

ORIGINAL RESEARCH ARTICLE

Multi-target modulation of the neuroinflammatory network by asiatic acid in ischemic stroke: Integrated network pharmacology, molecular docking, and in vitro validation

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Abstract

Asiatic acid (AA), a pentacyclic triterpenoid derived from *Centella asiatica*, has shown neuroprotective and anti-inflammatory activities, but the molecular network underlying its potential effects in ischemic stroke remains incompletely understood. Here, network pharmacology, protein-protein interaction (PPI) analysis, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, molecular docking, and *in vitro* experiments were integrated to define a multi-target neuroinflammatory framework for AA. A total of 194 AA-associated targets and 2,721 ischemic stroke-associated targets were identified, yielding 96 overlapping targets. PPI analysis prioritized IL-6, TNF, AKT1, and IL-1 β as prominent hub candidates, while enrichment analysis linked the shared target network to hypoxia responses, inflammatory regulation, apoptosis, HIF-1 signaling, and TNF-related pathways. Molecular docking predicted structurally plausible interactions between AA and AKT1, TNF, IL-1 β , and IL-6. In HT22 cells subjected to oxygen-glucose deprivation/reoxygenation (OGD/R), AA improved cell viability, reduced TNF- α , IL-6, and IL-1 β release, and attenuated OGD/R-induced increases in *Akt1*, *Tnf*, *Il1b*, and *Il6* mRNA. Collectively, these findings support a hypothesis in which AA modulates an interconnected neuroinflammatory and stress-response network rather than a single molecular target. IL-6, TNF, IL-1 β , and AKT1 therefore emerge as experimentally supported candidate nodes for further target-validation studies and, with appropriate *in vivo* and clinical validation, as potential biomarker candidates. This integrated framework expands the therapeutic rationale for AA in ischemic stroke while defining specific targets and pathways for future mechanistic investigation.

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Citation: Gong J, Tang KH. Multi-target modulation of the neuroinflammatory network by asiatic acid in ischemic stroke: Integrated network pharmacology, molecular docking, and in vitro validation. *Adv Neurol*. doi: 10.36922/AN026310047

Received: July 30, 2026**Revised:** September 11, 2026**Accepted:** September 17, 2026**Published online:** September 29, 2026

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Keywords: Asiatic acid; Ischemic stroke; Network pharmacology; Molecular docking; Oxygen-glucose deprivation/reoxygenation; Inflammation; Apoptosis

1. Introduction

Ischemic stroke is one of the major causes of death and long-term neurological disability worldwide and continues to impose a substantial medical and socioeconomic burden. Global Burden of Disease 2021 analyses indicate that, although age-standardized rates

have declined in several regions, the absolute number of affected individuals remains considerable.¹ The pathological process begins with an abrupt reduction in cerebral blood flow, resulting in insufficient delivery of oxygen and glucose to brain tissue. This initiates a complex cascade involving energy failure, excitotoxicity, ionic imbalance, mitochondrial dysfunction, excessive production of reactive oxygen species, inflammatory responses, and ultimately neuronal death.² Reperfusion is essential for tissue rescue, but the restoration of oxygen supply may also intensify oxidative and inflammatory injury. Therefore, ischemic stroke is increasingly understood as a dynamic, multi-factorial pathological process rather than a disorder driven by a single molecular event.

Neuroinflammation represents a major component of secondary brain injury after cerebral ischemia and reperfusion. Following ischemic insult, inflammatory signaling promotes the production and release of cytokines and other mediators that can amplify neuronal dysfunction and tissue damage. Experimental evidence has demonstrated an important role for interleukin-1 (IL-1) signaling in cerebral ischemic injury; combined deficiency of IL-1 α and IL-1 β reduces injury after transient middle cerebral artery occlusion, while IL-1 receptor antagonism can also decrease infarct volume under defined experimental conditions.³ Tumor necrosis factor (TNF) is likewise involved in ischemic inflammatory responses, although its biological effects may vary according to molecular form, cellular source, and stage of injury. Selective preservation of membrane TNF in the absence of soluble TNF has been associated with neuroprotection after focal cerebral ischemia.⁴ These findings highlight the complexity of inflammatory regulation and support the investigation of therapeutic agents capable of modulating multiple interconnected inflammatory targets.

Asiatic acid (AA) is a naturally occurring pentacyclic triterpenoid associated with *Centella asiatica* and has attracted attention because of its reported antioxidant, anti-inflammatory, mitochondrial-protective, and neuroprotective properties. Previous experimental studies provide a direct rationale for evaluating AA in cerebral ischemia. Krishnamurthy *et al.*⁵ reported that AA reduced cerebral injury in a mouse model of focal ischemia and improved cell viability and mitochondrial membrane potential in HT22 neuronal cells exposed to oxygen-glucose deprivation. Lee *et al.*⁶ subsequently demonstrated that AA reduced infarct volume, improved neurological outcomes, attenuated mitochondrial dysfunction, and reduced matrix metalloproteinase-9 induction in focal ischemia models. In a rat embolic stroke model, AA was also reported to decrease infarct volume and reduce

mitochondrial release of cytochrome c and apoptosis-inducing factor.⁷ Taken together, these observations suggest that AA has reproducible neuroprotective potential across different experimental systems. However, the molecular network through which AA may simultaneously influence inflammatory, hypoxic, and cell-death responses has not been fully clarified.

Because ischemic stroke involves multiple interacting pathological processes, a single-target approach may not fully explain the pharmacological actions of a multi-functional natural compound such as AA. Network pharmacology provides a systems-level strategy for mapping compound-target-disease relationships and identifying candidate hub proteins within complex biological networks. Protein-protein interaction (PPI) topology can further prioritize highly connected targets, whereas Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses can reveal biological functions and pathways statistically associated with the overlapping target set. Molecular docking provides an additional structural layer by predicting potential ligand-protein binding conformations. Nevertheless, these computational methods are primarily hypothesis-generating: network centrality does not establish direct pharmacological action, pathway enrichment does not demonstrate pathway activation or inhibition, and docking scores are not equivalent to experimentally measured binding affinities. Consequently, integrating these predictions with biological experiments is necessary to determine whether the computational predictions correspond to measurable cellular effects.

Hypoxia-responsive mechanisms, including hypoxia-inducible factor 1 (HIF-1)-related signaling, have been implicated in neuronal survival and apoptosis after cerebral ischemia.^{8,9} Accordingly, the present study integrated target-network analysis, functional enrichment, molecular docking, and HT22 oxygen-glucose deprivation/reoxygenation (OGD/R) validation to investigate whether AA-associated neuroprotection is accompanied by changes in inflammatory mediators and selected candidate-target transcripts.

Accordingly, the central objective of this study is not merely to determine whether AA produces a protective phenotype, but to define a testable multi-target neuroinflammatory network relevant to ischemic injury. We therefore examined whether computationally prioritized hub nodes converged with measurable inflammatory and transcriptional responses in OGD/R-injured neurons. Within this framework, IL-6, TNF, IL-1 β , and AKT1 were treated as candidate therapeutic nodes and hypothesis-generating biomarker candidates whose

translational value requires subsequent protein-level, *in vivo*, and clinical validation.

Previous studies have established that AA can reduce ischemic injury in animal and neuronal models, but they have primarily emphasized phenotypic neuroprotection, mitochondrial preservation, or apoptosis-related outcomes. The present study addresses a complementary gap by integrating compound–disease target mapping, PPI topology, GO/KEGG enrichment, molecular docking, and OGD/R validation of inflammatory and transcript-level endpoints within one hypothesis-generating framework. Table 1 summarizes the relationship between representative previous studies and the present work. On this basis, the specific contributions of the present study are: (i) construction of an AA–ischemic stroke target network and systematic prioritization of candidate hub nodes; (ii) integration of PPI topology, GO/KEGG enrichment, and molecular docking with HT22 OGD/R experiments so that computational predictions are linked to measurable viability, cytokine, and transcriptional endpoints; and (iii) definition of IL-6, TNF, IL-1 β , and AKT1 as a focused, experimentally supported candidate-node set for subsequent protein-level, target-engagement, *in vivo*, and biomarker-oriented validation. These contributions are intended to define a testable multi-target neuroinflammatory framework rather than to claim newly proven direct pharmacological targets.

