:

	Forward	Reverse
Actin	CGGTTCCGATGCCC TGAGGCTCTT	CGTCACACTTCATG ATGGAATTGA
GAPDH	AACGACCCCTTCAT TGAC	TCCACGACATACTC AGCAC
GCS	GGAGGCGATGTTCT TGAGAC	CAGAGGGTCGGATG GTTG
HO-1	CAGAAGATGTAGCC AGAGCA	CATAGGGCAAGCGGTCA
IL-1β	AATCTGTACCTGTC CTGCGTGTT	TGGGTAATTTTTGG GATCTACACTCT
iNOS	CGAAACGCTTCACT TCCAA	TGAGCCTATATTCG TGTGGCT
Nrf2	TGACTCTGACTCCG GCATTTCACT	TCCATTTCCCGAGT CACTGAACCCA
NQO-1	GGCTGTCAGAAAAG CACTGATC	ACAGTCTCGGCAGG ATACTGAA

GCS, γ-glutamylcysteine synthetase; *HO-1*, heme oxygenase 1; *NQO-1*, NADPH quinone dehydrogenase 1

Western Blot Analysis

IMG microglial cells were pre-treated with gedunin (0–7.5 μM) for an hour and followed by $A\beta_{1–42}$ oligomer (1 μM) exposure for 24 h in the presence of gedunin. Post-treatment culture media from the cells were collected for use in experiments described above, and the cells were subjected to trypsinization. The resulting cell pellets were washed with PBS, lysed in NP-40 buffer, and sonicated to obtain whole cell lysates. Protein concentrations were determined using Bradford protein assays. Protein (30 μg) was loaded on a

10% polyacrylamide Bis-Tris protein gel (Thermo Scientific, Waltham, MA, USA) and subjected to sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis. Protein was then transferred to a PVDF membrane, which was pre-soaked in methanol for 30 s. The membranes were then blocked with 5% milk buffer and incubated with primary antibodies overnight at 4 °C. Primary antibodies used in this experiment include the following: mouse anti-actin (1:2000), rabbit anti-GCS (1:1000), rabbit anti-HO-1 (1:1000), rabbit anti-NQO-1 (1:1000), rabbit anti-iNOS (1:1000) (Eurofins Scientific, Luxembourg), rabbit anti-phospho NF-κB p65 (1:1000), rabbit anti-piκB (1:1000), Cleaved Caspase-3 (Asp175) Antibody (1:1000), Lamin B1 (D4Q4Z), Rabbit mAb (1:1000), and rabbit anti-Nrf2 (1:500) (Cell Signaling Technology, Danvers, MA, USA). After incubation with a primary antibody, membranes were washed three times with PBST for 15 min. Following incubation with a secondary antibody for 1.5 h at room temperature, proteins on the membrane were visualized by ECL detection (Millipore Corporation, Billerica, MA, USA). Secondary antibodies used in this experiment include goat anti-rabbit HRP (1:2000) and goat anti-mouse horseradish peroxidase (HRP; 1:2000) (Millipore Corporation, Billerica, MA, USA). The blots were visualized using a ChemiDoc Imaging (Bio-Rad, Hercules, CA, USA) system.

Immunocytochemistry of Nrf2 and NF-κB

IMG microglial cells were seeded in eight-well chamber slides at a density of 10,000 cells/well in 0.5 mL. Cells intended for imaging of Nrf2 were treated with gedunin (0–7.5 μM) for 24 h, and cells intended for imaging of pNF-κB were pre-treated with gedunin 7.5 μM for 2 h, followed by co-treatment with $A\beta_{1-42}$ oligomer (1 μM) exposure for 24 h. Cells were then rinsed with Tris-buffered saline (TBS) and fixed with 4% paraformaldehyde for 20 min at room temperature, followed by three 1X TBS washes for 5 min each. Cells were permeabilized with 0.1% Triton solution for 15 min at room temperature, followed by two washes with 1X PBS for 5 min each. The permeabilized cells were then blocked with 10% goat serum for 1 h at room temperature. After blocking, permeabilized cells were incubated with rabbit anti-pNF-κB p65 primary antibody (1:500) and anti-Nrf2 primary antibody (1:500) (Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C overnight. The next day, cells were washed in 1X TBS three times for 5 min each and incubated at room temperature for 1 h with goat anti-rabbit ALEXA 488 green fluorescent secondary antibody (1:500). Following three 1X TBS washes for 10 min each, well chambers were removed, and the slide was air dried before mounting in ProlongGold with 4′,6-diamidino-2-phenylindole (DAPI). Cells were observed using a fluorescent confocal microscope (Zeiss, LSM 780, Dublin, CA, USA) and photographed at × 20 magnification.

