Research Paper

Therapeutic targets and molecular mechanism of calycosin for the treatment of cerebral ischemia/reperfusion injury

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ABSTRACT

This study was designed to understand the pivotal anti-cerebral ischemia/reperfusion injury (CIRI) targets and pathways of calycosin through network pharmacology and molecular docking analyses. In this study, bioinformatics tools were employed to characterize and identify the pharmacological functions and mechanisms of calycosin for CIRI management. The network pharmacology data identified potential, merged CIRI-associated targets of calycosin including tumor protein p53 (TP53), protein kinase B (AKT1), vascular endothelial growth factor A (VEGFA), interleukin 6, tumor necrosis factor (TNF), and mitogen-activated protein kinase 1 (MAPK1). Molecular docking analysis indicated the binding efficacy of calycosin with three of the targets, namely TP53, AKT1, and VEGFA. The biological processes of calycosin for the treatment of CIRI are mainly involved in the improvement of endothelial cell proliferation and growth, inflammatory development, and cellular metabolism. In addition, the anti-CIRI actions of calycosin were primarily through suppression of the toll-like receptor, PI3K-AKT, TNF, MAPK, and VEGF signaling pathways. Taken together, the current bioinformatic findings revealed pivotal targets, biological functions, and pharmacological mechanisms of calycosin for the treatment of CIRI. In conclusion, calycosin, a functional phytoestrogen, can be potentially used for the treatment of CIRI in future clinical trials.

INTRODUCTION

Cerebral ischemia/reperfusion injury (CIRI), a type of brain injury, can cause severe damage to encephalic cells and impair their function [1]. Some common pathological manifestations detected in patients with CIRI include hydrocephalus, intracephalic necrosis, and cellular inflammatory infiltration [2]. The histopathological changes in CIRI may be short term or extended based on the hypoxemic degree of hypoxia in brain tissue. Any oxygen-deficient condition will be detrimental and lethal for the tissue as the brain is highly sensitive to hypoxia [3]. Globally, the geriatric population has been on the rise in recent decades, and the incidence of CIRI in elderly people has increased significantly resulting in a high rate of cerebrovascular

disorders [4]. Epidemiological data show that cases of CIRI are mounting in hospitals, especially in less developed countries, where socioeconomic factors are poor [5]. In China, the number of cases of CIRI is higher than that in other countries and is characterized by a large economic burden [6]. Current drug therapies for CIRI management include free radical scavengers (such as edaravone), excitatory amino acid antagonists (such as coumarin), calcium channel blockers (such as nimodipine), and anti-inflammatory agents (such as lovastatin) [7]. However, the undesired effects of these pharmacotherapies commonly encountered during clinical treatment of CIRI patients are a big challenge. Natural anti-CIRI molecules can be good alternative treatment strategies to circumvent these complications and should be screened and identified accordingly. Historically, many traditional Chinese medicines have commonly been used for prophylaxis and treatment of endemic diseases, such as thyroncus and malaria [8]. Calycosin, a pharmacologically bioactive compound, has potent anti-inflammatory and antioxidant properties [9]. It has been reported that calycosin may exert potent anti-tumor activity against colorectal cancer [10]. Interestingly, the neuroprotective benefits of calycosin have been validated both *in vivo* and *in vitro* [11]. Guo et al. (2012) have established the role of calycosin as an anti-CIRI molecule in rats through induction of antioxidant effects [12]. However, the mechanism of calycosin action for treatment of CIRI remains unclear. An attractive methodology using network pharmacology and molecular docking has been applied to the discovery of therapeutic bio-targets and action pathways of bioactive compounds [13–14]. Our previous bioinformatics findings illustrate potential roles of some bioactive agents in the treatment of diseases, including calycosin against meningitis, vitamin C against COVID-19, curcumol against interstitial cystitis, and plumbagin against liver cancer [15–18].

In the present study, we used bioinformatics tools including network pharmacology and molecular docking to uncover the pharmacological functions and therapeutic mechanisms of calycosin as an anti-CIRI agent. A flow chart was created to summarize the main findings of calycosin action for the treatment of CIRI, obtained via bioinformatics analysis (Figure 1).

MATERIALS AND METHODS

Harvesting the genes of calycosin-anti-CIRI target

All calycosin-putative genes were identified through a series of traditional Chinese medicine systems pharmacology (TCMSP), Swiss Target Prediction, and SuperPred databases. Meanwhile, other CIRI-associated pathogenic genes were collected using the DisGeNET and Genecards databases. Calycosin and CIRI-related genes were merged using the UniProt database. After re-determination using the Funrich software, the calycosin-anti-CIRI targets were screened and specified as described in previous reports [19–20].

