

Systematic Investigation of *Ginkgo Biloba* Leaves for Treating Cardio-cerebrovascular Diseases in an Animal Model

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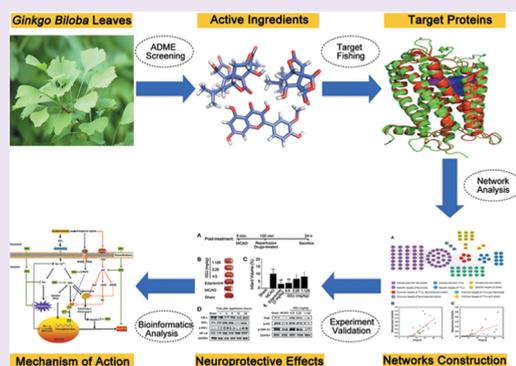
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Supporting Information

ABSTRACT: Globally, cardio-cerebrovascular diseases (CCVDs) are the leading cause of death, and thus the development of novel strategies for preventing and treating such diseases is in urgent need. Traditional Chinese medicine (TCM), used for thousands of years in Asia and other regions, has been proven effective in certain disorders. As a long-time medicinal herb in TCM, *Ginkgo biloba* leaves (GBLs), have been widely used to treat various diseases including CCVDs. However, the underlying molecular mechanisms of medicinal herbs in treating these diseases are still unclear. Presently, by incorporating pharmacokinetic prescreening, target fishing, and network analysis, an innovative systems-pharmacology platform was introduced to systematically decipher the pharmacological mechanism of action of GBLs for the treatment of CCVDs. The results show that GBLs exhibit a protective effect on CCVDs probably through regulating multiple pathways and hitting on multiple targets involved in several biological pathways. Our work successfully explains the mechanism of efficiency of GBLs for treating CCVDs and, meanwhile, demonstrates that GDJ, an injection generated from GBLs, could be used as a preventive or therapeutic agent in cerebral ischemia. The approach developed in this work offers a new paradigm for systematically understanding the action mechanisms of herb medicine, which will promote the development and application of TCM.



Cardio-cerebrovascular diseases (CCVDs), a class of disorders involving the heart and blood vessels, are the major cause of human death, affecting both the Western industrialized countries and developing ones.¹ Although many CCVDs are not fatal, they seriously disturb the individual's normal daily life, and the related costs on health care systems are enormous.² Consequently, the development of novel strategies to not only reduce the high cost of prevention and treatment of these ailments but also minimize the side effects of corresponding effective and alternative drugs is of great significance to public health. Also, a better understanding of the mechanisms by which the agents play their roles is needed.

Currently, with the increased incidence of CCVDs, more attention has been drawn to developing herbal drugs from traditional Chinese medicine (TCM), a whole medicinal system with clinical practice over thousands of years.³ Through the synergistic effect of multi-ingredients, multitargets, and multi-pathways, TCM produces its efficacy in a holistic way with fewer side effects, showing a significant advantage over a single drug treatment, especially in treating chronic complex, multifactorial diseases.⁴ The ever-increasing usage of TCM is a good indication of the public interest in such medicines.

For centuries, *Ginkgo biloba* leaves (GBLs) have been widely used in TCM for treating various medical conditions. In recent decades, a commercial standardized extract of GBLs (EGb 761) has been marketed as a therapeutic dietary supplement for counteracting a wide range of diseases and has demonstrated its neuroprotective and antioxidant properties⁵ against a variety of cardiovascular and neurological disorders.^{6,7} A growing body of evidence supported by both experimental and clinical studies has highlighted the beneficial role of GBLs on cognitive function, different types of cancer, and especially the treatment of CCVDs.^{8–14} Its actions are thought to be mediated mainly via its various active constituents, acting in a complementary manner. However, due to the multicomponents of the herb, up to now the molecular mechanisms of action and signaling pathways leading to the therapeutic effects of GBLs remain, still, poorly understood.

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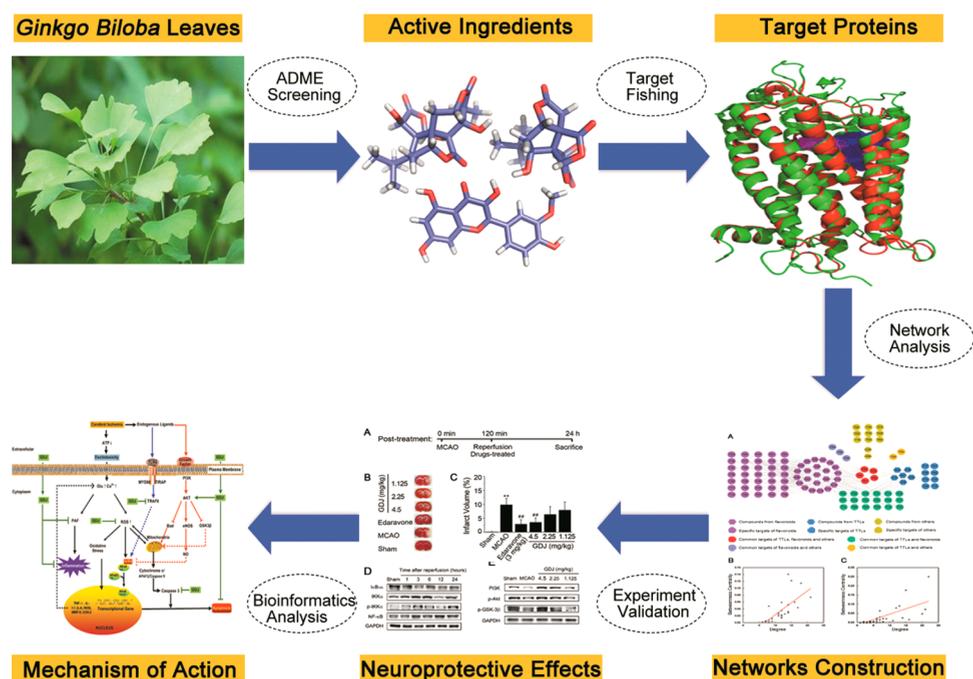


Figure 1. Workflow for systems-pharmacology approach. ADME: absorption, distribution, metabolism, and excretion.