Nuclear Fraction Isolation and Detection of Nrf2 Translocation

IMG microglial cells were seeded into T-25 flasks at a density of 2 million cells in 5 mL DMEM media. Cells were treated with gedunin (0–7.5 μM) for 2 h. Nuclear extracts of IMG cells were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Following gedunin treatment, cells were collected and washed with 1X PBS, then centrifuged at 500×*g* for 2 min. After the final wash, the cell pellet was dissolved in ice-cold CER I (100 μL) and vortexed until cells were thoroughly resuspended. Next, ice-cold CERII (5.5 μL) was added to the mixture and cells were mixed by vortexing and incubated for 1 min. Cells were centrifuged for 5 min at 16,000×*g* and the supernatant removed. To this cell pellet, ice-cold NER (35 μL) was added, and cells were resuspended by vortexing for 15 s every 10 min, for a total of 40 min. Finally, the tubes were centrifuged at 16,000×*g* for 10 min. The supernatant containing the nuclear fraction was immediately transferred to a pre-chilled tube and stored at − 80 °C for future use. Protein concentrations within the nuclear fraction were determined using the Bradford assay method. Western blot analysis was conducted using anti-Nrf2 primary antibody (1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA). After three PBS washes for 15 min, the membranes were incubated with goat anti-rabbit HRP secondary antibody, followed by ECL detection. The blots were visualized using the ChemiDoc Imaging system.

siRNA Transfection

Small interfering RNA (siRNA) for Nrf2 (Santa Cruz Biotechnology, Dallas, TX, USA) was used to knockdown Nrf2 mRNA levels in IMG microglial cells. IMG cells were seeded in two 6-well plates at a density of 500,000 cells per well and incubated at 37 °C until ~ 80% confluent. After 24 h, one plate of cells was transfected with Nrf2 siRNA primers (0–40 nM) using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the second plate was transfected with scrambled siRNA using Lipofectamine 3000 reagent for 24 h which served as a negative control. After 24 h, IMG cells were then pre-treated with gedunin for 1 h instead of 2h2 h (0–7.5 μM) and then co-treated with $A\beta_{1-42}$ oligomers (1 μM) in the presence of gedunin for 24 h. NO levels within the culture media of IMG cells transfected with scrambled siRNA or Nrf2 siRNA were detected using a Griess reagent as described above.

Neurotoxicity of Activated Microglial Conditioned Media in SHSY5Y Cells Expressing Tau

For neurotoxicity assays, SHSY5Y cells expressing tau were seeded at a density of 10,000 cells in 0.1 mL cell culture

medium per well in a 96-well plate. After 24-h incubation, the media was exchanged with the conditioned medium obtained from IMG microglial cells challenged with Oligomeric Abeta ± gedunin. After 72-h exposure, the cell viability was determined by MTT assay as described above.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. GraphPad Prism software (La Jolla, CA, USA) was used to conduct a one-way analysis of variance (ANOVA). Post hoc analyses were performed using Dunnett's tests for comparisons to control, and Tukey's tests for other pairwise comparisons. $P < 0.05$ was considered statistically significant. In the figures, * indicates significant differences from the control group, while # indicates significant differences from the oligomeric $A\beta_{1–42}$ treatment group.

Results

Effects of Gedunin on $A\beta_{1–42}$ Oligomer-Induced NO Production and iNOS Expression

One-way ANOVA confirmed significant differences between the treatment groups in eliciting the production of the proinflammatory mediator NO ($F[5, 12] = 22.28$, $P < 0.0001$; Fig. 1a). Post hoc Tukey's tests confirmed that the exposure of the microglial cell line IMG to $A\beta_{1–42}$ oligomers induced significant increases in NO levels ($P < 0.0001$; Fig. 1a). Notably, gedunin treatment suppressed $A\beta_{1–42}$ oligomer-induced NO secretion in a dose-dependent manner; post hoc Tukey's tests showed the protective effect of gedunin to be significant at concentrations of 5 and 7.5 μM ($P < 0.0005$ and $P < 0.0001$, respectively; Fig. 1a).

One-way ANOVA also showed significant differences between the experimental groups in the mRNA expression of the NO synthesizing enzyme, iNOS ($F[4, 10] = 24.94$, $P < 0.0001$). Post hoc analysis demonstrated the expected elevation in iNOS mRNA expression following exposure of microglial cells to $A\beta_{1–42}$ oligomers ($P < 0.0001$; Fig. 1b). Moreover, the $A\beta_{1–42}$-induced increases in iNOS mRNA were significantly attenuated by gedunin in a dose-dependent fashion (Fig. 1b); post hoc Tukey's tests confirmed significant reductions compared to $A\beta_{1–42}$ alone in the presence of both 3.75 and 7.5 μM gedunin ($P < 0.005$ and $P < 0.0005$). The changes observed in iNOS mRNA levels were also seen at the protein level (Fig. 1c), with $A\beta_{1–42}$-induced increases in iNOS being attenuated by gedunin.

Effects of Gedunin on $A\beta_{1–42}$ Oligomer-Induced IL-1β

One-way ANOVA revealed significant differences in the levels of the proinflammatory cytokine IL-1β amongst the treatment groups ($F[5, 11] = 45.70$, $P < 0.0001$; Fig. 2a). Post hoc Tukey's tests confirmed that the exposure of the microglial cells to $A\beta_{1–42}$ oligomers significantly increased secretion of the proinflammatory cytokine IL-1β into the culture media ($P < 0.0001$; Fig. 2a). Post hoc analysis also showed that gedunin significantly suppressed IL-1β secretion by $A\beta_{1–42}$ treated microglial cells ($P < 0.0001$; Fig. 2a).

Additionally, there were significant differences between the treatment groups when IL-1β mRNA expression was measured in microglial cells ($F[4, 10] = 68.87$, $P < 0.0001$; Fig. 2b). Post hoc analysis confirmed the expected increase in IL-1β mRNA expression in the microglial cells exposed to $A\beta_{1–42}$ oligomers ($P < 0.0001$; Fig. 2b). This $A\beta_{1–42}$-induced increase in IL-1β mRNA expression was dose-dependently attenuated in the presence of gedunin, with statistically significant reductions observed at the 3.75 and 7.5 μM concentrations ($P < 0.0005$ and $P < 0.0001$ respectively; Fig. 2b).