Ascertaining the pivotal targets of calycosin-anti-CIRI

All merged genes of calycosin-anti-CIRI targets were used for re-analysis to create a target-functional network via the STRING database following an associated algorithm [21]. Using Cytoscape software, all identified genes of calycosin-anti-CIRI targets were employed to plot a network of protein-protein interactions (PPI). After testing with topological parameters in the NetworkAnalyzer setting, all pivotal targets of calycosin-anti-CIRI were identified, and these key targets were visualized in a network diagram [22].

Analyses of key targets in biological functions and molecular mechanisms

The pharmacological functions and signaling pathways of all pivotal targets of calycosin-anti-CIRI were identified and uncovered using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) database. Subsequently, pivotal targets were determined using Cytoscape software to create a network diagram of anti-CIRI targets of calycosin. After determining the log P value using the OmicShare tool, the advanced bubble diagrams used for biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG)-based molecular pathways were detailed and visualized, as reported previously [23–24].

Molecular docking of calycosin with anti-CIRI targets

In brief, pivotal targets including tumor protein p53 (TP53), protein kinase B (AKT1), and vascular endothelial growth factor A (VEGFA) were screened and used for calycosin-associated molecular docking. The structure of these target proteins was retrieved from protein-data-bank (PDB) database with PDB IDs 2MWO, 3O96, and 5T89, respectively. Calycosin was docked to these proteins at their respective binding sites where their ligands are bound. The three-dimensional structure of calycosin prior to docking was demonstrated using ChemBio3D Draw setting of Chem Bio Office 2010 software. The software AutoDock Vina was used for all dockings and the molecular docked

structures of calycosin with the target proteins of TP53, AKT1, and VEGFA were viewed using MGLTools in Autodock Vina software. The accuracy of the docking parameters was determined and identified using the root mean square deviation (RMSD) of the ligand molecules. The setting with an RMSD <4 Å is denoted as the threshold to conform to ligand molecules [25–26].

RESULTS

Harvested targets of calycosin and CIRI

After analyses of databases, 407 anti-diseased targets of calycosin and 198 diseased targets of CIRI were identified. In addition, a total of 51 merged targets of calycosin and CIRI are shown in a Venn diagram. The correlation network connected with these merged targets is shown in Figure 2.

Cluster analysis findings

In the following analysis, the sub-network clusters obtained by the MCODE algorithm in Cytoscape software are shown in Figure 3. The predictive targets of RFC2, PFC3, RFC4, RFC5, FEN1, LIG1, PCNA, and APEX1 in calycosin against CIRI were clustered as a category. Other FADD, TNFRSF10A, TNFRSF10B, MDM2, PIK3CA, and ESR2 targets in calycosin against CIRI were grouped as another category.

Pivotal target findings

Based on the topological data and degree value to screen pivotal targets, the parameters of mean and large degrees of freedom in calycosin against anti-IRI target proteins were standardized. As a result, six core CIRI-associated targets of calycosin were screened and identified, including TP53, AKT1, VEGFA, interleukin 6 (IL6), tumor necrosis factor (TNF), and mitogen-activated protein kinase 1 (MAPK1) (Figure 4).

Biological functions and anti-CIRI pathways

We revealed the pharmacological mechanisms of calycosin for management of CIRI using enrichment analysis with pivotal targets. An advanced bubble chart showed top 20 biological processes of calycosin that can possibly be involved for treatment of CIRI, including its metabolic and apoptotic functions. The detailed mechanisms of calycocin action were determined from the enrichment analysis findings that revealed top 20 anti-CIRI pathways including modulation of cell proliferation and inflammation, and improvement of intracellular microenvironment (Figure 5). More data with a total of 29 biological processes and 78 molecular pathways of calycosin for CIRI management are provided in Supplementary Table 1 and Supplementary Table 2 respectively.