Presently, to uncover the action mechanisms of GBLs for treating CCVDs, we performed an innovative systems-pharmacology approach (Figure 1). First, the active ingredients of GBLs with favorable pharmacokinetic properties were screened out by a systems-ADME process. Second, multiple targets of these bioactive chemicals were determined and validated by a comprehensive method. Third, through the network analysis, the crucial disease-relevant biological pathways were captured and the multimechanisms of GBLs were also interpreted. Finally, to demonstrate the reliability of our method, the protective effects of Ginkgo diterpene lactone meglumine injection (GDJ) from GBLs on ischemic injury were explored using an animal model. Our results provide an important reference for insight into the efficiency of GBLs on treating CCVDs and offer a novel strategy to discovery of new drugs from plants as well.

MATERIALS AND METHODS

Data Preparation and ADME Screening. All chemical ingredients of GBLs were first obtained from the TCMSP.¹⁵ Then, three *in silico* ADME models (Supporting Information) including PreOB (predict oral bioavailability), PreDL (predict drug-likeness), and PreHL (predict half-life) were employed to explore the candidate compounds in GBLs. The threshold values for these screening models are set to OB \geq 40%, DL \geq 0.20, and HL \geq 4, respectively. The compounds which successfully satisfy all the criteria are treated as candidate molecules.

Target Fishing and Network Construction. Presently, the most likely protein targets of the bioactive compounds were first predicted using a comprehensive approach (more details in Supporting Information). Then, using Cytoscape 3.0,¹⁶ two types of global networks, i.e., compound–target (C-T) and compound–target–pathway (C-T-P) networks with their fundamental topological properties were constructed.

Experimental Procedures. Animals and Drug Treatment. Sprague–Dawley male rats (250–300g) were randomized and treated with distilled water, GDJ (1.125, 2.25, and 4.5 mg/kg); GA, GB, and GC (10, 20, and 40 mg/kg); edaravone (3 mg/kg); and EGb 761 (20 mg/kg) by tail intravenous injection (i.v.), respectively. Approved by

Laboratory Animal Association of Jiangsu, all work procedures are consistent with the Guidelines of NIH for the use of experimental animals.

Middle Cerebral Artery Occlusion (MCAO). With the use of a previously described method¹⁷ with minor modification (see Supporting Information), transient MCAO (tMCAO) surgery was performed on animals anesthetized with isoflurane. 2,3,5-Triphenyltetrazolium chloride (TTC) staining was used to assess the cerebral infarct volume.

Antibodies and Western Blotting. Antibodies used in the present work contain anti-I κ B- α , antiphospho-I κ B- α , anti-IKK α , antiphospho-IKK α , anti-NF- κ B, antiphospho-NF- κ B (Sigma), anti-COX-2, anti-c-myc antiphospho-PI3K, anti-PI3K, antiphospho-Akt, anti-Akt, antiphospho-GSK-3 β , anti-GSK-3 β , anti-cytochrome C (CST), anti-cleaved caspase 3, anti-¹³⁶p-bad, anti-bad, anti-Bcl-2, and anti-GAPDH (Abcam). Western blot assays were carried out by standard protocols on brain cortices of rats at the indicated time.

Measurement of TNF- α and IL-6. Following 22 h of reperfusion, rat serum was collected to measure the levels of TNF- α and IL-6 using the ELISA kits (eBioscience).

Statistical Analysis. All data are presented as mean values \pm SD, and the statistical results are analyzed by a one way ANOVA and Student's *t* test.

RESULTS AND DISCUSSION

ADME Screening and Pharmacological Analysis. Unfavorable ADME properties are considered the major reason for the failures of candidate molecules of reaching the market. Using three *in silico* prescreening models, 31 ingredients of GBLs with favorable pharmacokinetic characteristics were determined (Table S1). On the basis of structural elucidation, these molecules are mainly grouped into two categories: terpene trilactones (TTLs) and flavonoids. The compounds which cannot be assigned into any of these two classes are grouped into “others.” TTLs, as a unique kind of constituent, occurring widely in GBLs, contain diterpene lactones (ginkgolides) and a sesquiterpene (bilobalide). Obviously, the OB and DL of TTLs are relatively higher than those of

Table 1. ADME Parameters of TTLs, Flavonoids, and Other Compounds in GBLs

class	subclass	mean OB (%)	range of OB (%)	mean DL	range of DL	mean HL	range of HL
TTLs	diterpene lactones	45.68	42.85–48.69	0.74	0.73–0.75	5.54	4.08–7.61
	sesquiterpene	86.51	86.51	0.36	0.36	5.90	5.90
flavonoids	flavonol glycosides	49.81	41.43–67.09	0.27	0.24–0.31	14.91	13.87–16.37
	aglycones	35.96	7.02–69.61	0.26	0.21–0.31	14.86	14.05–16.81
others	steroids	47.43	43.83–51.03	0.66	0.56–0.76	11.21	5.68–16.75