Effects of Gedunin on $A\beta_{1–42}$ Oligomer-Induced NF-κB Phosphorylation and Nuclear Translocation

An increasing body of evidence suggests that amyloid beta (Aβ) peptides and other inflammatory stimuli are known to induce iNOS expression in glia cells via the activation of the transcription factor NF B. Therefore, we investigated whether the observed anti-inflammatory effects of gedunin were due to blockade of NF- B activation within IMG cells. NF- B is normally activated by phosphorylation of I B proteins, directing them for rapid degradation through the ubiquitin-proteasome pathway. The released NF- B subunit enters into the nucleus where it regulates gene expression. Western blot analysis of whole cell lysates demonstrated that $A\beta_{1–42}$ oligomers induced phosphorylation of p65 subunit which was significantly inhibited by pre-treatment with gedunin (Fig. 3a). The relevance of the phosphorylation of NF-κB was further demonstrated in nuclear translocation studies which showed that gedunin attenuated $A\beta_{1–42}$ oligomers induced nuclear translocation of NF-κB (Fig. 3b). These data suggest that gedunin can prevent NF B-mediated induction of inflammatory pathways.

Effects of Gedunin on Nrf2 Activation

Previous studies with a high-throughput screening of a spectrum library for Nrf2 activators in a SHSY5Y neuronal cell line demonstrated that gedunin is a potent inducer of Nrf2 activity [14]. Recently, numerous studies have suggested that Nrf2 plays a critical role in counteracting inflammatory responses in a variety of experimental models via inhibition of

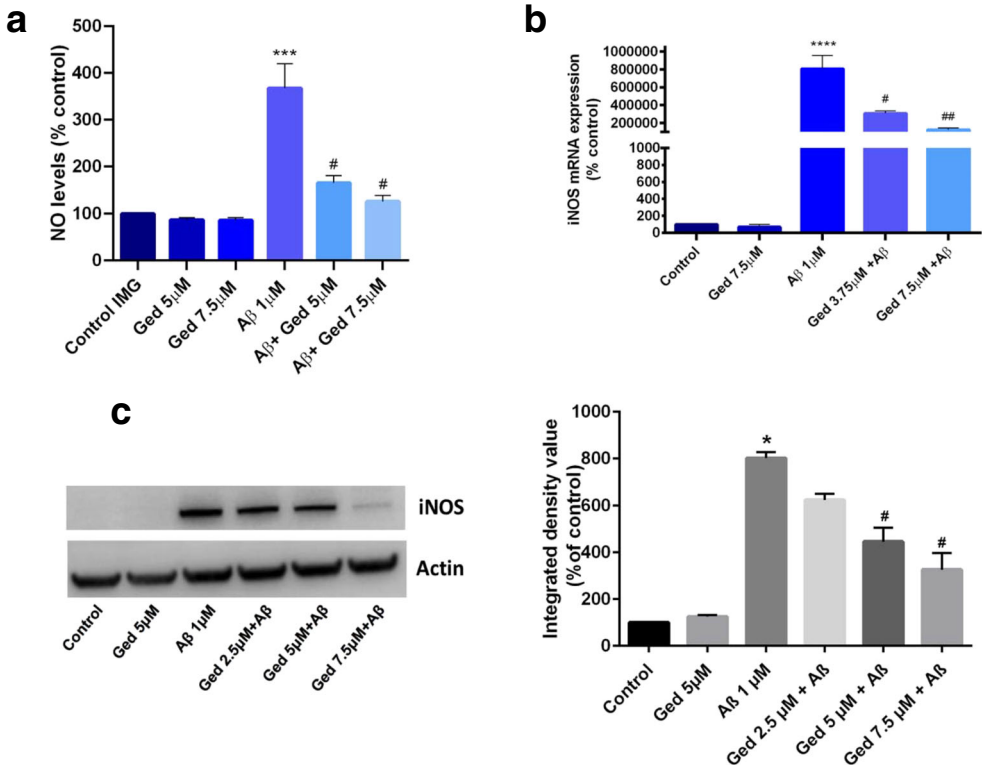

Fig. 1 Gedunin (Ged) inhibits inflammatory production of NO levels induced by $A\beta_{1–42}$ oligomers in IMG cells. **a** NO levels in culture media were measured following pre-treatment with gedunin for 2 h and co-treatment with $A\beta_{1–42}$ oligomers for 24 h. **b** Total RNA was isolated from IMG cells pre-treated with gedunin for 2 h and co-treated with $A\beta_{1–42}$ for 6 h, and relative mRNA levels of iNOS were analyzed using qPCR. **c** The whole cell lysate of IMG cells pre-treated with gedunin for 2 h and co-treated with $A\beta_{1–42}$ for 24 h was subjected to western blot analysis for iNOS expression. **d** The band density of INOS enzyme (integrated density value) is expressed as a percentage ratio of the densitometric optical density of the iNOS protein to that of Actin. Data are expressed as mean ± SEM for three independent experiments. $^{****}P < 0.0001$, Aβ vs control; $^{\#}P < 0.0005$, – Ged+AB vs. AB+Ged 5 μM; $^{\#\#}P < 0.0001$ – Ged+AB vs. AB+Ged 7.5 μM

NF-κB activity. Furthermore, multiple lines of evidence suggest that genetic disruption or pharmacologic inhibition of Nrf2 signaling augments the expression and activity of proinflammatory mediators via regulation of NF-κB activity indicating that there is functional crosstalk between these two important pathways [16]. We, therefore, examined whether the anti-inflammatory properties of gedunin in IMG cells may be due to Nrf2 induction.