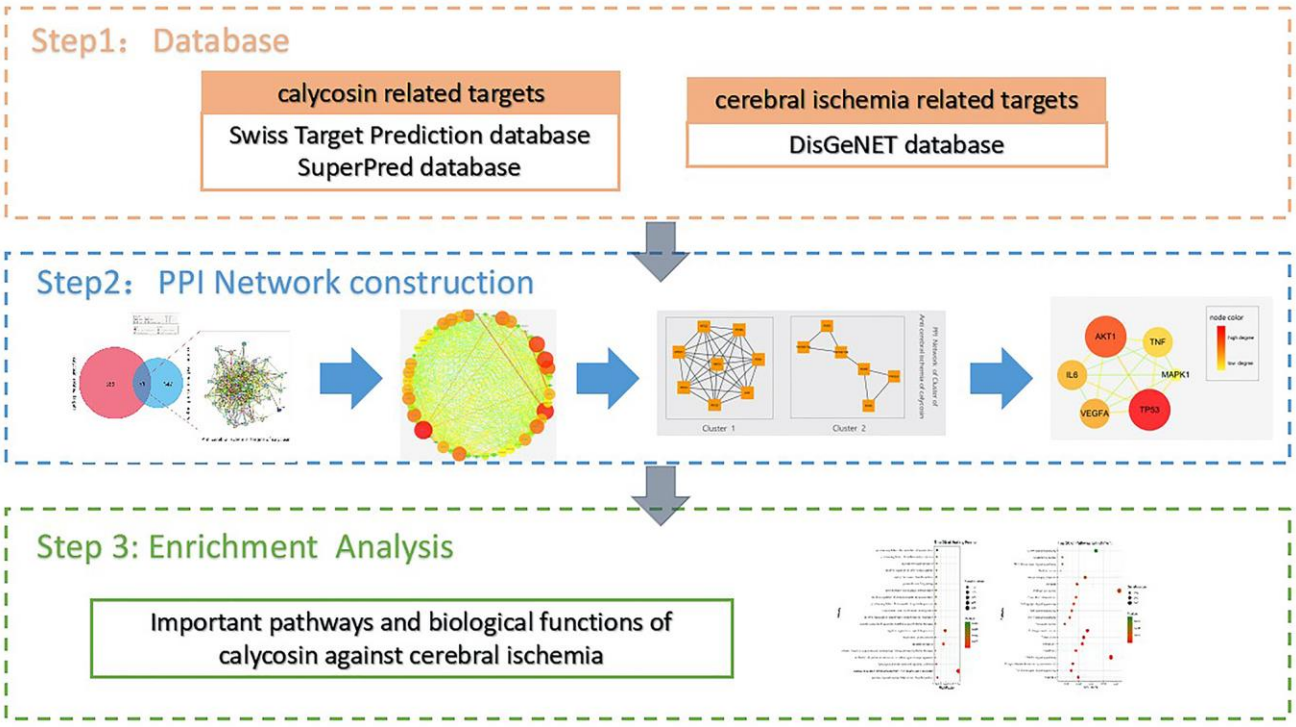

Figure 1. Flowchart of the current bioinformatics tools used for this study to reveal the pivotal targets and molecular mechanisms underlying the anti-CIRI action of calycosin through network pharmacology and molecular docking methods.

Molecular docking findings

The RMSD value of MLY, the original bound ligand in the TP53 protein (2MWO) was 2.675 Å. The ligand was bound at the target site through hydrogen bonding with the amino acid residues of the protein including ASP-1521 (2.7 Å), LYS-372 (2.6 Å), GLN-375 (1.8 Å), and LYS-373 (2.3 Å). In the calycosin-docked protein, calycosin was found to make hydrogen bonds with amino acid residues of ASP-1521 (2.5 Å), LYS-373 (2.4 Å), and SER-371 (2.5 Å) (Figure 6A). In AKT1 (3O96), the RMSD value of IQ0, the original ligand, was 1.024 Å. The hydrogen bonding of the ligand with the binding site amino acid residues of the protein included VAL-271 (3.2Å), ASN-54 (2.9 Å), LYS-268 (2.6 Å), SER-205 (2.5 Å), and TYR-272 (3.2 Å).