2. Materials and methods

2.1. Study design

Workflow/algorithm: (i) retrieve and standardize AA-associated targets; (ii) retrieve ischemic stroke–associated genes using the predefined GeneCards threshold; (iii) intersect the two target sets; (iv) construct the STRING PPI network and calculate Cytoscape topological metrics; (v) perform GO and KEGG over-representation analyses; (vi) prioritize representative hub nodes that align with the experimental endpoints; (vii) perform molecular docking as a structural plausibility screen; and (viii) test the resulting hypotheses in HT22 OGD/R cells using viability, cytokine, and quantitative real-time polymerase chain reaction (qRT-PCR) endpoints. This workflow is a deterministic analysis pipeline rather than a predictive machine-learning algorithm (Figure 1).

The study used a sequential computational-to-experimental design. AA-associated targets and ischemic stroke–associated genes were collected independently and standardized before intersection. Overlapping genes were subjected to PPI network construction and topological analysis, followed by GO and KEGG enrichment. Candidate hub proteins were selected for molecular docking. The resulting computational hypotheses informed the design of an HT22 OGD/R experiment evaluating cell viability, inflammatory cytokines, and selected gene-expression endpoints.

Table 1. Representative previous studies and the research gap addressed by the present study

Study	Dataset/Model	Method/Model used	Objective	Main result	Conclusion/Relevance to research gap
Krishnamurthy <i>et al.</i> ⁵	Mouse focal ischemia; HT22 OGD	<i>In vivo</i> + <i>in vitro</i>	Evaluate AA neuroprotection	Reduced cerebral injury; improved HT22 viability and mitochondrial membrane potential	Established protective phenotype; did not define an integrated multi-target inflammatory network
Lee <i>et al.</i> ⁶	Focal cerebral ischemia models	<i>In vivo</i>	Assess infarct, mitochondrial dysfunction, and MMP-9	Reduced infarct volume and mitochondrial dysfunction; reduced MMP-9 induction	Supported neuroprotection but did not integrate network pharmacology with target-prioritized validation
Lee <i>et al.</i> ⁷	Rat focal embolic stroke	<i>In vivo</i>	Assess AA in embolic stroke	Reduced infarct volume and mitochondrial release of cytochrome c/AIF	Extended <i>in vivo</i> evidence; target-network relationships remained unresolved
Present study	Target databases + HT22 OGD/R	Network pharmacology + PPI + GO/KEGG + docking + ELISA/qRT-PCR	Define and experimentally probe a multi-target neuroinflammatory network	Prioritized IL6, TNF, IL-1 β , AKT1; AA improved viability and attenuated cytokine/transcript changes	Provides an integrated, hypothesis-generating network for protein-level, target-engagement, and <i>in vivo</i> validation

Abbreviations: AA: Asiatic acid; AIF: Apoptosis-inducing factor; ELISA: Enzyme-linked immunosorbent assay; GO: Gene Ontology; IL-6: Interleukin-6; KEGG: Kyoto Encyclopedia of Genes and Genomes; MMP-9: Matrix metalloproteinase 9; OGD/R: Oxygen-glucose deprivation/reoxygenation; PPI: Protein–protein interaction; qRT-PCR: Quantitative real-time polymerase chain reaction; TNF: Tumor necrosis factor.

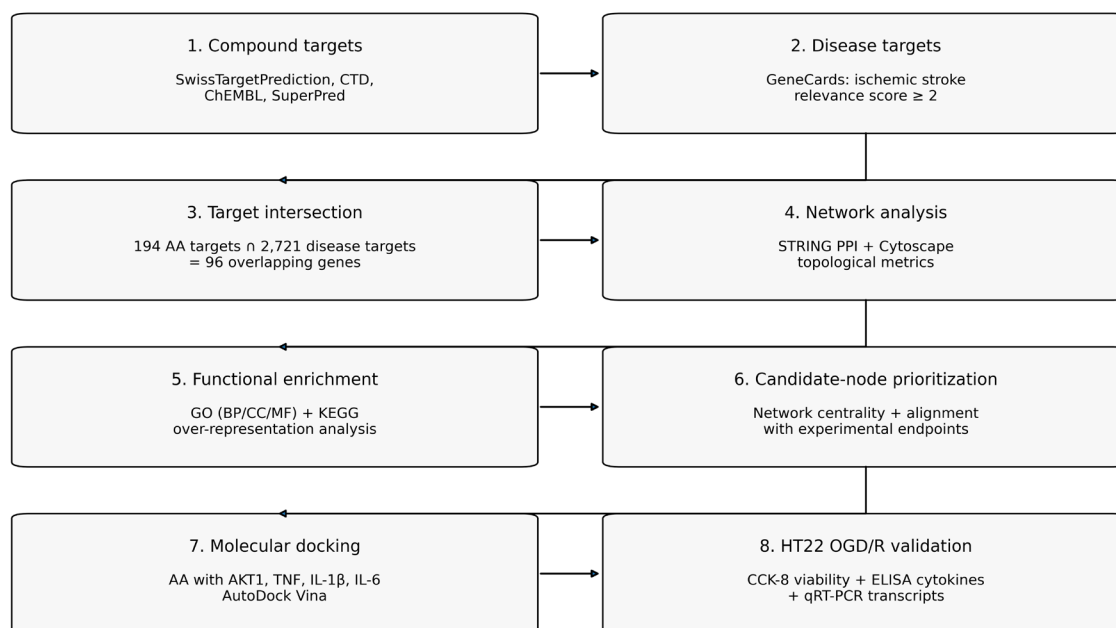


Figure 1. Study workflow linking target retrieval, network/enrichment analysis, docking, and HT22 OGD/R experimental validation

Abbreviations: AA: Asiatic acid; BP: Biological process; CC: Cellular component; CCK-8: Cell Counting Kit 8; CTD: Comparative Toxicogenomics Database; ELISA: Enzyme-linked immunosorbent assay; GO: Gene Ontology; IL-6: Interleukin-6; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecular function; OGD/R: Oxygen-glucose deprivation/reoxygenation; PPI: Protein-protein interaction; qRT-PCR: Quantitative real-time polymerase chain reaction; TNF: Tumor necrosis factor.

2.2. Identification of asiatic acid-associated targets

Candidate targets were collected from SwissTargetPrediction¹⁰, the Comparative Toxicogenomics Database (CTD)¹¹, ChEMBL¹², and SuperPred¹³. SwissTargetPrediction targets with probability > 0 were retained (104 targets). CTD records were restricted to *Homo sapiens* (11 targets). For ChEMBL, records annotated as “single protein” or “protein complex group” with *Homo sapiens* listed as the organism were retained; UniProt accessions were mapped to gene symbols using org.Hs.eg.db, yielding 13 gene targets. SuperPred records with probability > 0 were retained, and UniProt identifiers were mapped to gene symbols, yielding 89 targets. Integration and deduplication resulted in 194 unique AA-associated targets.

2.3. Identification of ischemic stroke-associated targets

GeneCards¹⁴ was searched using the term “Ischemic Stroke.” The initial search returned 7,061 records. Genes with a GeneCards relevance score ≥ 2 were retained, and duplicates were removed, resulting in 2,721 ischemic stroke-associated targets.

2.4. Identification of overlapping targets

The 194 AA-associated targets were intersected with the 2,721 ischemic stroke-associated targets. Ninety-six shared genes were identified and were retained for PPI and enrichment analyses. A Venn diagram was generated to visualize the overlap.

2.5. Protein-protein interaction network and topological analysis

The 96 overlapping targets were submitted to STRING¹⁵ with the organism restricted to *Homo sapiens*. The interaction network was imported into Cytoscape version 3.10.4¹⁶ for visualization and topological analysis. The final connected network contained 95 nodes. Degree, betweenness centrality, closeness centrality, clustering coefficient, and stress were calculated to characterize network position. Highly connected proteins were interpreted as candidate hub nodes rather than validated pharmacological targets.

2.6. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses

Gene Ontology and KEGG enrichment analyses were performed in R version 4.5.2 using clusterProfiler¹⁷ and the org.Hs.eg.db human gene annotation database. All

96 overlapping genes were successfully mapped to Entrez identifiers. The original analysis retained enriched terms/pathways with nominal $p < 0.05$. clusterProfiler also generated multiple-testing-adjusted p -values (p_{adjust}), which are displayed in the KEGG bubble plot; dot size represents the number of overlapping genes assigned to a pathway. A total of 1,769 GO terms (1,574 biological process, 51 cellular component, and 144 molecular function terms) and 146 KEGG pathways were obtained. Representative terms and pathways were visualized using bar and bubble plots. Enrichment results were interpreted as statistical over-representation, not as direct evidence of pathway activation or inhibition.