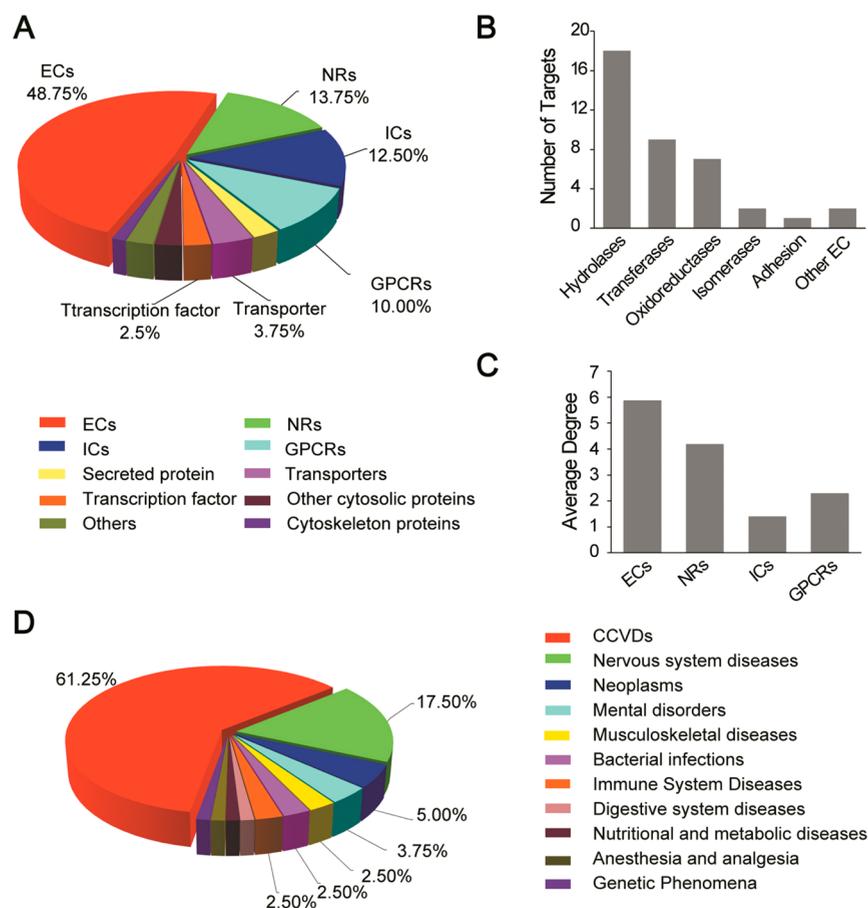


Figure 2. (A) Distribution of drug targets according to their biochemical criteria. (B) Classification of targets in enzyme. (C) Average degree of four main kinds of protein targets, i.e., ECs, NRs, GPCRs, and ICs. (D) Fractions of drug targets according to their therapeutic areas. EC, enzyme; GPCR, G protein-coupled receptor; NR, nuclear receptor; IC, ion channel; CCVDs, cardio-cerebrovascular diseases.

flavonoids, whereas the half-life ($t_{1/2}$) of TTLs is shorter than that of flavonoids (Table 1).

Ginkgolides, including GA, GB, and GC, are potent and selective antagonists of platelet activating factor (PAF) with good OBs ranging from 42.85% to 48.69% and high DL values of 0.73–0.75 (Table 1). Due to the specific and potent antagonist activity against PAF, ginkgolides are able to improve blood circulation and prevent and treat thrombosis and illness of blood vessels of the heart and brain.¹⁸ Interestingly, all ginkgolides have similar absorption behaviors due to their similarity in molecular structures. Actually, the fact that GBLs were enriched for GB, which has a longer half-life than GA, may be attributed to the prolonged residence time and greater bioavailability (Table S1). With the highest OB of 86.51% and a DL of 0.36, bilobalide also presents important therapeutic activities of GBLs.

Flavonoids, a class of polyphenolic compounds ubiquitously existing in the plant kingdom, include flavonol glycosides and aglycones and are getting increasing attention for their

antioxidant, improved cardiovascular well-being, and anticancer activities.¹⁹ Pharmacokinetic studies have shown that many flavonoids are poorly absorbed, leading to a low OB.²⁰ For instance, quercetin with a poor OB of 9.45% shows cardio-protective, radical-scavenging, and anti-inflammatory effects²¹ and is thus retained for future analysis. But for another flavonol aglycone, kaempferol exhibits a significantly high OB of 69.61% and favorable DL of 0.24 (Table S1). All results indicate that different types of flavonoids can result in different bioavailability, which is possible due to their different molecular structures. The DL prediction of these aglycones is in the range of 0.27–0.35. With respect to $t_{1/2}$, the flavonoids show relatively long half-life, about 13.87–16.81 h, which are significantly longer than those of TTLs (4.08–7.61 h).

Attributing to a combination of flavonoids and TTLs, as well as their antioxidant and free-radical-scavenging properties, the therapeutic effects of GBLs can be observed in various CCVDs.

Detection of Drug–Target Interactions. Presently, based on the comprehensive method (Supporting Informa-