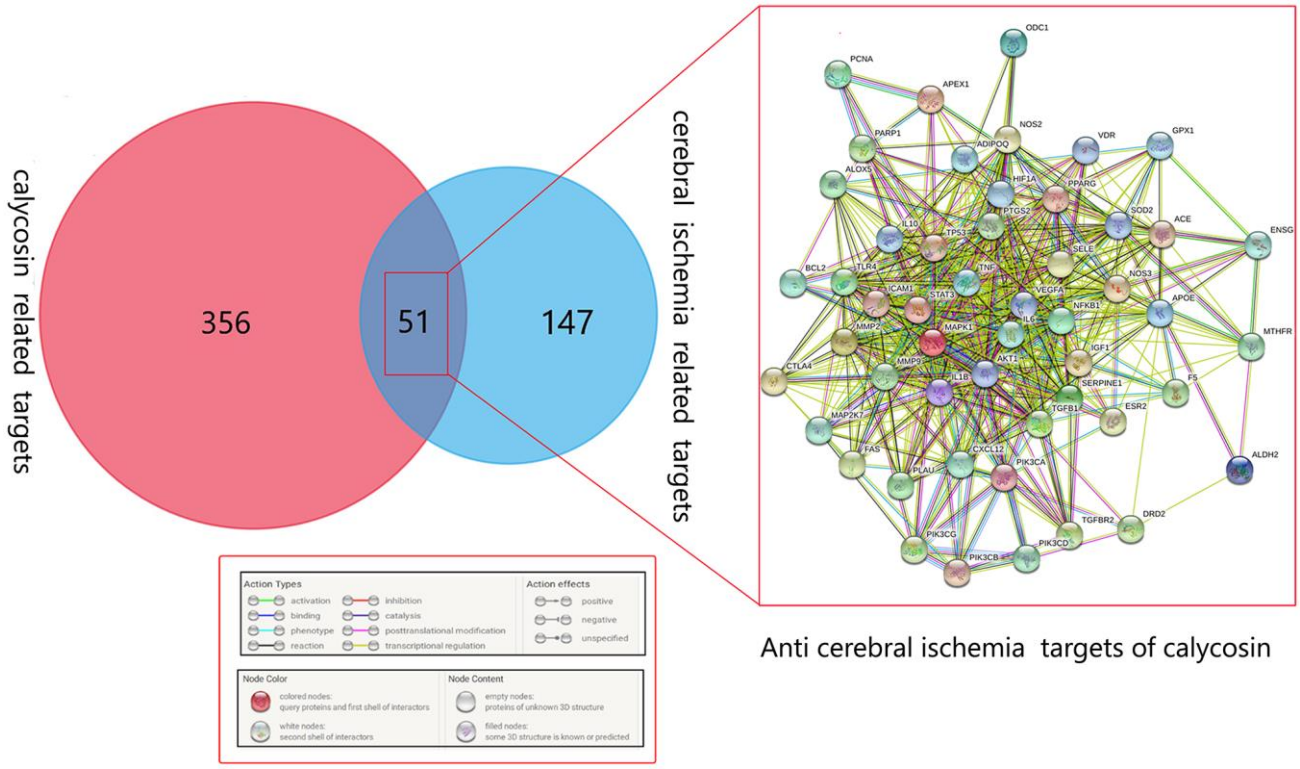

Figure 2. Venn diagram showing all the common, merged targets of calycosin and CIRI. All merged targets are interrelated in the network diagram.

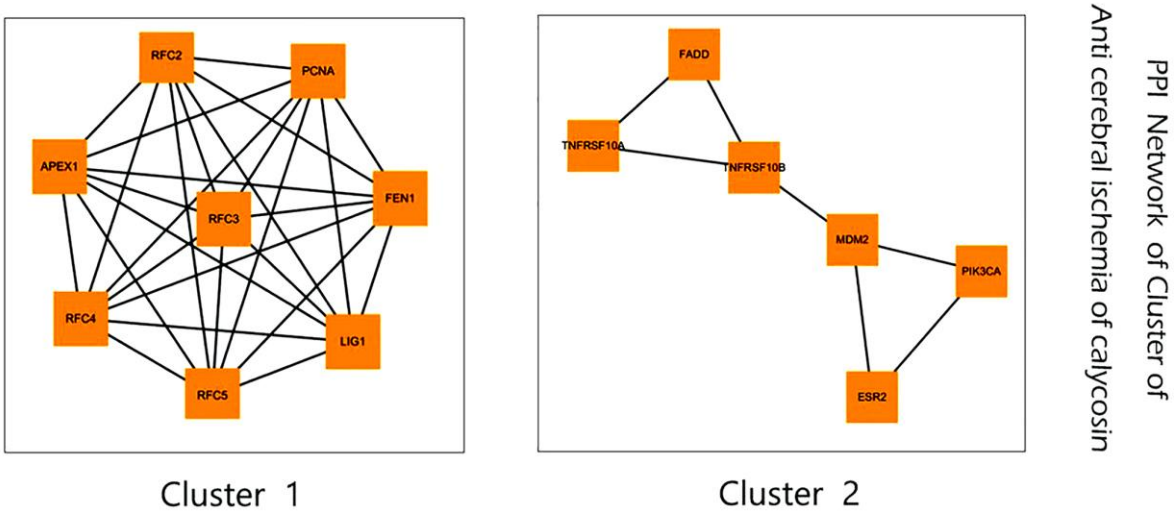

Figure 3. Subnetwork clusters of identified targets for calycosin against CIRI obtained using MCODE algorithm.

Calycosin formed hydrogen bonds with the amino acid residues of VAL-271 (3.2 Å), ASN-54 (2.9 Å), LYS-268 (2.4 Å), SER-205 (2.4 Å), TYR-272 (3.3 Å), and ASP-292 (2.3 Å) (Figure 6B). In the VEGFA protein (5T89), the RMSD value of the original ligand, NAG, was 3.130 Å. The amino acid residue of the protein involved in hydrogen bonding with the ligand was GLN-342 (3.2 Å). Calycosin was involved in docking