2.7. Molecular docking

The three-dimensional structure of AA (PubChem CID 119034) was obtained from PubChem.¹⁸ Protein structures were obtained from the RCSB Protein Data Bank.¹⁹ AKT1, TNF, IL-1 β , and IL-6 were selected for docking because they were among the highly connected inflammatory/survival-related candidate hub nodes in the PPI network and, unlike several other central nodes, they were also aligned with the planned experimental endpoints: TNF- α , IL-6, and IL-1 β were quantified using enzyme-linked immunosorbent assay (ELISA), and the corresponding *Tnf*, *Il6*, and *Il1b* transcripts, together with *Akt1*, were measured using qRT-PCR. The receptor structures used in the completed docking workflow were AKT1 (PDB ID: 1H10), TNF (4TSV), IL-1 β (1L2H), and IL-6 (1ALU). Structure-specific limitations were considered: 1H10 contains the isolated pleckstrin-homology domain of AKT1 bound to an inositol phosphate ligand; 4TSV is a human TNF- α mutant structure; 1L2H is an IL-1 β F42W/W120F double-mutant structure; and 1ALU is a human IL-6 structure. These experimentally resolved entries were retained to preserve consistency with the completed docking dataset, not because they are uniquely optimal receptors or demonstrably the most frequently used docking templates. Receptor structures were prepared in PyMOL 3.0 (Schrödinger, Inc., United States of America [USA]) by removing water molecules and non-relevant components. AutoDockTools (MGLTools version 1.5.7, The Scripps Research Institute, USA) was used for receptor/ligand preparation and docking-box definition. Docking was performed with AutoDock Vina (version 1.2.7, The Scripps Research Institute, USA) using 50 runs in the completed workflow.²⁰ The selected poses were visualized in PyMOL. Vina scores were interpreted as computational estimates of structural compatibility rather than experimental affinity.

2.8. Cell culture and oxygen-glucose deprivation/reoxygenation model

Fetal bovine serum was obtained from ExCell Bio (China). High-glucose Dulbecco's modified Eagle medium (DMEM) and penicillin-streptomycin were obtained from Solarbio (China). Cell Counting Kit-8 (CCK-8) reagent was obtained from GLP BIO Technology LLC (GK10001, USA). TNF- α (BMS607-3), IL-6 (BMS603-2), and IL-1 β (BMS6002) ELISA kits were obtained from Thermo Fisher Scientific (USA). qRT-PCR-related reagents were obtained from Nanjing Jiancheng Bioengineering Institute (China).

Mouse hippocampal neuronal HT22 cells were kindly provided by Professor Xueli Shi and her research team at the School of Traditional Chinese Medicine, Guangxi University of Chinese Medicine (Nanning, China). Cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells in the logarithmic growth phase were used for experiments.

To establish the OGD/R model, the normal culture medium was removed, and HT22 cells were washed with phosphate-buffered saline (PBS). Cells were transferred to glucose-free DMEM and incubated at 37°C under hypoxic conditions (1% O₂, 5% CO₂, and 94% N₂) for 6 h. The glucose-free medium was then replaced with complete high-glucose DMEM, and cells were returned to normoxic conditions (37°C, 5% CO₂) for 24 h of reoxygenation. Cells continuously maintained under normal culture conditions served as the control group.

2.9. Asiatic acid treatment and experimental groups

Asiatic acid was evaluated at 2.5, 5, 10, and 20 μ M in the CCK-8 concentration-screening experiment. Because 10 μ M produced the greatest recovery of cell viability, this concentration was selected for subsequent ELISA and qRT-PCR analyses. The validation groups were Control, OGD/R Model, and Asiatic acid group.

2.10. Cell viability assay

Cell viability was assessed using a CCK-8 assay. HT22 cells were seeded in 96-well plates and subjected to the indicated treatments. CCK-8 reagent was added according to the manufacturer's instructions, absorbance was measured at 450 nm using a microplate reader (SpectraMax M5, Molecular Devices, USA), and viability was expressed relative to the Control group. Six biological replicates were included in each group ($n = 6$).

2.11. Enzyme-linked immunosorbent assay measurement of inflammatory cytokines

After treatment, culture supernatants from the Control,

OGD/R Model, and OGD/R + AA (10 μ M) groups were collected. TNF- α , IL-6, and IL-1 β concentrations were determined using ELISA kits according to the manufacturer's instructions. Cytokine concentrations were calculated from the corresponding standard curves and expressed as pg/mL.

2.12. Quantitative real-time polymerase chain reaction

Total RNA was extracted from HT22 cells in the Control, OGD/R Model, and OGD/R + AA (10 μ M) groups using RNA extraction reagents (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. RNA concentration and purity were assessed before reverse transcription. For each sample, 500 ng of total RNA was used for reverse transcription into complementary DNA (cDNA) using reverse-transcription reagents from Nanjing Jiancheng Bioengineering Institute. qRT-PCR was performed using qPCR reagents from the same manufacturer on an ABI real-time PCR system (7500 Real-Time PCR System, Applied Biosystems, USA). The amplification program consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by melt-curve analysis. The messenger RNA (mRNA) expression levels of *Akt1*, *Tnf*, *Il1b*, and *Il6* were determined, with *Gapdh* used as the internal reference gene. Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are provided in Table 2. Six biological replicates were included in each group ($n = 6$); technical replicate measurements were averaged before statistical analysis.

2.13. Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed on GraphPad Prism (version 9.0.0, GraphPad Software Inc., USA) using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test for pairwise comparisons among groups. Six biological replicates were included per group ($n = 6$) for the cellular validation experiments. A two-sided p -value < 0.05 was considered statistically significant.

3. Results

3.1. Identification of asiatic acid-associated and ischemic stroke-associated targets

Potential targets associated with AA were collected from SwissTargetPrediction, CTD, ChEMBL, and SuperPred and were standardized and deduplicated before subsequent analysis. A total of 194 unique AA-associated targets were identified. In parallel, 2,721 ischemic stroke-associated genes meeting the predefined GeneCards relevance criterion were identified. Cross-comparison of the two target sets yielded 96 overlapping genes, representing the candidate target set through which AA may exert effects relevant to ischemic stroke (Figure 2). The presence of these shared targets provided the basis for subsequent PPI network construction, functional enrichment analysis, and molecular docking.

3.2. Protein-protein interaction network and candidate hub nodes

The 96 overlapping targets were submitted to STRING to examine potential PPIs and were subsequently imported into Cytoscape for topological analysis. After removal of the isolated node, the final PPI network contained 95 connected nodes (Figure 3). The network showed a non-uniform distribution of connectivity, indicating that a subset of proteins occupied relatively central positions within the interaction structure. Among these targets, IL-6, TNF, AKT1, and IL-1 β exhibited high connectivity and were prioritized as representative candidate hub proteins because they also converged with the inflammatory and gene-expression endpoints selected for experimental validation. Other highly connected proteins, including caspase 3 (CASP3), peroxisome proliferator-activated receptor gamma (PPAR γ), BCL-2, nuclear factor-kappa B1 (NF- κ B1), and nuclear factor NF- κ B p65 subunit (RelA), were also located within the central region of the network. PPAR γ was not included in the present docking/qRT-PCR panel because the experimental validation was deliberately restricted to a tractable set that directly linked the cytokine measurements (TNF- α , IL-6, and IL-1 β) with one survival-related hub (AKT1). Its omission does

Table 2. Primer sequences used for quantitative real-time polymerase chain reaction

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Akt1	ACGAGGTCAAGTCCTTCTGG	CGTAGTCCTTGGTGTCGTTTC
Tnf	GACCCTCACACTCAGATCATTTCTCT	CCTCCACTTGGTGGTTTGCT
Il1b	TTCAGGCAGGCAGTATCACTC	GAAGGTCCAACGCCAACTGA
Il6	AGGATAACCACTCCCTCCAGAAA	TTGCCATTGCACAACCTCTTTTCT
Gapdh	AGGTCGGTGTGAACGGATTGT	TGTAGACCATGTAGTTGAGGTCA



Figure 2. Venn diagram showing the intersection of 194 asiatic acid-associated targets and 2,721 ischemic stroke (IS)-associated targets, yielding 96 shared candidate genes

not imply lower biological importance; PPAR γ remains a candidate node for future mechanistic validation. Overall, the topology suggests that inflammatory regulation, cell survival, metabolism, and apoptosis-related signaling may be closely interconnected in the predicted AA–ischemic stroke target network.