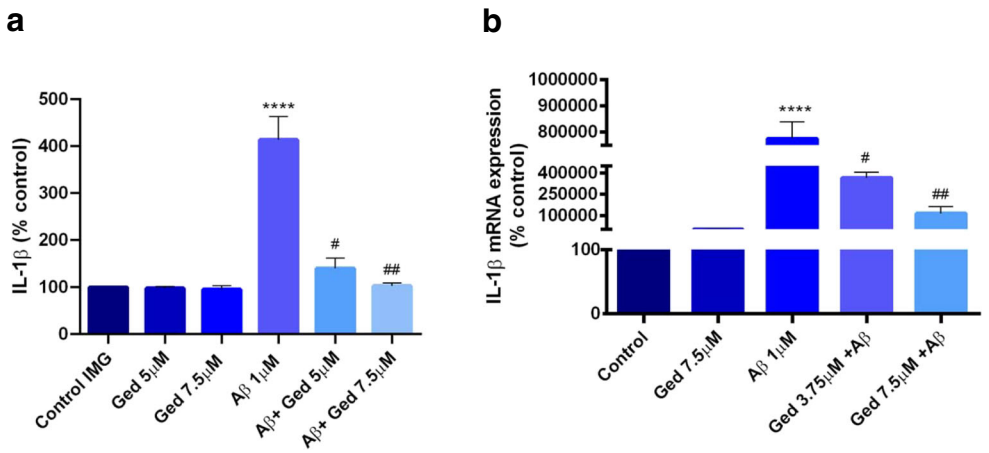

Fig. 2 Gedunin (Ged) inhibits oligomeric $A\beta_{1–42}$-induced proinflammatory IL-1β levels in IMG cells. **a** IL-1β in culture media were measured following pre-treatment with gedunin for 2 h and co-treatment with $A\beta_{1–42}$ oligomers for 24 h. **b** Total RNA was isolated from IMG cells pre-treated with gedunin for 2 h and co-treated with $A\beta_{1–42}$ for 6 h, and relative mRNA levels of IL-1β were analyzed using qPCR. Data is expressed as mean ± SEM for three independent experiments. $^{****}P < 0.0001$, Aβ vs control; $^{\#}P < 0.0002$, – Ged+AB vs. AB+Ged 5 μM; $^{\#\#}P < 0.0001$ – Ged+AB vs. AB+Ged 7.5 μM

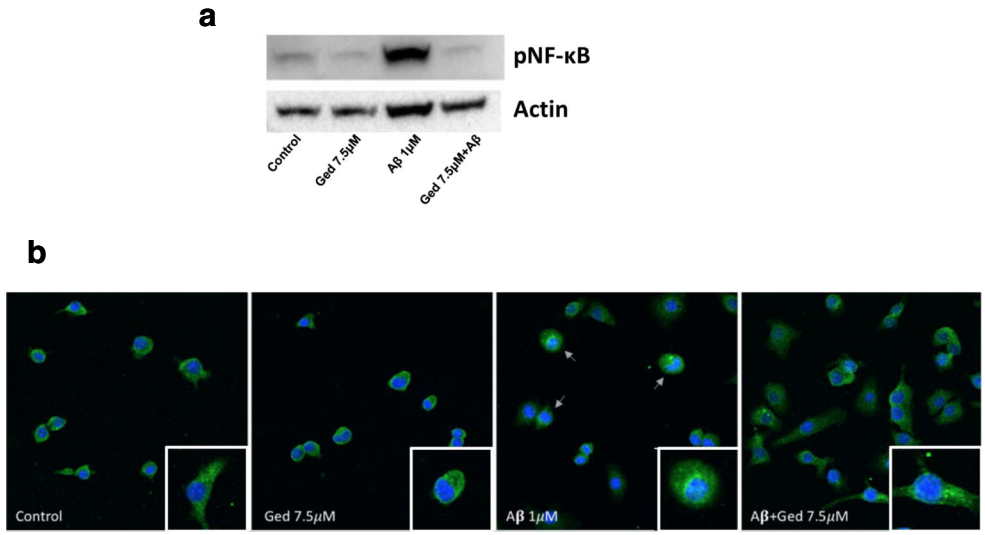

Fig. 3 $A\beta_{1–42}$ oligomers induce the activation and nuclear translocation of NF-κB. **a** The whole cell lysate of IMG cells pre-treated with gedunin (Ged) for 2 h and co-treated with $A\beta_{1–42}$ for 1 h was subjected to western blot analysis for pNF-κB expression. **b** Nuclear translocation of NF-κB in IMG cells pre-treated with gedunin for 2 h and co-treated with $A\beta_{1–42}$ for 1 h. IMG cells were immunostained for phosphorylated NF-κB (p NF-κB) and visualized by immunofluorescent confocal microscopy

Immunofluorescent confocal microscopy demonstrated that gedunin elicited nuclear translocation of the transcription factor and NF-κB regulator, Nrf2 in IMG microglia (Fig. 4a). Nuclear translocation of Nrf2 was further validated by the presence of increased Nrf2 protein in the nuclear fraction of microglial cells treated with gedunin, compared to control cells that were not exposed to gedunin (Fig. 4b).