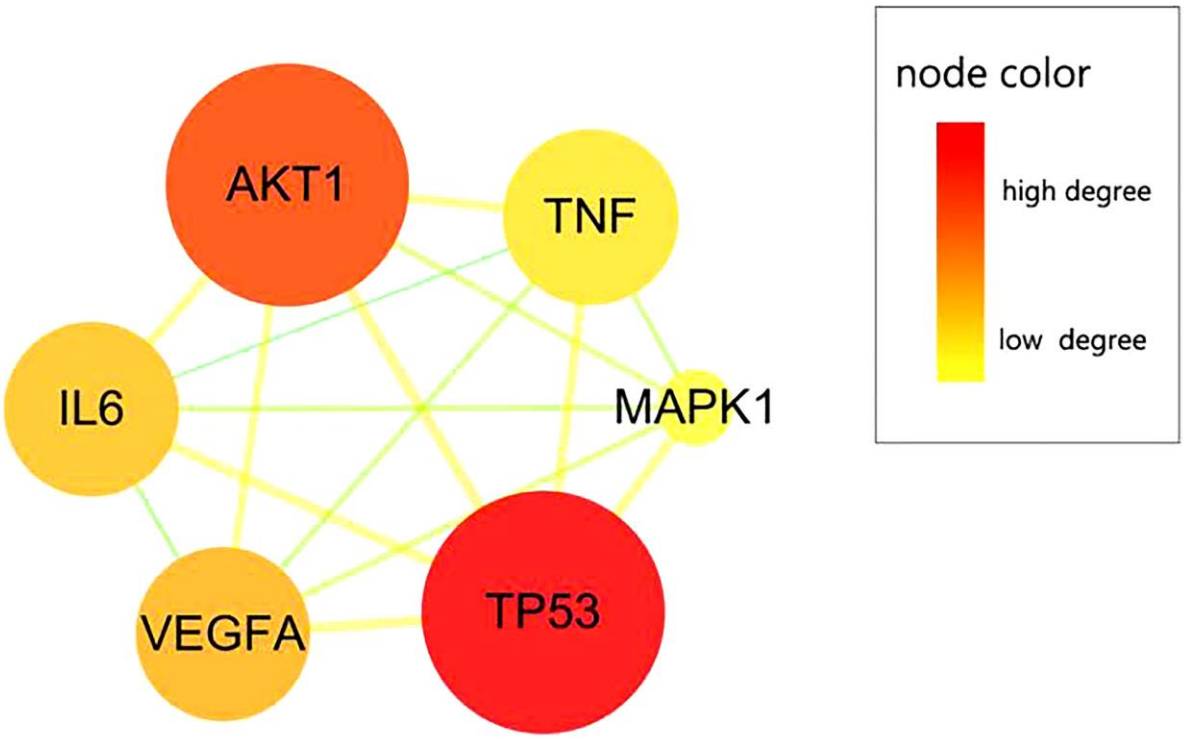

Figure 4. CIRI-related pivotal targets of calycosin. Six pivotal targets were screened and identified from merged targets, namely TP53, AKT1, VEGFA, IL6, TNF, and MAPK1.

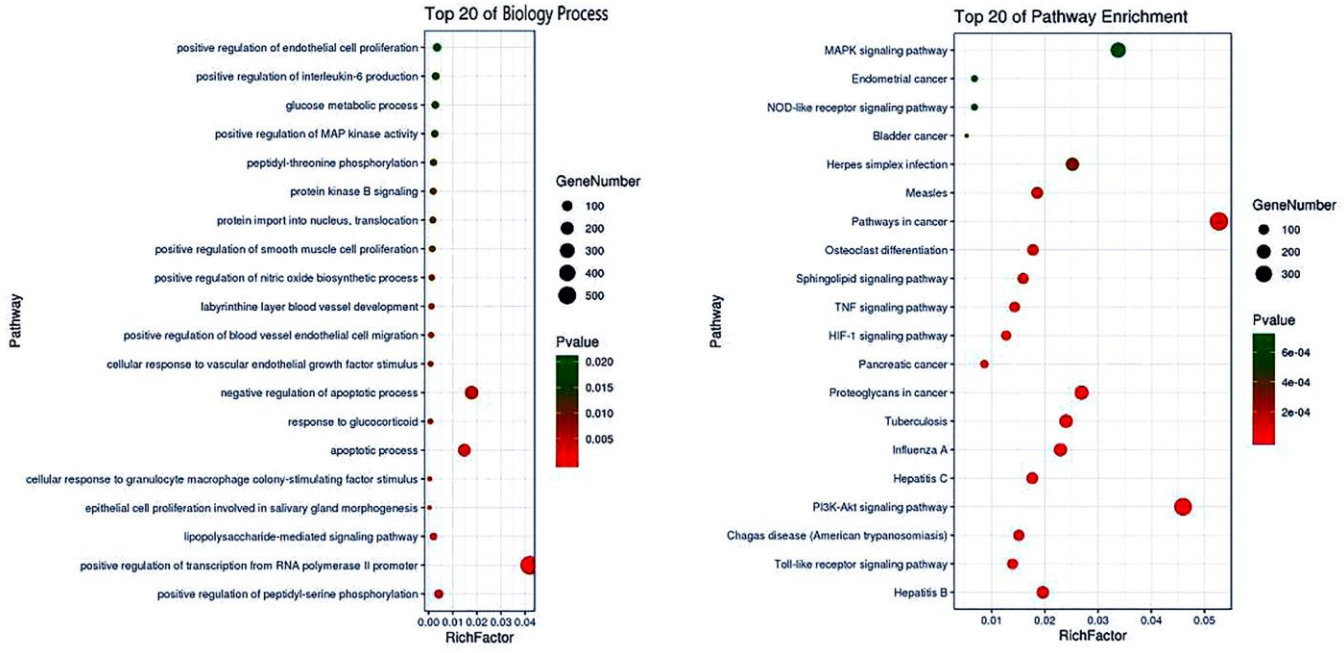

Figure 5. Top 20 pharmacological processes and molecular pathways of calycosin for CIRI management. Based on enrichment analysis, both pharmacological processes and molecular pathways were revealed to understand the mechanism of action of calycosin for the treatment of CIRI.