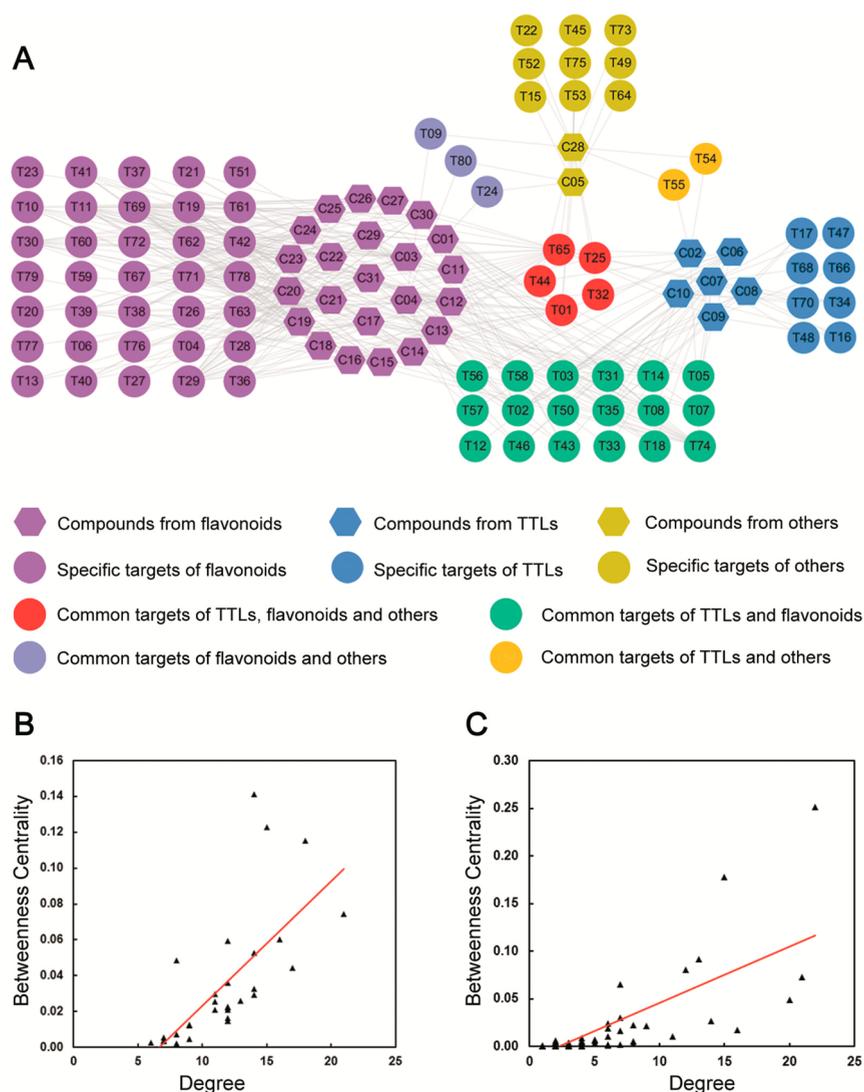


Figure 3. (A) A global view of C-T network. (B and C) The correlation plots of the betweenness centrality and degree for drug and target sets, respectively. TTLs: terpene trilactones.

tion), 80 potential targets (Table S2) for 31 candidate compounds were rapidly fished out, generating 363 ligand–target interactions. The distribution of the biochemical classification indicates that the target space mainly consists of enzymes, nuclear receptors (NRs), ion channels (ICs), G protein-coupled receptors (GPCRs), transporters, and transcription factors (Figure 2A). Remarkably, the obtained drug targets are enriched in enzymes (48.75%), highlighting the critical roles of enzyme targets in drug discovery. Among these enzymes, 18 targets are hydrolases (including 11 proteases), nine are transferases (including eight kinases), seven are oxidoreductases, two are isomerases, one is adhesion, and two are other enzymes (Figure 2B).

NRs, acting as ligand-activated transcription factors, account for the second largest predicted drug targets (13.75%). By regulating the activation of various target proteins, these receptors are important drug targets in terms of potential therapeutic application.²² The other common receptors are GPCRs, a class of integral membrane proteins which have seven trans-membrane domains, comprising 10% of all protein targets. They are a drug-dense family in many diseases, particularly in the neuroscience and cardiovascular fields.²³

For instance, ADRA1B and ADRB2, belonging to the guanine nucleotide-binding GPCRs superfamily, exert various cardiovascular effects, including improvement of cardiac contractility, hastened cardiac relaxation, and accelerated atrioventricular conduction.²⁴

For ICs, they are a large superfamily of membrane proteins that pass ions across membranes. Due to their pivotal role in regulating cardiac electric activity and vascular tone, ICs represent a major target for the induction of cardioprotective and antihypertensive effects.²⁵ Taking sodium channel protein for an example, it is proven to be highly related to cardiac arrhythmias.²⁶ Moreover, by inspection of the average degree of these four types of drug targets (Figure 2C), the enzyme, GPCR, and NR targets present more connected components than that of ICs, suggesting that they have strong binding specificity with their ligands, compared with ICs.

Also, when studying the functional distribution of these candidate targets according to the therapeutic areas, it is observed that these drug targets may be categorized into 11 types (Figure 2D). Among targets with explicit therapeutic functions, the drug target related CCVDs are most populated, followed by the targets associated with nervous system diseases.

C-T Network Analysis. Using 31 candidate compounds and their 80 potential targets, a global view of the C-T network was generated, which consists of 111 nodes and 363 edges (Figure 3A). The mean number of targets per compound is 2.6, showing the multitarget features and poly pharmacology properties of constituents in GBLs. In these ingredients, 21 have high degree distributions, and each of them hits more than 10 potential targets, indicating their crucial roles. For instance, quercetin has the highest degree, followed by ginkgolide B with 18 drug–target interactions and kaempferol possessing 17 target proteins. Moreover, the relationships between degree and betweenness centrality in the drug and target sets (Figures 3B and C) demonstrate that the distribution of degree and betweenness centrality is strongly correlated, and the most highly connected vertices have high centrality scores.

By further observation of the C–T network, we find that many targets are hit by different numbers of compounds, implying the multicomponent characteristics of herbs. Remarkably, five targets (6.25%) are found to be shared by flavonoids, TTLs, and others. Among them, the common target DPP4 with the highest degree can be modulated by 16 compounds in flavonoids, five in TTLs, and one in another kind, indicating its vital role in helping to treat CCVDs. Similarly, prothrombin, the key enzyme in the blood coagulation system, has been identified by 12 chemicals in flavonoids, one in TTLs, and two in another kind. It is proved to have a relationship with MI, stroke, and venous thrombosis in a large cohort of U.S. men.²⁷ All these results suggest that individual drugs can act on the same targets, thus exerting a synergistic therapeutic effect on CCVDs.