3.3. Gene Ontology enrichment analysis

Gene Ontology enrichment analysis was performed on the 96 overlapping targets to characterize their potential biological functions. A total of 1,769 enriched GO terms were identified, including 1,574 biological process terms, 51 cellular component terms, and 144 molecular function terms. Biological-process enrichment prominently included responses to oxygen levels, decreased oxygen levels, hypoxia, inflammatory responses, rhythmic processes, membrane-potential regulation, catabolic regulation, and environmental stimuli. The cellular-component results distributed the shared targets across mitochondrial respiratory-chain complexes, postsynaptic and synaptic membranes, neuronal cell bodies, membrane rafts/microdomains, dendritic/neuron spines, and related membrane specializations. Molecular-function terms included nuclear-receptor and ligand-activated transcription-factor activity, transcription-coregulator/coactivator binding, ligand-gated ion-channel activity, peptide binding, amyloid-beta binding, and neurotransmitter-receptor-related activities (Figure 4).

Thus, the overlapping target set spans multiple cellular compartments and molecular functions rather than representing a single localized pathway, which is consistent with the multi-process nature of ischemia/reperfusion injury.

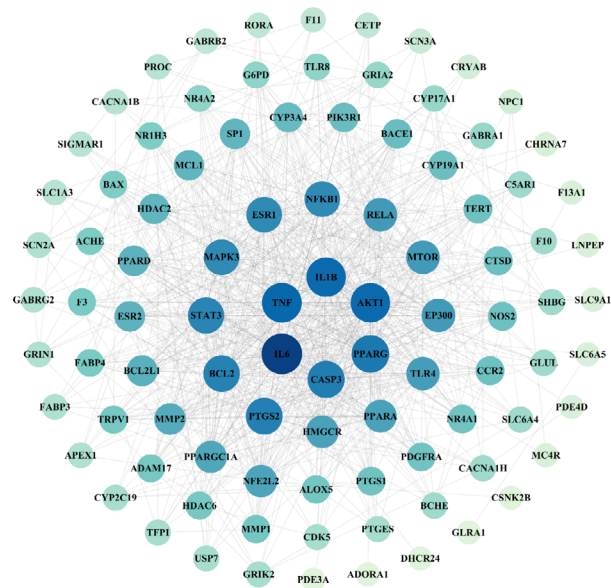


Figure 3. Topological visualization of the protein–protein interaction network. Darker/larger central nodes indicate highly connected candidate hub proteins, including IL6, TNF, AKT1, IL1B, CASP3, PPARG, BCL2, and related targets.

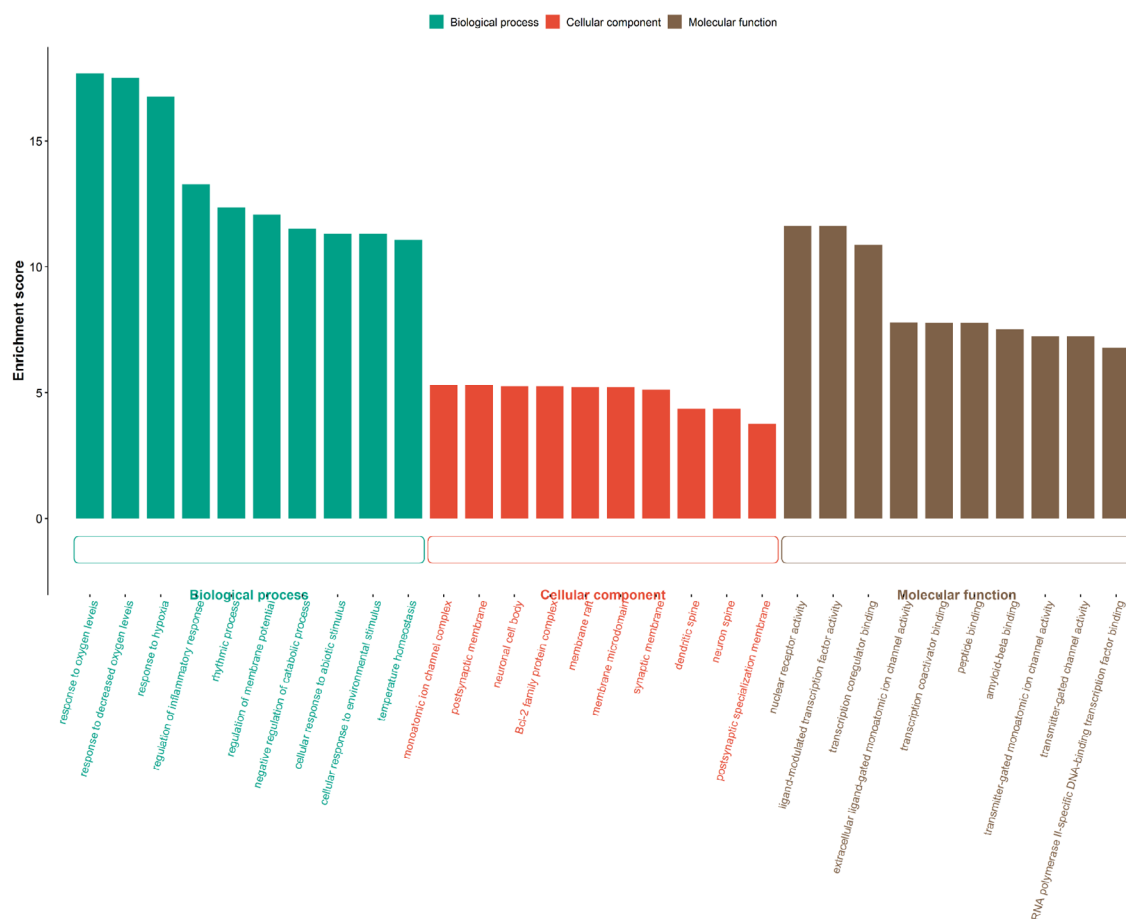


Figure 4. Gene Ontology enrichment analysis of overlapping asiatic acid–ischemic stroke targets. Representative biological process, cellular component, and molecular function terms are shown.

3.4. KEGG pathway enrichment analysis

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis identified 146 pathways associated with the overlapping AA–ischemic stroke targets. The top 20 bubble plot summarizes pathway gene count (dot size) together with clusterProfiler-adjusted p -value, p_{adjust} (color), whereas the original enrichment screen retained pathways at nominal $p < 0.05$ (Figure 5). Among the enriched categories, HIF-1 signaling, TNF signaling, apoptosis, and several neurodegeneration-related pathways were particularly relevant to cerebral ischemia/reperfusion, a condition characterized by oxygen/glucose deprivation, energy failure, oxidative stress, inflammatory signaling, mitochondrial dysfunction, and cell-death responses. Construction of the target–pathway network further demonstrated that several genes, including *AKT1*, *TNF*, *IL1B*, *IL6*, *CASP3*, *NFKB1*, *RELA*, and *BCL2*, participated

in more than one enriched pathway (Figure 6). This pattern supports a coordinated network interpretation rather than an isolated single-pathway mechanism.