interaction with the amino acid residues, GLN-342 (2.6 Å) and THR-421 (2.0 Å) after being docked to VEGFA protein (Figure 6C).

DISCUSSION

Our current findings via bioinformatics analyses using network pharmacology and molecular docking revealed pivotal targets, biological functions, and molecular pathways of calycosin involved in CIRI management. We identified a total of six pivotal CIRI-associated targets of calycosin, including TP53, AKT1, VEGFA, IL6, TNF, and MAPK1. Moreover, molecular docking analysis demonstrated efficient binding of calycosin with thee of the targets, namely TP53, AKT1, and VEGFA. These findings strongly implicate an anti-CIRI

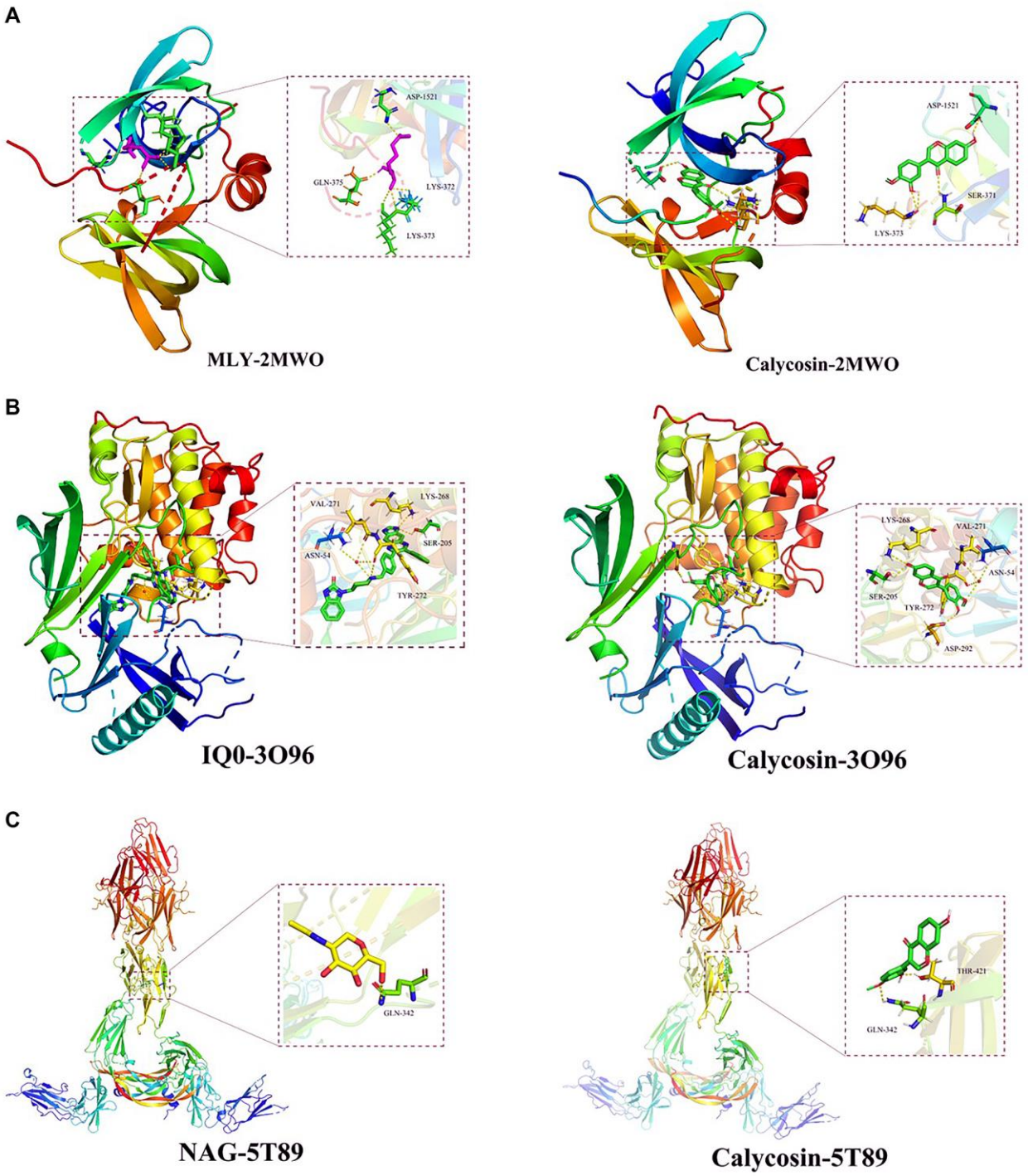

Figure 6. Docking poses of calycosin on three identified targets. By using molecular docking analysis, the data demonstrated that effective binding capacities of calycosin with CIRI were identified in (**A**) TP53 (2MWO), (**B**) AKT1 (3O96), and (**C**) VEGFA (5T89) targets.