Nrf2 target genes were also upregulated by gedunin, as measured using qPCR (Fig. 4c). One-way ANOVA, followed by post hoc Dunnett's tests, showed that gedunin dose-dependently increased mRNA expression of GCS ($F[2, 6] = 53.86$, $P < 0.0001$), HO-1 ($F[2, 5] = 33.26$, $P < 0.005$), and NQO-1 ($F[2, 4] = 304.80$, $P < 0.0001$) in microglial cells. Western blot analysis further confirmed increased protein expression of HO-1 and NQO-1 by gedunin (Fig. 4d).

To further test whether the anti-inflammatory effects of gedunin following $A\beta_{1–42}$ oligomer exposure were mediated via Nrf2 activation, siRNA was used to effectively knock down 70% of Nrf2 within the microglial cells (data not shown). One-way ANOVA confirmed a significant difference between the treatment groups on NO production in microglial cells treated with Nrf2 or scrambled siRNA ($F[5, 9] = 51.58$, $P < 0.0001$; Fig. 5). In control cells treated with scrambled siRNA, post hoc Tukey's tests confirmed the expected $A\beta_{1–42}$-induced increase in NO ($P < 0.0005$) and prevention in the presence of gedunin ($P < 0.005$), similar to the pattern observed in normal microglial cells not receiving control siRNA (Fig. 1a). In microglial cells treated with Nrf2 siRNA, there was no difference in the basal level of NO produced compared to control, scrambled siRNA-treated cells (n.s.). However, when exposed to $A\beta_{1–42}$ oligomers, the Nrf2 siRNA–treated microglial cells exhibited an enhanced pro-inflammatory response ($P < 0.0001$), as measured by a significantly higher increase in NO production compared to the responses elicited in cells receiving scrambled siRNA ($P < 0.005$). Although gedunin was able to attenuate the $A\beta_{1–42}$ oligomers-induced increase in NO production in Nrf2 siRNA–treated microglial cells ($P < 0.005$), the protective effect of gedunin was significantly weaker in Nrf2 siRNA–treated microglial cells compared to microglial cells treated with scrambled siRNA ($P < 0.001$).

Effects of Gedunin on Secondary Neurotoxicity of SH-SY5Y Cells Overexpressing Tau

One-way ANOVA revealed a significant difference in cell viability exhibited amongst the treatment groups in human neuronal SH-SY5Y cells overexpressing full-length human wild-type tau protein ($F[3, 12] = 217.4$, P 0.001; Fig. 6a). When SH-SY5Y neuronal cells overexpressing tau were exposed to conditioned media from microglial cells exposed to $A\beta_{1–42}$ oligomers, there was a significant decrease in cell viability as confirmed using post hoc analysis ($P < 0.0001$; Fig. 6a). Interestingly, conditioned media from gedunin-supplemented and Aβ-exposed microglial cells were significantly less cytotoxic to SH-SY5Y neuronal cells overexpressing tau ($P < 0.0001$; Fig. 6a). The quantitative results revealed by the MTT assays were visually confirmed using fluorescence microscopy of GFP expressing WT tau cells (Fig. 6b).

To further characterize the type of neurotoxicity experienced by SH-SY5Y neuronal cells overexpressing tau following exposure to conditioned media from $A\beta_{1–42}$ oligomers-treated microglial cells, cleaved caspase-3, a proteinase associated with apoptosis, was measured and shown to differ amongst the experimental groups (Fig. 6c). The western blot analysis confirmed a significant increase in cleaved caspase-3