Furthermore, 18 targets (22.5%) are commonly modulated flavonoids and TTLs, exhibiting a similar therapeutic mechanism of these two major groups of constituents of GBLs. Examples of such compounds are ginkgolide B and quercetin, sharing seven protein targets, and it has been reported that both compounds can show their neuroprotective properties by blockage of the early signaling cascades, leading to $A\beta$ toxicity.²⁸ Apart from the common targets, flavonoids and TTLs have their own targets, showing their specific mechanism of action. There are 35 targets identified only by 23 chemicals in flavonoids, and eight targets are specifically targeted by four compounds in TTLs, implying that a combination of these compounds probably presents a better effect than a single drug. All the findings imply the probably different binding properties of both groups of compounds with the active substances. Additionally, it is intriguing to note that two chemicals GB and bilobalide have no specific targets, suggesting that these molecules may be replaced by other compounds.

C-T-P Network Analysis. To understand the underlying therapeutic mechanism of GBLs, presently, 40 biological pathways (Table S3), which have high correlations with CCVDs, were extracted from KEGG.²⁹ As a result, 10 pathways have significant strengths of association with signal transduction. Among these, several pathways are associated with inflammation, and the PI3K-Akt signaling pathway is the most important one. Some other pathways like TNF, MAPK, and NF- κ B signaling pathways have been known to be related with CCVDs.^{30–32} Also, there are a number of important and well-known signaling pathways involved in other subclasses including the endocrine related pathway PPAR signaling, controlling lipid metabolism and blood glucose uptake,³³ and the excretory system pathway aldosterone-regulated sodium

reabsorption, playing a crucial role in regulating blood pressure.³⁴

Then, we mapped all candidate compounds with their corresponding targets onto these pathways. After discarding 18 targets without participating in any related pathway, a multilevel C-T-P network was constructed, which consists of 133 nodes (31 compounds, 62 genes, and 40 pathways) and 497 edges (Figure S1). Clearly, most pathways possess a few target proteins, but about 35% of targets are involved in multipathways (≥ 8), presenting the key roles in treating CCVDs. For instance, RELA,³⁵ which is associated with the cell cycle, proliferation, and cell death, is influenced by six compounds through 12 biological pathways.

Furthermore, we find that four pathways, i.e., PI3K-Akt, neuroactive ligand–receptor interaction, estrogen, and calcium signaling pathways, are significantly enriched for these target proteins, implying the key roles of these pathways. Among them, the neuroactive ligand–receptor interaction pathway has the highest degrees and the key proteins in this pathway like ADRA1B and F2 are highly associated with the cardiac function.²⁴ Also, we find that many ingredients of GBLs participated in these pathways, providing a basis for the treatment and prevention of CCVDs. For instance, 23 chemicals such as apigenin, bilobalide, ginkgolides, and stigmasterol are referred to as regulating main targets in the neuroactive ligand–receptor interaction pathway.

In addition, six signaling pathways including TNF, metabolic, VEGF, toll-like receptor (TLR), MAPK, and NF- κ B signaling pathways are the second most important of the main pathways capable of regulating anti-inflammatory, neuroprotective, and antioxidative effects.^{30–32} Interestingly, all of these pathways are shared by flavonoids and TTLs but present different importance for these two groups of compounds. For flavonoids, neuroactive ligand–receptor interaction, estrogen, calcium, TNF, metabolic, VEGF, and MAPK signaling pathways show relatively higher associations with their corresponding targets, whereas TTLs intensively influence PI3K-Akt, TLR, and NF- κ B signaling pathways. This suggests that different ingredients of GBLs may be involved in different multipathways, showing their specific mechanism of action.

Identification of Compounds for Animal Experiments.

On the basis of the above computational analysis, we know that two main groups of ingredients, i.e., TTLs and flavonoids, occurring in GBLs hit on various target proteins through regulating several biological pathways. These proteins denote different functions, which are significantly enriched for CCVDs. Presently, to confirm the efficiency of the *in silico* screening protocol, we randomly singled out a few molecules from flavonoids or TTLs and selected a class of diseases from CCVDs for further experimental validations. For convenience, we choose GA, GB, and GC from TTLs and tested the therapeutic efficacy of ingredient combinations GDJ in an animal model. Actually, GDJ, which is mainly constituted of three compounds, GA, GB and GC, was obtained from our cooperative partners (Jiangsu Kanion Pharmaceutical CO. LTD). For a comparison, the efficacy of GDJ's main molecules was also investigated.

Protective Effects of GDJ Post-Treatment in tMCAO.

To evaluate the effects of GDJ on preventing stroke injury, 24 h after tMCAO, we assess the infarct volume in response to ischemic injury. Figure 4A shows the experimental protocol. Obviously, there was no infarction in the sham group, whereas a large area of infarction was observed in the brain areas of rats

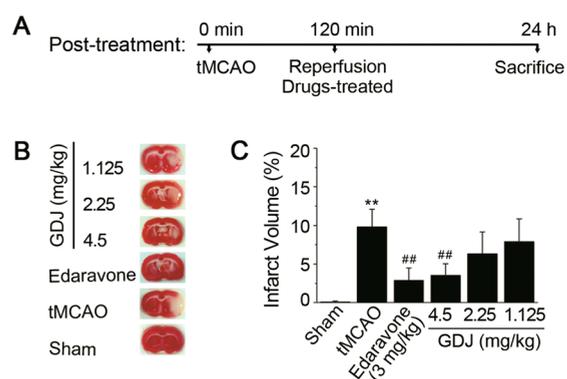


Figure 4. Neuroprotective effects of GDJ post-treatment on ischemic injury. (A) Experimental protocol. (B) TTC staining of brain slices. The ischemic area remained white, while the intact area was stained red. (C) Infarct volumes. Data are presented as mean \pm SD ($n = 6$). ** $p < 0.01$ compared to sham group, ### $p < 0.01$ compared to tMCAO group. GDJ: Ginkgo diterpene lactone meglumine injection; tMCAO: transient middle cerebral artery occlusion.