3.5. Molecular docking

To further evaluate the structural plausibility of the candidate targets identified through network analysis, AA was docked with *AKT1*, *TNF*, *IL-1 β* , and *IL-6*. The predicted Vina scores ranged from -7.0 to -6.5 kcal/mol, with *IL-1 β* showing the lowest predicted docking score among the four proteins (Table 3). The docking models suggested that AA could be accommodated within the selected binding regions through combinations of hydrogen-bond and hydrophobic interactions. Representative predicted interactions included *ASN54* for *AKT1*; *THR105* and *GLU107* for *TNF*; *GLU37*, *GLN39*, and *VAL41* for *IL-1 β* ; and *THR137* for *IL-6*. The corresponding binding

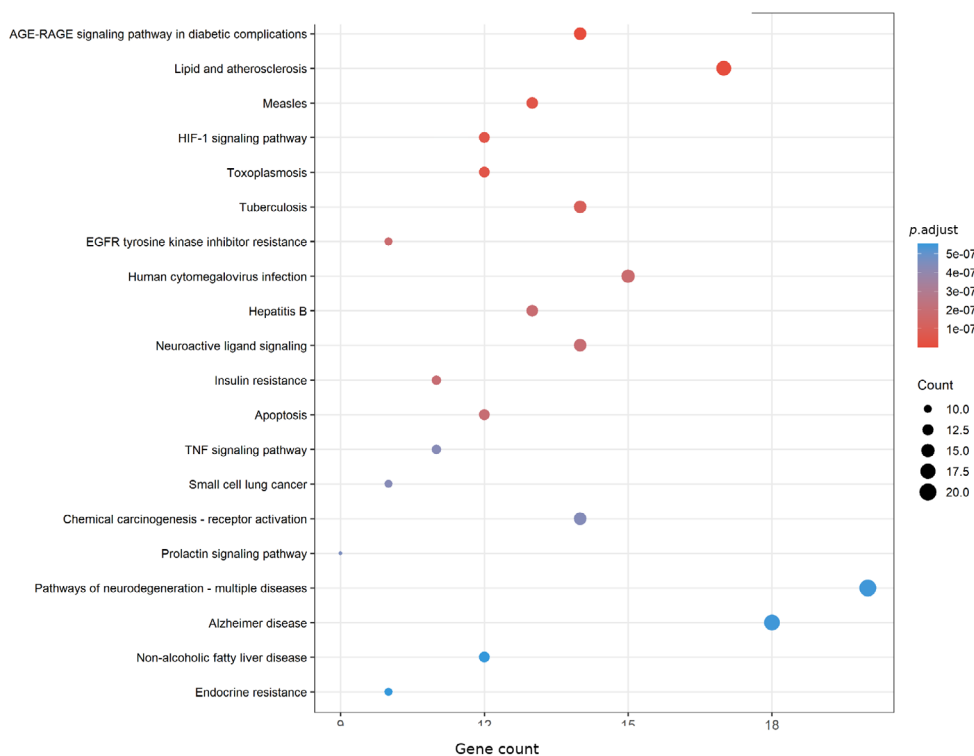


Figure 5. Kyoto Encyclopedia of Genes and Genomes pathway enrichment bubble plot (top 20). Dot size represents gene count, and color represents the multiple-testing-adjusted p-value (p.adjust) reported by clusterProfiler.

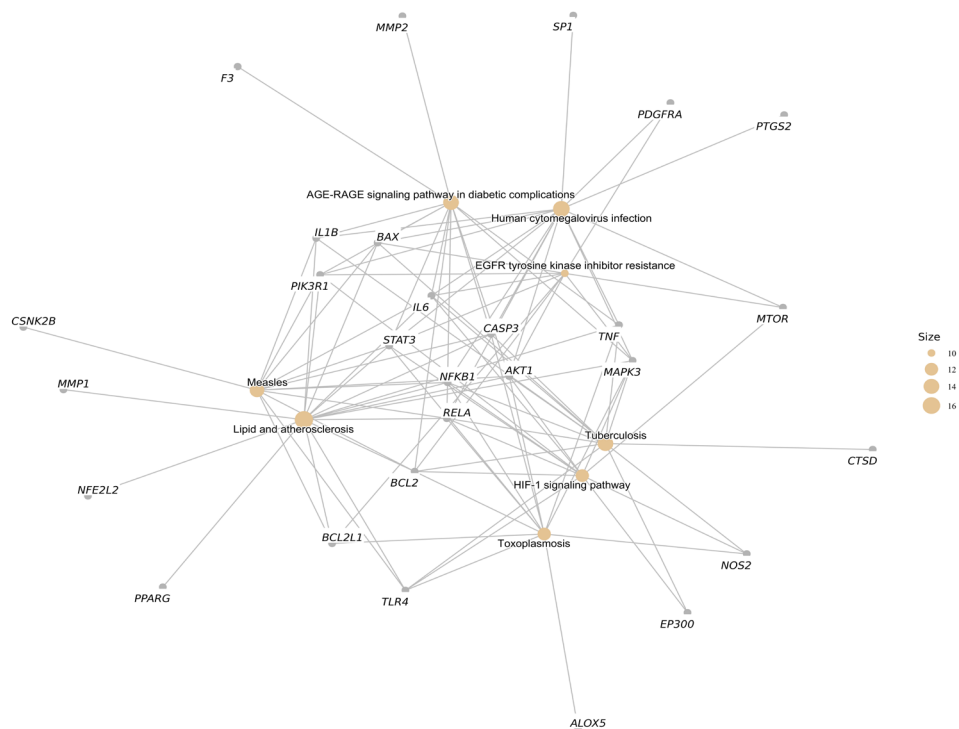


Figure 6. Target-pathway network illustrating shared genes across selected enriched Kyoto Encyclopedia of Genes and Genomes pathways. AKT1, TNF, IL1B, IL6, CASP3, NFKB1, RELA, BCL2, and related genes connect multiple pathway categories.

conformations are shown in **Figure 7**. These results provide structural support for the selection of the four proteins as candidate targets, although the predicted interactions require direct experimental confirmation.

3.6. In vitro experimental validation

3.6.1. Effect of asiatic acid on oxygen-glucose deprivation/reoxygenation-induced HT22 cell viability

To determine whether AA could protect neuronal cells against ischemic-like injury, HT22 cells were subjected to OGD/R and treated with different concentrations of AA. OGD/R markedly reduced cell viability to $71.85 \pm 4.08\%$ of the Control level, confirming successful establishment of a substantial cellular injury model. AA treatment improved cell viability across the tested concentration range, with progressively greater recovery from 2.5 to 10 μM . In particular, 10 μM AA restored cell viability to $94.85 \pm 3.28\%$, showing a significant protective effect compared with the OGD/R Model group ($p < 0.01$). At 20 μM , viability remained significantly higher than in the Model group ($90.12 \pm 3.95\%$; $p < 0.01$) but was lower than

at 10 μM . Because the present screening experiment was not designed to establish a toxicity mechanism and no dedicated cytotoxicity assays were performed, this modest decrease should not be interpreted as evidence of toxicity. Rather, the data indicate that the protective response did not increase monotonically beyond 10 μM within the tested range. Therefore, 10 μM , which produced the greatest observed recovery, was selected for the subsequent ELISA and qRT-PCR experiments (**Table 4**; **Figure 8**).

3.6.2. Effect of asiatic acid on TNF- α , IL-6, and IL-1 β release

The inflammatory response to OGD/R was evaluated by measuring TNF- α , IL-6, and IL-1 β in the culture supernatant. OGD/R produced a pronounced increase in all three pro-inflammatory cytokines compared with the Control group (all $p < 0.001$), indicating that the injury model was accompanied by a strong inflammatory response. Treatment with 10 μM AA significantly reduced TNF- α , IL-6, and IL-1 β levels relative to the OGD/R Model group (all $p < 0.001$; **Table 5**; **Figure 9**). Although cytokine concentrations remained above Control levels, the consistent reduction across all three inflammatory

Table 3. Molecular docking results of asiatic acid with the selected core targets

Target	PDB ID	Vina score (kcal/mol)	Predicted interactions
AKT1	1H10	-6.5	H-bond: ASN54; hydrophobic: ARG48, ILE36
TNF	4TSV	-6.8	H-bonds: THR105, GLU107; hydrophobic: CYS69, LYS112
IL-1 β	1L2H	-7.0	H-bonds: GLU37, GLN39, VAL41; hydrophobic: LYS63, VAL151
IL-6	1ALU	-6.9	H-bond: THR137; hydrophobic: LEU147

Abbreviations: H-bond: Hydrogen bond; IL-1 β : Interleukin-1 β ; PDB: Protein Data Bank; TNF: Tumor necrosis factor.