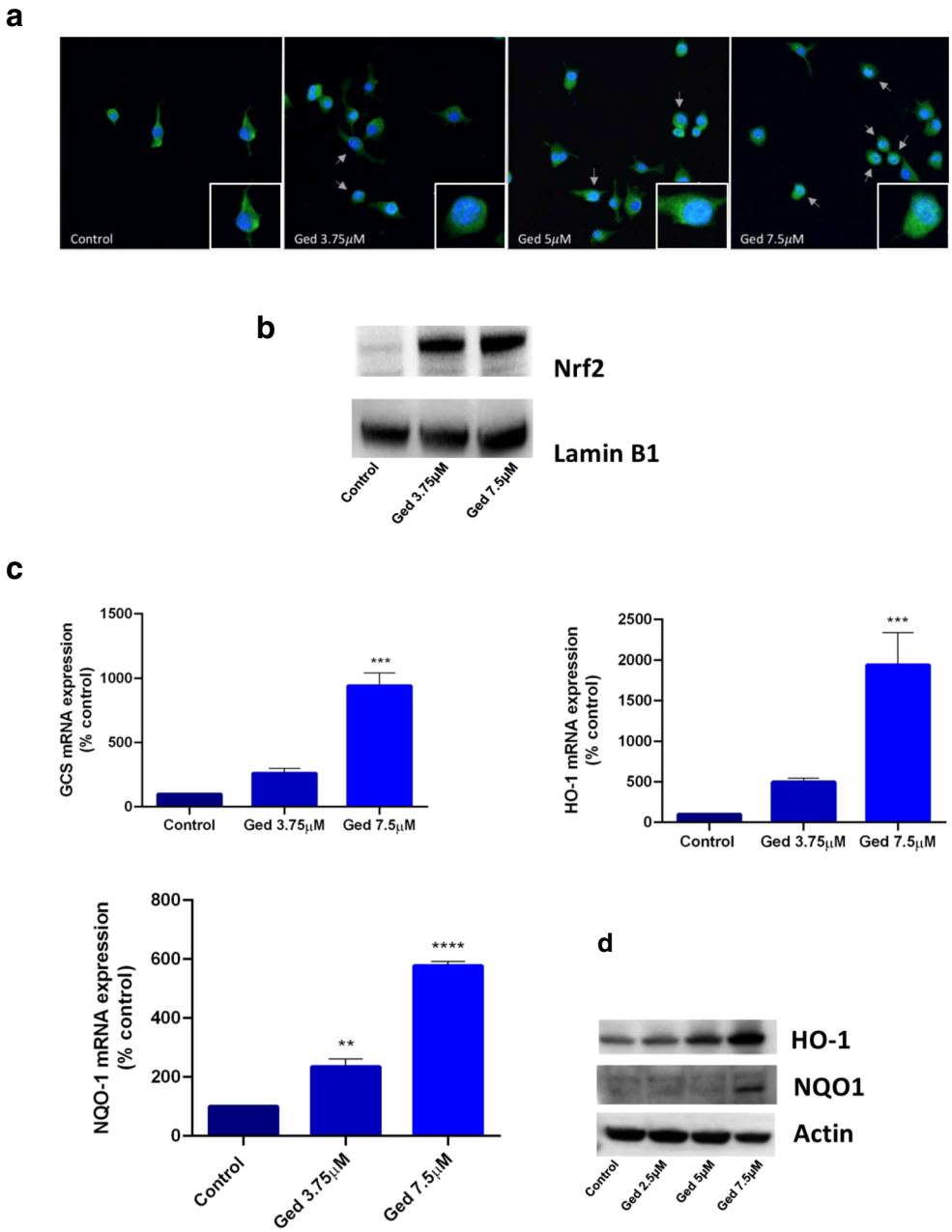

Fig. 4 Gedunin (Ged) induces the translocation of Nrf2 to the nucleus, leading to increased transcription of Nrf2's downstream target genes and translation of their respective proteins. **a** Nuclear translocation of Nrf2 in IMG cells treated with gedunin for 2 h. IMG cells were immunostained for Nrf2 and visualized by immunofluorescent confocal microscopy. **b** The nuclear fraction of IMG cells treated with gedunin for 2 h was extracted and subjected to western blot analysis for nuclear Nrf2. **c** Total RNA was isolated from IMG cells treated with gedunin for 6 h, and relative mRNA levels of Nrf2 target genes were analyzed using qPCR. **d** The whole cell lysate of IMG cells treated with gedunin for 16 h was subjected to western blot analysis for Nrf2 target gene expression. Data is expressed as mean ± SEM for three independent experiments. $**P < 0.02$, $***P < 0.002$, $****P < 0.0001$ of the untreated control sample

protein levels in SH-SY5Y neuronal cells exposed to conditioned media from $A\beta_{1-42}$-treated microglial cells. In contrast, conditioned media from $A\beta_{1-42}$-treated microglial cells that were also exposed to gedunin elicited significantly less cleaved caspase-3 expression in SH-SY5Y neuronal cells overexpressing tau, further supporting a protective effect of gedunin.

Discussion

Our study is the first report of the ability of the natural product gedunin to mitigate $A\beta_{1-42}$ oligomers-induced microglial activation and associated inflammatory cascades. Moreover, the anti-inflammatory effects of gedunin appear mediated via Nrf2-NF-κB signaling.

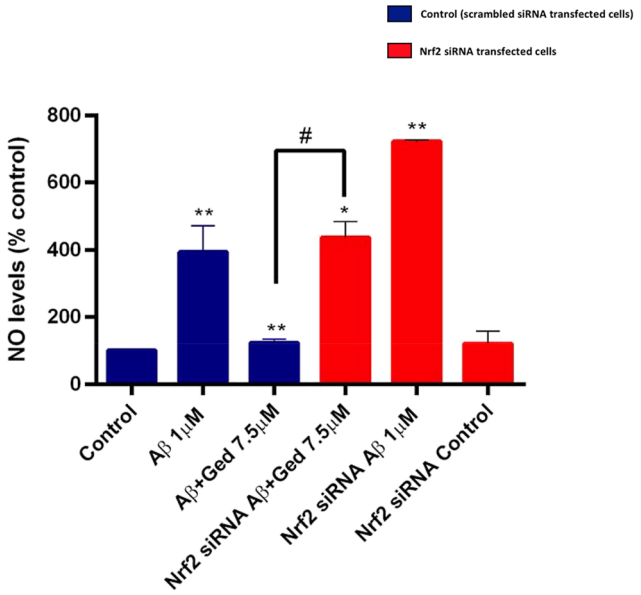

Fig. 5 Effects of Nrf2 silencing using siRNAs on NO production from IMG microglia cells treated with gedunin and $A\beta_{1–42}$ oligomers. IMG cells were transfected with scrambled siRNA (blue) or Nrf2 siRNA primers (red) for 24 h. Transfected IMG cells were then pre-treated with gedunin for 2 h and co-treated with $A\beta_{1–42}$ in the presence of gedunin for 24 h. The production of NO within the culture media was measured as previously described. Data are expressed as mean ± SEM for three independent experiments. $*P < 0.03$, $**P < 0.02$. $^{\#}P < 0.001$, Aβ+Ged 7.5 μM vs. Nrf2 siRNA Aβ+Ged 7.5 μM