subjected to tMCAO (Figure 4B). Post-treatment with edaravone or GDJ decreased infarct volume induced by I/R (Figure 4B). However, in infarct size, no obvious difference was found in both the tMCAO group and the GDJ 1.125 mg/kg group due to the low concentration of this drug (Figure 4C). Furthermore, the dose of 4.5 mg/kg of GDJ was more protective than the dose of 2.25 mg/kg on the cerebral injury, indicating that the neuroprotection of GDJ is dose-dependent, so that this dosage was used for further analysis.

Subsequently, the protections of GDJ and its main constituents were compared. A significantly lower infarct

volume was observed in the treatment groups of GA (40 mg/kg) and GB (10 mg/kg; Figure 5A and B). However, GA post-treatment at 10 and 20 mg/kg did not show any beneficial effects on infarcted tissue (Figure 5A), while GB (20 and 40 mg/kg) presented few effects (Figure 5B). Similarly, a limited protection was seen in infarct rate if administrating with GC (10 and 40 mg/kg; Figure 5C), and there is no neuroprotection observed in the GC (20 mg/kg) group. All these results demonstrate that the protection of GA, GB, and GC was in a dose-dependent manner.

Additionally, EGb 761, as a popular product obtained from GBLs, also shows the neuroprotective properties in ischemic brain injury.⁵ Therefore, we selected EGb 761 as another positive control, and the protection of EGb 761 and GDJ as well as its main ingredients GA, GB, and GC were compared. The results show that although the group post-treated with EGb 761 (20 mg/kg) provides better protection than the group treated with GDJ (4.5 mg/kg), both groups present significantly smaller infarct volumes as compared to the tMCAO group (Figure 5D). Moreover, there is no statistically significant difference in protection on cerebral infarction observed in GA (40 mg/kg), GB (10 mg/kg), and GC (10 mg/kg) groups. Therefore, we assume that the therapeutic effects of GDJ may arise from synergistic actions of herbal ingredients, which is probably more effective than the single drug. The detailed mechanisms underlying this synergy will be discussed in the following.

The Neuroprotective Mechanisms of GDJ. In the previous work, we present that the protective effect of GDJ is more effective than single drug and is also dose-dependent (Figure 5D). Only at 4.5 mg/kg did we observe the greatest effects on infarct size. On the basis of the previous pathway

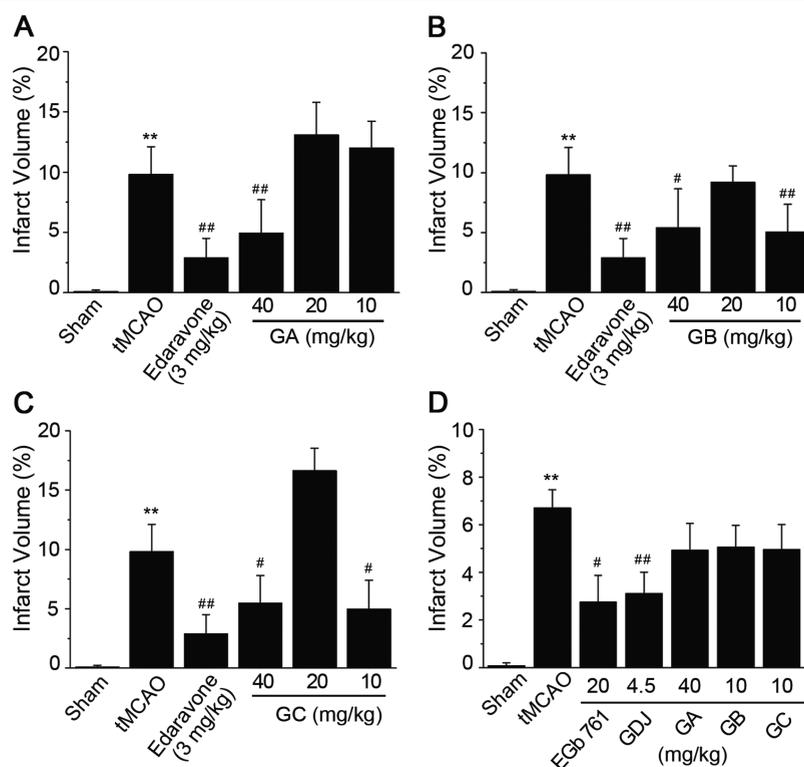


Figure 5. (A, B, and C) Protective effects of GA, GB, and GC on infarct volume. (D) Post-treatment with EGb 761 (20 mg/kg), GDJ (4.5 mg/kg), and GDJ's main components in the optimal dose reduces damage from stroke. Data are shown as mean \pm SD ($n = 6$). ** $p < 0.01$ compared to sham group, # $p < 0.05$, ### $p < 0.01$ compared to tMCAO group. GA, ginkgolide A; GB, ginkgolide B; GC, ginkgolide C.