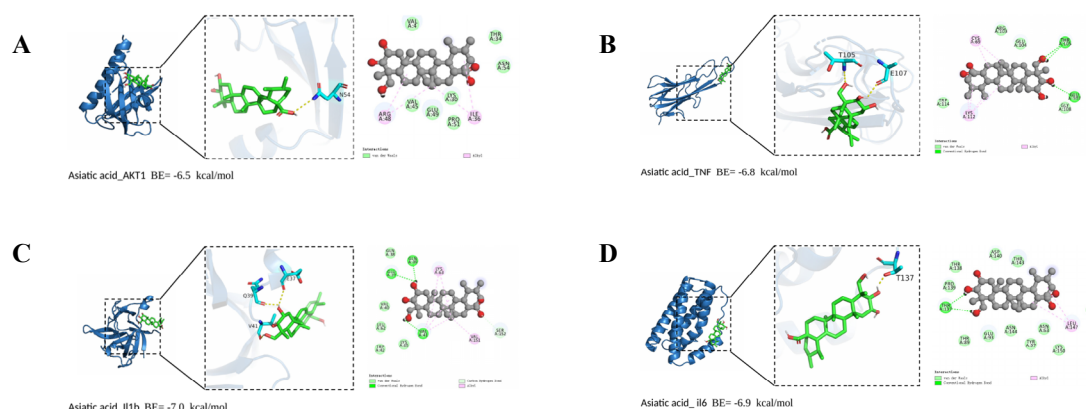
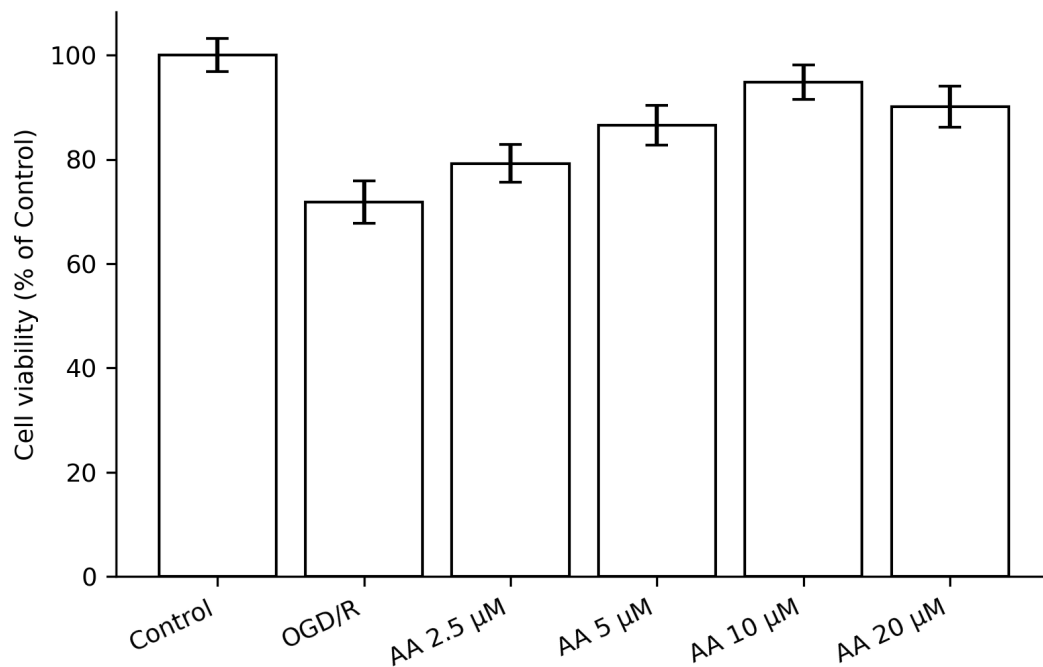


Figure 7. Predicted docking poses of asiatic acid with (A) AKT1, (B) TNF, (C) IL-1 β , and (D) IL-6. Predicted binding energies are indicated within the panels; receptor-template limitations are described in Section 2.7.

Abbreviations: IL-1 β : Interleukin-1 β ; TNF: Tumor necrosis factor.

Table 4. Effects of asiatic acid (AA) on HT22 cell viability after oxygen-glucose deprivation/reoxygenation (OGD/R) (*n* = 6)

Group	AA concentration (μ M)	Cell viability (%)	Statistical comparison
Control	0	100.00 $\pm$ 3.15	–
OGD/R Model	0	71.85 $\pm$ 4.08	$p < 0.01$ vs. Control
OGD/R + AA	2.5	79.24 $\pm$ 3.65	$p < 0.05$ vs. Model
OGD/R + AA	5	86.50 $\pm$ 3.82	$p < 0.01$ vs. Model
OGD/R + AA	10	94.85 $\pm$ 3.28	$p < 0.01$ vs. Model
OGD/R + AA	20	90.12 $\pm$ 3.95	$p < 0.01$ vs. Model

**Figure 8.** Effects of asiatic acid (AA) on the viability of oxygen-glucose deprivation/reoxygenation (OGD/R)-injured HT22 cells. Data are presented as mean $\pm$ SD. The control group was normalized to 100%.**Table 5. Inflammatory cytokine levels in cell supernatants of each group (*n* = 6)**

Indicator	Control	OGD/R Model	Asiatic acid group	ANOVA
TNF- α (pg/mL)	42.50 $\pm$ 4.83	385.20 $\pm$ 28.88	248.60 $\pm$ 17.11	$F(2,15)=465.73; p<0.001$
IL-6 (pg/mL)	28.32 $\pm$ 3.43	520.80 $\pm$ 34.95	315.40 $\pm$ 19.73	$F(2,15)=678.73; p<0.001$
IL-1 β (pg/mL)	15.20 $\pm$ 2.29	186.40 $\pm$ 14.15	118.50 $\pm$ 9.77	$F(2,15)=444.55; p<0.001$

Abbreviations: IL-1 β : Interleukin-1 β ; OGD/R: Oxygen-glucose deprivation/reoxygenation; TNF: Tumor necrosis factor.

mediators indicates that AA substantially attenuated OGD/R-induced inflammatory cytokine release in HT22 cells.

3.6.3. Effect of asiatic acid on candidate target-gene expression

The qRT-PCR analysis revealed clear expression changes in the four candidate genes under the different treatment conditions (Table 6; Figure 10). Gene symbols are presented using mouse nomenclature (*Akt1*, *Tnf*, *Il1b*, and *Il6*), whereas the corresponding cytokine proteins are written as TNF- α , IL-1 β , and IL-6. Compared with the Control group, OGD/R significantly increased the mRNA expression of *Akt1*, *Tnf*, *Il1b*, and *Il6* (all $p < 0.001$), demonstrating a coordinated transcriptional response to ischemic-like injury. AA treatment effectively attenuated these OGD/R-induced alterations. Specifically, the elevated expression levels of *Tnf*, *Il1b*, and *Il6* were significantly reduced after AA treatment, consistent with the changes observed in the corresponding inflammatory cytokine measurements. *Akt1* mRNA expression was also significantly lower in the AA-treated group than in the OGD/R Model group ($p < 0.001$). Collectively, these results indicate that AA partially reversed the OGD/R-induced transcriptional changes in the selected network-predicted targets, providing experimental support for the computational target-screening results.

4. Discussion

The present study employed an integrated strategy combining network pharmacology, PPI topology, GO and KEGG enrichment analyses, molecular docking, and *in vitro* validation to investigate the potential mechanisms of AA in ischemic stroke. The computational analysis identified 96 overlapping targets between AA and ischemic stroke, with IL-6, TNF, AKT1, and IL-1 β emerging as prominent candidate hub proteins. Functional enrichment further connected these targets with hypoxia responses, inflammatory regulation, apoptosis, HIF-1 signaling, and TNF-related pathways. Molecular docking predicted structurally plausible interactions between AA and the selected hub proteins. Importantly, the subsequent HT22 OGD/R experiments showed that AA improved cell viability, reduced inflammatory cytokine release, and attenuated OGD/R-induced transcriptional changes in the selected candidate genes. Thus, the experimental findings were directionally consistent with the major biological themes identified by the computational analyses.