action of calycosin. TP53, a well-reported anti-oncogene, can suppress intracellular DNA injury or genomic aberrations that are responsible for cell cycle arrest and cell growth [27]. When mutated, the variant TP53 in diseased tissues may induce tumorigenesis, causing human tumor growth [28]. Increasing evidence shows a neuroprotective effect of activated TP53 against spiral ganglion neuron injury in mice through regulation of the Wnt signaling pathway [29]. AKT1, a protein kinase, plays an essential role in controlling cell survival and apoptosis [30]. It has been experimentally found that activation of AKT1 signaling may inhibit neuro-degeneration in amyloid β-deposited brains in rats [31]. VEGFA, a vascular endothelial growth factor, is involved in regulating vascular endothelial cell growth, vascular permeability, and angiogenesis [32]. Some data indicate that VEGFA overexpression in Müller cells, the principal glial cells, may promote retinal dysfunction [33]. Although current evidence establishes a role of these targets in neuroprotection, reports indicating roles of their genes in anti-CIRI actions are limited. Therefore, it is reasoned that TP53, AKT1, and VEGFA genes may function as effective neuroprotection agents against CIRI. Enrichment analysis revealed that the biological processes of calycosin for CIRI management are involved in the amelioration of endothelial cell proliferation and growth, inflammatory development, and cellular metabolism. These functions might be primarily responsible for the pharmacological action of calycosin in the treatment of CIRI. Mechanically, the anti-CIRI action of calycosin is via inhibition of the toll-like receptor, PI3K-AKT, TNF, MAPK, and VEGF signaling pathways. These data indicate that calycosin might contribute to the suppression of neuroinflammation, neural lesion/necrosis, and vascular degeneration. Despite absence of any experimental validation, our current bioinformatics findings indicate that calycosin may be a promising candidate for the treatment of CIRI.

CONCLUSION

In conclusion, the pivotal targets, biological functions, and molecular mechanisms of calycosin related to the treatment of CIRI are revealed through bioinformatics tools using network pharmacology and molecular docking. Furthermore, pharmacological targets, including TP53, AKT1, and VEGFA, have been identified before any experimental validation.

AUTHOR CONTRIBUTIONS

Bin Yang contributed to the conception, design of the manuscript. Songzuo Yu, Ka Wu, Yujia Liang, Haitao Zhang, Chao Guo contributed to the acquisition, analysis, and interpretation of data in this manuscript. Songzuo Yu, Bin Yang drafted this manuscript. Bin Yang revised this manuscript. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to this study.

FUNDING

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SUPPLEMENTARY MATERIALS

Supplementary Tables

Supplementary Table 1.

Term	Pop Hits	Pop Total	*P* Value
positive regulation of peptidyl-serine phosphorylation	58	13824	1.39E-06
positive regulation of transcription from RNA polymerase II promoter	580	13824	4.30E-05
lipopolysaccharide-mediated signaling pathway	28	13824	5.90E-05
epithelial cell proliferation involved in salivary gland morphogenesis	5	13824	0.002169
cellular response to granulocyte macrophage colony-stimulating factor stimulus	6	13824	0.002602
apoptotic process	204	13824	0.003126
response to glucocorticoid	10	13824	0.004333
negative regulation of apoptotic process	246	13824	0.004513
cellular response to vascular endothelial growth factor stimulus	11	13824	0.004766
positive regulation of blood vessel endothelial cell migration	14	13824	0.006062
labyrinthine layer blood vessel development	16	13824	0.006926
positive regulation of nitric oxide biosynthetic process	18	13824	0.007789
positive regulation of smooth muscle cell proliferation	21	13824	0.009082
protein import into nucleus, translocation	24	13824	0.010373
protein kinase B signaling	26	13824	0.011234
peptidyl-threonine phosphorylation	28	13824	0.012094
positive regulation of MAP kinase activity	35	13824	0.015098
glucose metabolic process	38	13824	0.016383
positive regulation of interleukin-6 production	40	13824	0.017239
positive regulation of endothelial cell proliferation	48	13824	0.020657
cellular response to hypoxia	50	13824	0.02151
response to exogenous dsRNA	51	13824	0.021936
humoral immune response	56	13824	0.024065
positive regulation of protein phosphorylation	59	13824	0.02534
positive regulation of sequence-specific DNA binding transcription factor activity	65	13824	0.027887
glucose homeostasis	86	13824	0.036757
peptidyl-serine phosphorylation	112	13824	0.047646
defense response to virus	125	13824	0.053051
positive regulation of apoptotic process	133	13824	0.056365

Supplementary Table 2.