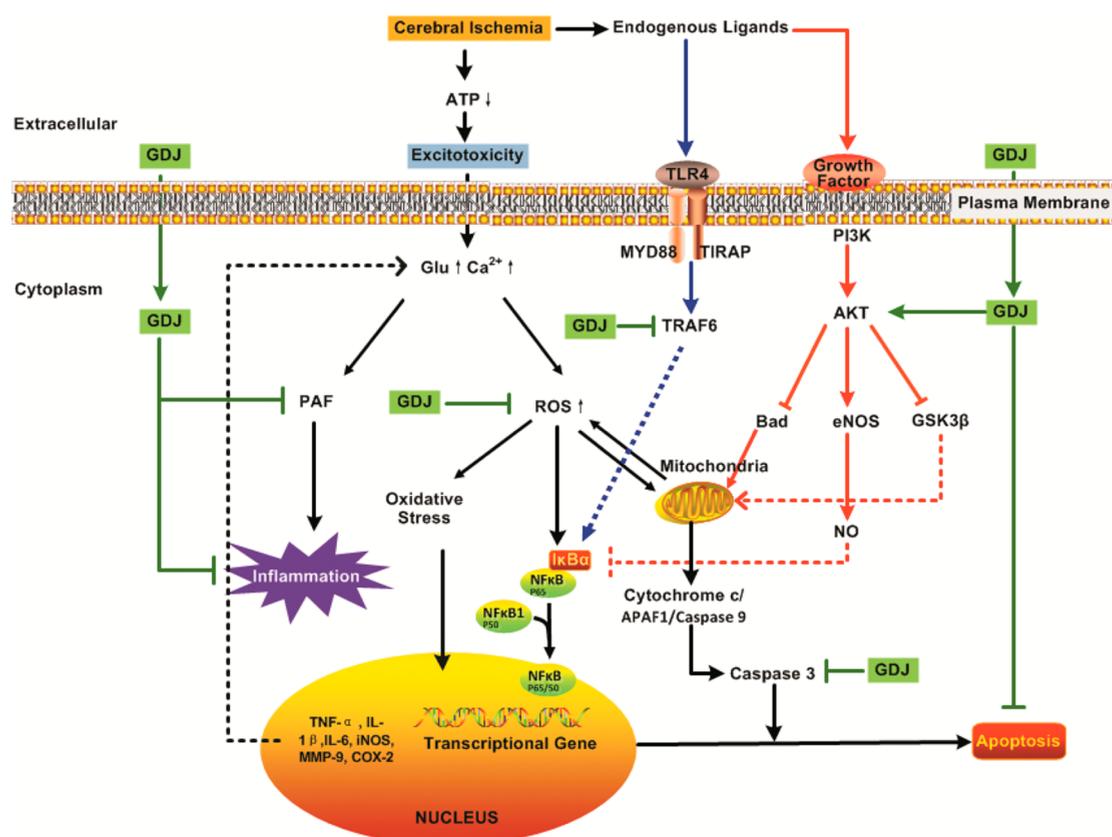


Figure 6. Hypothetical neuroprotective mechanisms of GDJ on ischemic injury. Glu, Glutamate; PAF, platelet activating factor; ROS, reactive oxygen species; NF- κ B, nuclear factor- κ B; PI3K, phosphoinositide-3-kinase; AKT, serine/threonine protein kinase; GSK3 β , glycogen synthase kinase3 β ; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; MMP-9, matrix metalloproteinase-9; TLR4: Toll-like receptor 4; MyD88: myeloid differentiation protein 88; TIRAP, toll-IL-1 receptor domain-containing adapter protein; TRAF6, TNF receptor-associated factor 6; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6. Lines with arrowheads denote activation, and lines with bars on the end represent inhibition.

enrichment analysis, we assume that this highest protection might be that herbal ingredients of GDJ simultaneously target multiple pathways like PI3K-Akt, TLR, and NF- κ B signaling pathways, thereby exerting synergistic effects in the ischemic brain. Actually, many studies^{32,36} have shown the important roles of NF- κ B, PI3K-Akt, and TLR4 signaling pathways in protecting cerebral ischemic injury.

As we all know, after ischemia, massive production of excitatory amino acids, mainly glutamate, results in microglia activation and an inflammatory response, leading to an increased degree of ischemic damages.³⁶ NF- κ B, an important transcription factor involved in these inflammatory processes, regulates pro-apoptotic, antiapoptotic, and pro-inflammatory gene expression, thereby showing a dual role in the survival of neurons.³⁷ Several pro-inflammatory NF- κ B target genes, containing TNF- α , IL-6, iNOS, MMP-9, and COX-2 are harmful to neurons in ischemic conditions.³⁸ By inhibiting NF- κ B activity, a therapeutic effect has been shown in strokes and traumatic brain injury.³⁸ A previous study³⁹ has confirmed that GKAB treatment significantly blocked NF- κ B translocation and down-regulated the expression of the NF- κ B target gene in the ischemic cortex, implying the neuroprotective effect of this drug, as demonstrated by our results.

Additionally, after cerebral I/R, lots of neurons in the ischemic area will experience apoptosis in the next few hours or days.⁴⁰ Many antiapoptosis agents are thus used for the protection of ischemic brain injury.⁴¹ The PI3K-Akt signaling, a

central mediator of signal transduction pathways, modulates cellular proliferation and survival.⁴² Through phosphorylating the membrane phosphoinositides, PI3K activates the downstream Akt.⁴² Activation of Akt can inhibit apoptosis by directly phosphorylating a series of molecules, containing GSK3 β and Bcl-2-associated death protein, thereby preventing cytochrome c release to cytoplasm and in turn blocking downstream caspase activation.⁴³ A past study⁴⁴ has suggested that in ischemic neurons, GB significantly increased cell viability and ¹³⁶p-Bad and p-GSK3 β expression, attenuated the level of caspase-3, and consequently reduced apoptotic cell death, supporting the critical role of the PI3K-Akt pathway.