The protective effect observed in the HT22 OGD/R model is consistent with previous preclinical evidence supporting the neuroprotective activity of AA. Earlier studies reported that AA reduced cerebral ischemic injury *in vivo* and protected neuronal cells exposed to oxygen-glucose deprivation, with beneficial effects

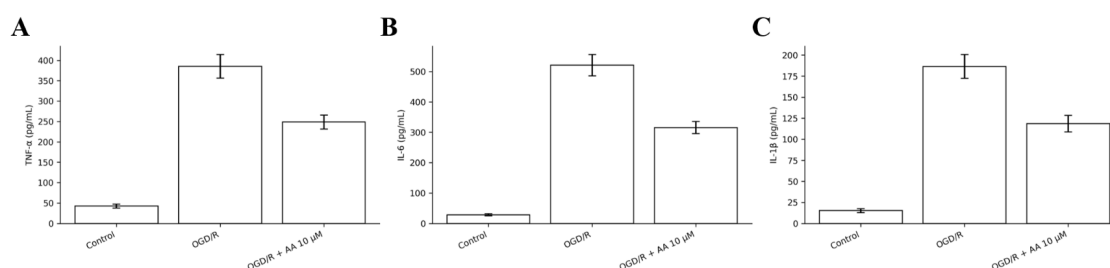


Figure 9. Effects of asiatic acid (AA) on inflammatory cytokine release in oxygen-glucose deprivation/reoxygenation (OGD/R)-injured HT22 cells. (A) Tumor necrosis factor- α (TNF- α), (B) interleukin-6 (IL-6), and (C) IL-1 β concentrations are shown as mean $\pm$ SD ($n = 6$ per group).

Table 6. mRNA expression levels of *Akt1*, *Tnf*, *Il1b*, and *Il6* in cells of each group ($n = 6$)

Gene	Control	OGD/R Model	Asiatic acid group	ANOVA
<i>Akt1</i>	1.000 $\pm$ 0.087	3.850 $\pm$ 0.261	2.418 $\pm$ 0.184	$F(2,15)=333.94; p<0.001$
<i>Tnf</i>	1.000 $\pm$ 0.108	18.420 $\pm$ 1.334	10.150 $\pm$ 0.812	$F(2,15)=557.86; p<0.001$
<i>Il1b</i>	1.000 $\pm$ 0.084	12.650 $\pm$ 0.987	7.483 $\pm$ 0.556	$F(2,15)=474.85; p<0.001$
<i>Il6</i>	1.000 $\pm$ 0.089	25.300 $\pm$ 1.813	13.800 $\pm$ 1.075	$F(2,15)=597.43; p<0.001$

Abbreviations: mRNA: Messenger RNA; OGD/R: Oxygen-glucose deprivation/reoxygenation.

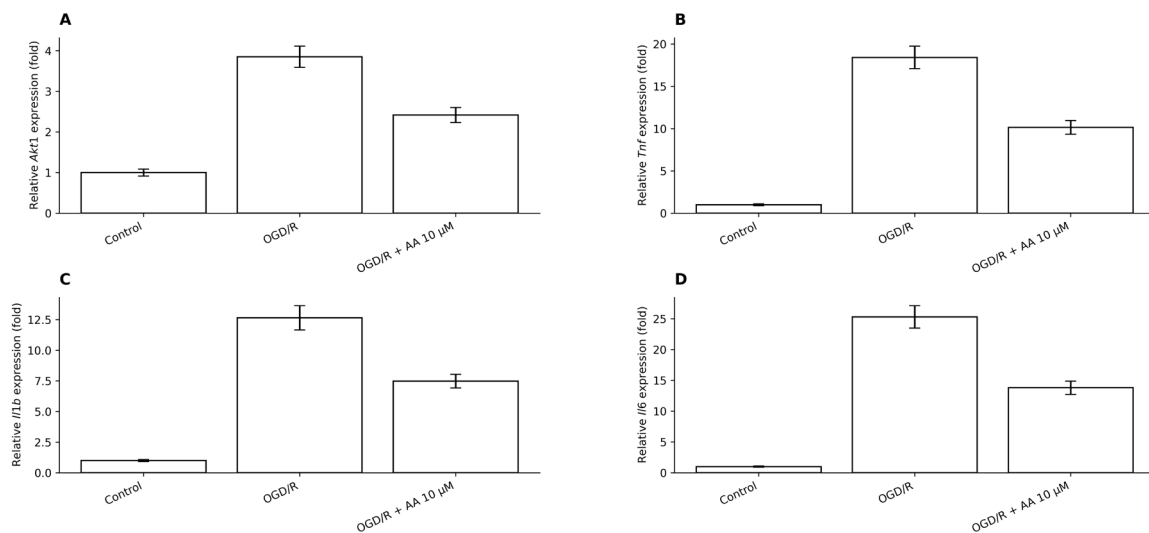


Figure 10. Effects of asiatic acid (AA) on candidate target-gene expression in oxygen-glucose deprivation/reoxygenation (OGD/R)-injured HT22 cells. Relative expression of (A) *Akt1*, (B) *Tnf*, (C) *Il1b*, and (D) *Il6* was determined using quantitative real-time polymerase chain reaction and is shown as mean $\pm$ SD ($n = 6$ per group).

involving mitochondrial function and cell survival.^{5,6} The present results extend these observations by showing a concentration-dependent recovery of HT22 cell viability after OGD/R, with 10 μ M producing the greatest recovery among the tested concentrations. Rather than attributing this cytoprotective effect to a single molecular target, the network results suggest that AA may act through an interconnected set of inflammatory and stress-response proteins. Such a multi-target pattern is biologically plausible in ischemic injury because oxygen-glucose deprivation simultaneously disrupts mitochondrial energy production, redox balance, ion homeostasis, and hypoxia-responsive signaling, while reperfusion can amplify reactive oxygen species generation and inflammatory signaling. These processes are not independent: mitochondrial dysfunction can promote danger signaling and cell-death pathways, inflammatory mediators can further impair mitochondrial and vascular function, and hypoxia/stress-response pathways can modify both survival and inflammatory programs. Accordingly, a compound that influences several connected nodes may produce a coordinated phenotype even when no single node is sufficient to explain the entire response.

Neuroinflammation is a central component of secondary injury following ischemic stroke and involves complex interactions among neurons, glial cells, vascular components, and peripheral immune cells.²¹ Peripheral immune-cell recruitment and blood-brain barrier disruption further contribute to the inflammatory

microenvironment after ischemia.²² In the present study, OGD/R markedly increased TNF- α , IL-6, and IL-1 β release, whereas AA significantly reduced all three cytokines. A similar pattern was observed at the transcriptional level for *Tnf*, *Il6*, and *Il1b*. The concordance between cytokine secretion and mRNA expression strengthens the interpretation that suppression of excessive inflammatory responses is an important component of the protective phenotype observed after AA treatment. These findings are also consistent with the PPI analysis, in which TNF, IL-6, and IL-1 β occupied highly connected positions, indicating that the experimentally measured inflammatory mediators were directly aligned with the principal targets prioritized by the network analysis. Importantly, the coordinated change in several inflammatory mediators emphasizes that post-ischemic inflammation is a network-level process rather than a single-cytokine event. This complexity provides a rationale for investigating therapeutic agents that can modulate multiple interacting inflammatory and stress-response components while avoiding the assumption that suppression of one mediator alone will reproduce the overall protective phenotype.

The HT22 OGD/R model provides a useful neuronal system for examining cellular responses to transient oxygen and glucose deprivation followed by reoxygenation. Published *in vitro* studies have shown that OGD/R can induce neuronal injury together with increased production of TNF- α , IL-1 β , and IL-6.²³ The marked reduction in viability and the strong elevation of these

cytokines in the present model group therefore indicate that the experimental system reproduced key injury and inflammatory features expected under ischemia/reperfusion-like conditions. The ability of AA to improve viability while simultaneously reducing inflammatory mediator release suggests that its effect was not limited to a single biochemical endpoint. Nevertheless, because HT22 cells represent a neuronal cell line, this model cannot reproduce the full multicellular environment of ischemic brain tissue, particularly the contributions of microglia, astrocytes, endothelial cells, and infiltrating immune cells.