Using an immortalized microglial cell line (IMG) as a model system, we demonstrated the ability of $A\beta_{1–42}$ oligomers to induce microglial activation through the NF-κB pathway, with resulting functional responses in proinflammatory cytokine targets such as IL-1β and iNOS. Under normal physiological conditions, the nuclear transcription factor NF-κB is localized to the cytosol where it is inactive while bound to the inhibitory protein iκB kinase [21]. In response to cellular stress, the NF-κB complex is activated following dissociation from iκB [22]. Activated NF-κB translocates to the nucleus where it binds to DNA and promotes the expression of downstream proinflammatory target genes [23]. In the present study, $A\beta_{1–42}$ oligomers elicited activation of microglial cells, as evidenced by (1) enhanced NF-κB nuclear translocation, (2) increased production and expression of the proinflammatory cytokine IL-1β, and (3) increased production of NO, along with enhanced expression of the NO synthesizing enzyme iNOS, a known target of NF-κB activation. The natural product gedunin was shown to significantly attenuate all of these effects, suggesting that it can produce anti-inflammatory actions by inhibiting the NF-κB pathway in microglia.

One target of activated microglia through the NF-κB pathway is iNOS [24]. The ability of gedunin to inhibit iNOS mRNA and protein expression in $A\beta_{1–42}$ oligomers exposed microglial cells suggests that gedunin suppresses NO production as a result of the decreased synthesis of NO, rather than increased clearance. Since iNOS is a recognized target of the NF-κB pathway in microglia, it supports the involvement of this pathway in the mechanism of action of gedunin.

Elevated levels of the proinflammatory cytokine IL-1β is also closely linked to neuroinflammation during AD, and more recently with associated cognitive decline [25]. Several

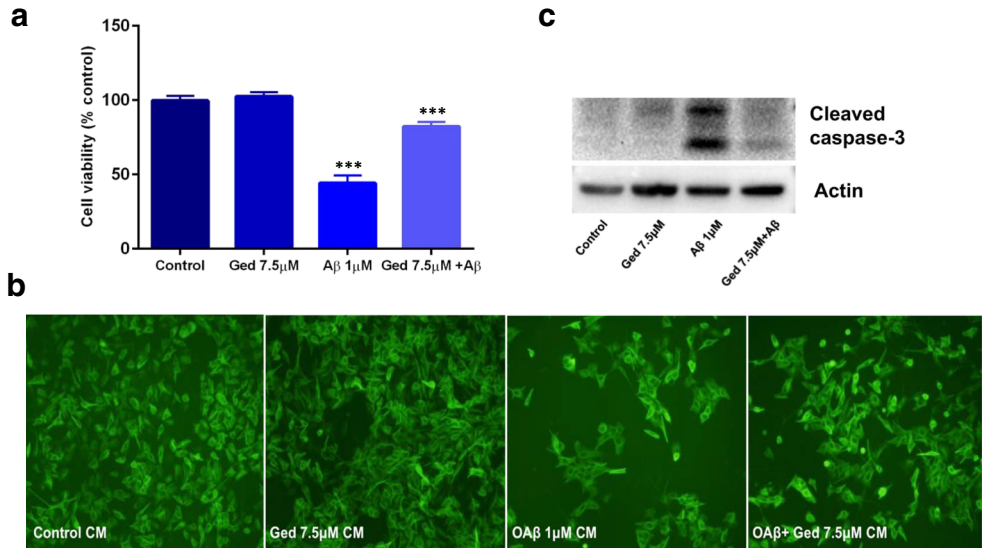

Fig. 6 Gedunin (Ged) confers protection to SH-SY5Y neuronal cells overexpressing tau against microglial toxicity. **a** SH-SY5Y neuronal cells overexpressing tau were exposed to conditioned media from IMG microglia cells treated with gedunin and oligomeric $A\beta_{1–42}$ for 72 h. Cell viability was assessed by MTT assay. **b** Representative images of GFP-Tau overexpressing SH-SY5Y neuronal cells exposed to conditioned media from IMG microglia treated with control, gedunin, oligomeric Aβ, and oligomeric Aβ with gedunin. **c** Western blot analysis of cleaved caspase-3 in whole cell lysate collected from SH-SY5Y Tau neuronal cells exposed to microglial conditioned media from various groups for 72 h. Data are expressed as mean ± SEM for three independent experiments. $****P < 0.0001$ – Ged+AB vs untreated control; $^{\#}P < 0.001$ – Ged+AB vs. AB+Ged 7.5 μM

reports show increased IL-1β levels in brains of aged mouse tissue and microglia surrounding amyloid plaques [26]. Therefore, it is noteworthy that in the present study, exposure of microglial cells to $A\beta_{1-42}$ oligomers was shown to produce IL-1β, which could be attenuated by the presence of gedunin.