Term	Pop Hits	Pop Total	*P* Value
Hepatitis B	148	7550	1.60E-08
Toll-like receptor signaling pathway	105	7550	5.19E-07
Chagas disease (American trypanosomiasis)	114	7550	7.23E-07
PI3K-Akt signaling pathway	347	7550	1.15E-06
Hepatitis C	133	7550	1.34E-06
Influenza A	173	7550	3.85E-06
Tuberculosis	181	7550	4.62E-06
Proteoglycans in cancer	203	7550	7.30E-06
Pancreatic cancer	65	7550	1.20E-05
HIF-1 signaling pathway	96	7550	3.88E-05
TNF signaling pathway	108	7550	5.52E-05
Sphingolipid signaling pathway	120	7550	7.56E-05
Pathways in cancer	398	7550	1.05E-04
Osteoclast differentiation	134	7550	1.05E-04
Measles	140	7550	1.20E-04
Herpes simplex infection	190	7550	2.97E-04
Bladder cancer	40	7550	4.05E-04
NOD-like receptor signaling pathway	51	7550	6.60E-04
Endometrial cancer	51	7550	6.60E-04
MAPK signaling pathway	255	7550	7.06E-04
Non-small cell lung cancer	56	7550	7.95E-04
HTLV-I infection	267	7550	8.08E-04
mTOR signaling pathway	59	7550	8.83E-04
VEGF signaling pathway	59	7550	8.83E-04
Apoptosis	62	7550	9.74E-04
Central carbon metabolism in cancer	62	7550	9.74E-04
Glioma	65	7550	0.001071
Renal cell carcinoma	66	7550	0.001104
Colorectal cancer	66	7550	0.001104
Fc epsilon RI signaling pathway	67	7550	0.001137
Melanoma	72	7550	0.001312
Chronic myeloid leukemia	73	7550	0.001349
Pertussis	77	7550	0.0015
Prostate cancer	86	7550	0.001867
Rheumatoid arthritis	95	7550	0.002274
T cell receptor signaling pathway	105	7550	0.002771
Insulin resistance	111	7550	0.003092
Thyroid hormone signaling pathway	113	7550	0.003202
Toxoplasmosis	113	7550	0.003202
Natural killer cell mediated cytotoxicity	117	7550	0.003429
Neurotrophin signaling pathway	125	7550	0.003905
FoxO signaling pathway	132	7550	0.004346
Jak-STAT signaling pathway	151	7550	0.005654
Non-alcoholic fatty liver disease (NAFLD)	160	7550	0.00633
Focal adhesion	208	7550	0.010532
Rap1 signaling pathway	214	7550	0.011126

Cytokine-cytokine receptor interaction	218	7550	0.01153
Ras signaling pathway	235	7550	0.013322
Thyroid cancer	31	7550	0.024392
Prion diseases	32	7550	0.025171
African trypanosomiasis	37	7550	0.029056
Graft-versus-host disease	42	7550	0.032927
Type II diabetes mellitus	47	7550	0.036786
Malaria	53	7550	0.0414
Amyotrophic lateral sclerosis (ALS)	54	7550	0.042168
Acute myeloid leukemia	56	7550	0.0437
Legionellosis	57	7550	0.044466
Cytosolic DNA-sensing pathway	61	7550	0.047524
B cell receptor signaling pathway	70	7550	0.054373
Inflammatory bowel disease (IBD)	70	7550	0.054373
Leishmaniasis	71	7550	0.055132
Adipocytokine signaling pathway	71	7550	0.055132
Prolactin signaling pathway	74	7550	0.057404
RIG-I-like receptor signaling pathway	76	7550	0.058917
Hypertrophic cardiomyopathy (HCM)	80	7550	0.061936
TGF-beta signaling pathway	83	7550	0.064194
Salmonella infection	83	7550	0.064194
Fc gamma R-mediated phagocytosis	85	7550	0.065698
ErbB signaling pathway	86	7550	0.066449
Small cell lung cancer	87	7550	0.067199
Progesterone-mediated oocyte maturation	88	7550	0.067949
Hematopoietic cell lineage	92	7550	0.070944
Estrogen signaling pathway	98	7550	0.075421
Choline metabolism in cancer	99	7550	0.076165
Cholinergic synapse	110	7550	0.084321
Amoebiasis	112	7550	0.085798
Epstein-Barr virus infection	118	7550	0.090215
Platelet activation	127	7550	0.096808