Besides, AKT1 was identified as a central PPI node because it occupied a highly connected position in the Cytoscape network and was prioritized by the same topology assessment used for the other candidate hubs (degree, betweenness centrality, closeness centrality, clustering coefficient, and stress). In **Figure 3**, this high-connectivity status is reflected by its prominent central visualization. AKT1 was additionally selected because it provided a survival/stress-response counterpart to the three cytokine-related nodes used for cross-layer validation. The phosphoinositide 3-kinase (PI3K)/AKT signaling axis has been widely implicated in neuronal survival, oxidative stress regulation, inflammation, and apoptosis after cerebral ischemia/reperfusion.²⁴ In the present study, OGD/R increased *Akt1* mRNA expression, whereas AA partially reduced this elevation. This transcriptional change indicates that *Akt1*-associated regulation may participate in the cellular response to OGD/R and AA treatment. However, AKT signaling is strongly affected by protein abundance, subcellular localization, and phosphorylation status. Consequently, the present mRNA data should not be interpreted as direct evidence that AA inhibits or activates the PI3K/AKT pathway. Measurement of total AKT, phosphorylated AKT, and downstream pathway proteins will be necessary to determine the functional direction of this signaling response.

Hypoxia-related biological processes were strongly represented in the GO analysis, and HIF-1 signaling was among the KEGG pathways considered most relevant to ischemic neuronal injury. HIF-1 α -dependent responses have been associated with neuronal adaptation, survival, metabolic regulation, and apoptosis after cerebral ischemia.^{8,9} The enrichment of hypoxia-related terms is therefore consistent with the biological context of the OGD/R model used in this study. At the same time, enrichment analysis identifies statistical overrepresentation of genes and does not demonstrate actual activation of HIF-1 signaling. The current findings should therefore be interpreted as evidence that hypoxia-responsive mechanisms are plausible components of the

AA-associated target network, rather than proof that HIF-1 is a directly regulated pathway.

Molecular docking was used to determine whether the network-prioritized proteins could also show potential structural interactions with AA. The four-protein panel was chosen to align computational prioritization with the experimental validation strategy: TNF, IL-6, and IL-1 β corresponded to the inflammatory mediators measured at both protein and transcript levels, while AKT1 represented a highly connected survival/stress-response hub. PPAR γ and other central proteins were not excluded on biological grounds; rather, the experimental scope was limited to a focused panel that could be evaluated coherently across network, docking, cytokine, and qRT-PCR layers. The receptor templates also impose important interpretive limitations: 1H10 represents an isolated AKT1 pleckstrin-homology domain, whereas 4TSV and 1L2H are mutant TNF- α and IL-1 β structures, respectively. These structural choices are therefore suitable only for a hypothesis-generating compatibility screen and do not establish binding to full-length wild-type proteins. All four selected proteins produced negative Vina scores within a relatively narrow range, and the predicted poses included hydrogen-bond and hydrophobic contacts. These results provide a structural rationale for considering AKT1, TNF, IL-1 β , and IL-6 as candidate AA-associated proteins. However, docking scores are computational estimates and should not be interpreted as experimentally measured binding affinities or as definitive evidence of direct binding.²⁵ As an interpretive benchmark, the present docking results should be compared primarily within the same protocol rather than against absolute affinity thresholds, because predicted scores and poses can change with receptor structure, protonation and preparation, binding-site definition, grid placement, and the conformational states allowed for the ligand and receptor. No experimentally validated positive-control ligand was docked under identical settings in the present workflow; therefore, the current scores are not a calibrated affinity benchmark. A stronger future benchmark would include re-docking of co-crystallized ligands where applicable, comparison across alternative receptor conformations or binding-site definitions, and assessment against experimentally measured binding or target-engagement data. Direct target-engagement studies, such as biochemical binding assays or other orthogonal approaches, will therefore be required to confirm whether AA physically interacts with these proteins under biological conditions.

More broadly, the present study illustrates the value of combining network pharmacology with experimental validation. Network pharmacology can organize complex

compound–target–disease relationships and prioritize candidate mechanisms for further testing, but network connectivity alone cannot establish pharmacological causality.²⁶ In this study, the major advantage of the integrated design is the convergence of several independent levels of evidence: inflammatory targets were highly connected in the PPI network, inflammation-related pathways were enriched, AA showed potential docking interactions with representative hub proteins, and the cellular experiments demonstrated reductions in both inflammatory cytokine release and corresponding gene expression. This convergence strengthens the biological coherence of the proposed mechanism. On this basis, a testable multi-target neuroinflammatory network can be operationally defined as the focused set of IL-6, TNF, IL-1 β , and AKT1 nodes together with their linked inflammatory, hypoxia/stress-response, and survival/cell-death processes. The model generates specific falsifiable predictions: AA-associated protection should be accompanied by coordinated changes at these nodes; perturbation of individual nodes or pathway branches should modify part of the AA response; and protein-level or target-engagement measurements should determine whether the transcript/cytokine changes correspond to direct or downstream regulation. This framework converts the broad network prediction into a tractable experimental program while recognizing that additional nodes such as PPAR γ , CASP3, NF κ B1, RelA, and BCL-2 may contribute. Nevertheless, the present data support a multi-target mechanistic hypothesis rather than a fully established causal pathway, and further pathway-specific experiments are needed before individual targets can be defined as direct mediators of AA action.

From a translational perspective, the convergence of IL-6, TNF, IL-1 β , and AKT1 across network topology, pathway enrichment, docking, cytokine measurements, and transcript-level validation provides a more informative framework than a binary conclusion that AA is simply effective. The three cytokine-related nodes define a coherent inflammatory module, whereas AKT1 links this module to broader survival and stress-response signaling. These molecules can therefore be prioritized as candidate therapeutic targets for mechanistic perturbation studies and as hypothesis-generating biomarker candidates for future studies examining pharmacodynamic response or disease stratification. Importantly, the present cell-based data are insufficient to establish biomarker performance, specificity, or clinical utility. Their value lies in narrowing a complex predicted network to a tractable set of experimentally supported nodes that can be tested in multicellular systems, animal models, and patient-derived samples. This network-oriented interpretation also creates a rationale for future combination or multi-

target strategies designed to modulate inflammatory injury without assuming that any single node fully accounts for the effects of AA.

Several limitations should be considered when interpreting the present findings. First, network pharmacology and molecular docking are prediction-based approaches and cannot independently establish direct target engagement or causal pathway regulation. Second, the experimental validation was limited to a single HT22 OGD/R model. Although this model is suitable for examining neuronal injury, it does not reproduce the multicellular neurovascular and immune environment of ischemic stroke. Third, the mechanistic validation focused on inflammatory cytokine release and mRNA expression. Protein expression, phosphorylation status, apoptosis-related proteins, and direct pathway activity were not assessed. Finally, the protective effect of AA has not yet been confirmed in an *in vivo* ischemic stroke model within the present study. Future work should therefore incorporate protein-level validation, target or pathway perturbation, direct binding studies, additional cellular systems, and animal models to establish a more complete mechanistic evidence chain.

5. Conclusion

In conclusion, the integrated network pharmacology, enrichment analysis, molecular docking, and *in vitro* validation performed in this study support a multi-target model of AA-associated neuroprotection against OGD/R-induced neuronal injury. Rather than indicating a single dominant mechanism, the convergent evidence identifies IL-6, TNF, IL-1 β , and AKT1 as interconnected candidate nodes linking neuroinflammation, hypoxia-responsive signaling, apoptosis, and cell-survival processes. In HT22 cells, AA improved viability while reducing inflammatory cytokine release and partially reversing OGD/R-induced transcriptional changes in these network-prioritized targets. These findings nominate IL-6, TNF, IL-1 β , and AKT1 for further evaluation as therapeutic targets and hypothesis-generating biomarker candidates, but they do not establish direct target engagement or clinical biomarker validity. Protein-level validation, pathway perturbation, direct binding studies, multicellular and *in vivo* models, and ultimately clinical studies will be required to determine their mechanistic and translational relevance. By defining a testable neuroinflammatory network rather than simply demonstrating a protective phenotype, this study provides a framework for developing and evaluating multi-target therapeutic strategies for ischemic stroke.

Acknowledgments

None.

Funding

The authors received no specific funding for this work.

Conflict of interest

The authors declare no conflicts of interest.

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Conceptualization: Kok Hong Tang

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Formal analysis: Junjun Gong

Investigation: Junjun Gong

Methodology: Junjun Gong

Supervision: Kok Hong Tang

Visualization: Junjun Gong

Writing—original draft: Junjun Gong

Writing—review & editing: Kok Hong Tang

Both authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Further disclosure

Generative AI tools were used during manuscript preparation for language editing, organization, and formatting assistance. The authors are responsible for verifying the scientific content, source data, references, analyses, interpretations, and final manuscript.

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