In light of increasing evidence in the literature of crosstalk between NF-κB and Nrf2 pathways [16, 27], together with recent studies of gedunin having astrocyte-dependent neuroprotective effects through the activation of Nrf2 and actions as a potent Nrf2 activator in a high-throughput screen [14], we confirmed the involvement of Nrf2 in the effects observed herein. Similar to NF-κB, inactive Nrf2 is localized to the cytosol where it is bound by its inhibitory protein, Kelch-like ECH-associated protein 1 (Keap1) [28]. Oxidative or electrophilic stress leads to conformational changes of Keap1 and its dissociation from Nrf2 [28]. This dissociation allows Nrf2 activation and translocation to the nucleus where it then binds to the antioxidant response element (ARE) region of DNA to promote the expression of downstream cytoprotective, antioxidant genes [29, 30]. In the current study, gedunin was shown to stimulate the nuclear translocation of Nrf2 and upregulate the target antioxidant genes GCS, HO-1, and NQO-1 in microglial cells. This pattern of results is consistent with the previously reported strong protective role of Nrf2 in counteracting inflammatory responses via inhibition of NF-κB activity [29, 31, 32].

Functionally, the Nrf2 pathway regulates the transcription of antioxidant genes including HO-1, GCS, and NQO1. The production of the Nrf2 antioxidant target gene HO-1, specifically, has been previously shown to inhibit the degradation of iκB and activation of the NF-κB pathway [33]. Using siRNA transfection methods to knock down the production of Nrf2 in IMG microglia cells activated by $A\beta_{1-42}$ oligomers, our study confirmed that gedunin exerts anti-inflammatory effects through modulation of the Nrf2 pathway and that silencing it exacerbates the inflammatory response to $A\beta_{1-42}$ oligomers in IMG microglia cells. These results are consistent with previous studies using human monocytes and Nrf2 knockout mice, in which knockdown of Nrf2, its downstream target genes, and the production of their respective antioxidant proteins all resulted in the induction of inflammation when stimulated with the proinflammatory cytokine, tumor necrosis factor alpha (TNF-α) [34]. Although, siRNA methods helped us demonstrate that an upstream target of Nrf2 exhibits antioxidant and anti-inflammatory properties, we have yet to identify the important downstream Nrf2 target genes that contribute to the suppression of NF-κB activity.

The sustained activation of microglial cells and the resultant release of proinflammatory cytokines and reactive oxygen and nitrogen species can generate a cytotoxic environment that impacts neighboring neurons and other cell types [35]. To confirm that in our IMG microglial model system, activation by $A\beta_{1-42}$ oligomers also generates a cytotoxic milieu, we tested the effects of conditioned media from our microglial cultures on neuronal viability. Since the increased accumulation of NFTs formed from tau and Aβ plaques is a common feature of AD, we selected as a model neuronal system for these studies human-derived SH-SY5Y cells overexpressing human full-length wild-type tau protein. When these neuronal cells were exposed to conditioned media from microglial cells exposed to $A\beta_{1-42}$ oligomers, there was an expected decrease in neuronal viability. In contrast, conditioned media from microglial cells exposed to $A\beta_{1-42}$ oligomers in the presence of gedunin was much less neurotoxic, indicating that the protective effect of gedunin extended to secondary neurotoxicity that could result from harmful microglial activation. The neurotoxicity exhibited by SH-SY5Y cells overexpressing human full-length wild-type tau in response to conditioned media from $A\beta_{1-42}$-activated microglial cells was shown to be associated with alterations in cleaved caspase-3, a proteinase associated with apoptosis [36]. Aβ has long been known to induce neuronal apoptosis in transgenic mice and neuronal cultures [37], contributing to the neurodegeneration associated with AD. In addition to its well-established role in apoptosis, caspase-3 also plays a lesser recognized role in Aβ-induced synaptic dysfunction [38], which could also contribute to cognitive deficits in AD separate from the death of neurons.

Recent evidence suggests the inclusion of natural products in an individual's diet, which can be obtained via the consumption of vegetables, fruits, and leaves or roots of plants and may delay the development or slow the progression of neurodegenerative diseases [39]. Given that current medications for AD are limited in number and offer only symptomatic relief by targeting the regulation of neurotransmitters in the brain, an alternative therapeutic strategy is targeting the neuroinflammation associated with AD using natural products [40, 41]. Our study demonstrates the anti-inflammatory and antioxidant properties of the natural product gedunin through modulation of Nrf2-NF-κB signaling. Suppression of Aβ-induced microglial activation and subsequent inflammatory cascades with gedunin also has secondary protective effects on neurons in vitro. Future studies aimed at further delineating the mechanisms by which gedunin conveys neuroprotective actions and further exploring its efficacy under in vivo conditions are warranted.

Conclusion

Our results demonstrate that gedunin is able to reduce inflammation by suppressing NO levels and the activation of NF-κB. Furthermore, we show that the anti-inflammatory properties of gedunin are due to the increased nuclear translocation of Nrf2, and the subsequent increase in expression of downstream antioxidant Nrf2 target genes. These results suggest that gedunin's modulation of the Nrf2 pathway plays an

important role in upregulating an anti-inflammatory signaling cascade in microglial cells, and further suggests that gedunin may be a potential natural therapeutic candidate against neuroinflammation associated with AD. Subsequent studies on SH-SY5Y neuronal cells overexpressing tau suggest that gedunin also protects against $A\beta_{1-42}$ oligomer-induced neurotoxicity. These findings support a potential role for gedunin as a natural alternative therapeutic against AD progression.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and Animals This article does not contain any studies with human participants or animals performed by any of the authors.

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