As for TLRs, they belong to the type I transmembrane protein family and act as signal transduction molecules that respond to an array of microbial products.⁴⁵ Until now, there were more than 10 kinds of TLRs,⁴⁵ among which TLR4 may play a more important role than others in the pathological progression of stroke-mediated brain injury. Several studies have suggested that TLR4 is involved in the neuronal death, brain edema, and inflammatory response to cerebral ischemic injury.⁴⁶ It has been reported that *G. biloba* extracts prevent neuronal cell apoptosis after neurotraumatic injury by suppressing TLR4/NF- κ B-dependent inflammatory responses.³²

Currently, our data demonstrate the inhibitory effect of GDJ on neurons against cerebral I/R. Since the main constituents of GDJ are GA, GB, and GC, we speculate that neuroprotective

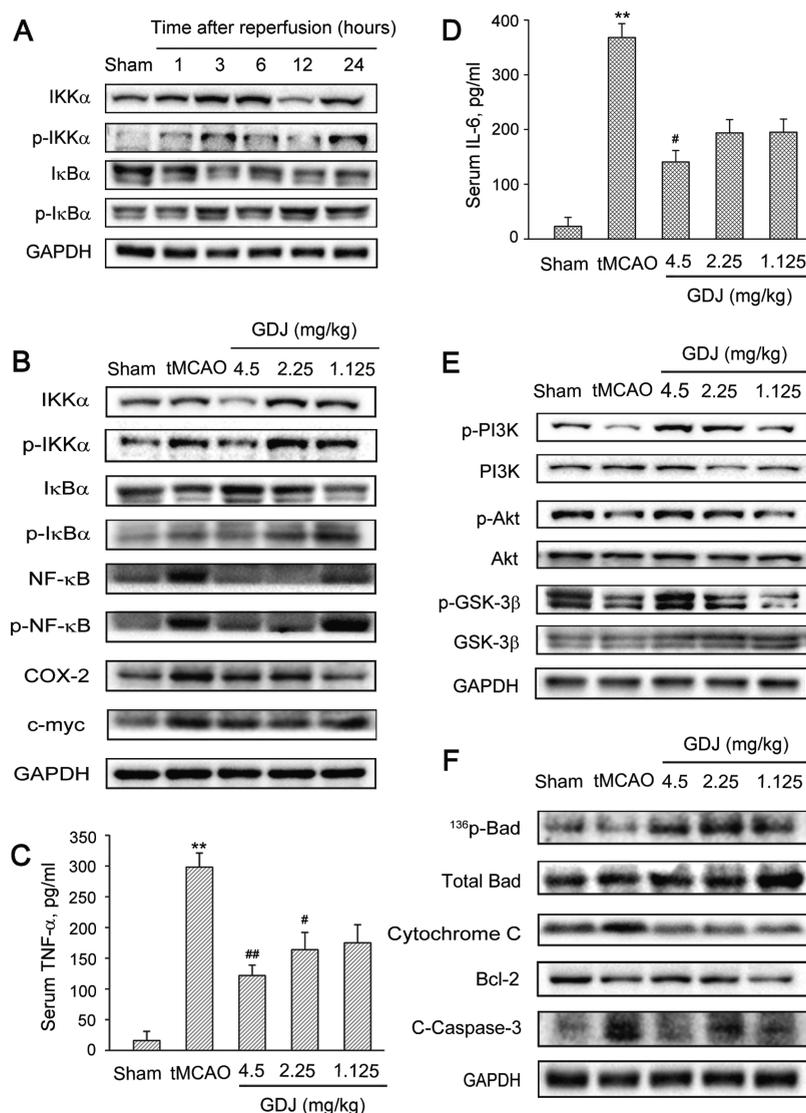


Figure 7. Effects of GDJ on the NF- κ B and PI3K-Akt signaling pathways induced by ischemic injury. (A) Time-dependent expression changes of phospho-I κ B- α , I κ B- α , IKK α , and phospho-IKK α after ischemia. (B) GDJ blocked the decrease of I κ B- α and the increase in phospho-I κ B- α , IKK α , NF- κ B, and phospho-NF- κ B 3h after reperfusion. Additionally, GDJ inhibited the expression of COX-2 and c-myc 12 h after reperfusion. (C and D) TNF- α and IL-6 concentrations in serum as measured by ELISA kits 24 h after tMCAO. (E) GDJ increased the expressions of PI3K, phospho-Akt, and phospho-GSK-3 β after I/R. (F) GDJ attenuated the increases of cytochrome c, cleaved-caspase 3, and improved the levels of ¹³⁶p-bad and Bcl-2 24 h after I/R. GAPDH is used as the control. Results are denoted as means \pm SD. ** p < 0.01 compared to sham group, and # p < 0.05, ## p < 0.01 compared to tMCAO group.

action of GDJ may be attributed to the combined effects of various constituents rather than to any single component. These combined beneficial effects could be synergistic. On the basis of these findings, we assume that the potential synergy of GDJ may be through inhibiting NF- κ B, activating PI3K-Akt and suppressing TLR4 signaling pathways, and subsequently inhibiting inflammation and neuronal apoptosis. The main molecular mechanisms involved in the neuroprotective effect of GDJ on ischemic injury are summarized in Figure 6.

Experimental Validation. For confirming the speculation of the neuroprotective mechanism of GDJ experimentally, the effects of GDJ on NF- κ B and PI3K-Akt signaling pathways induced by ischemic injury were chosen for testing. The expression levels of GDJ related proteins in these two pathways were examined by Western blot, and the inflammatory cytokines were analyzed through ELISA kits.

Our results show that 3 h after reperfusion, an increase in IKK α , p-IKK α , and p-I κ B α levels as well as a decrease in the level of I κ B α was detected (Figure 7A), presenting the activation of NF- κ B. Interestingly, GDJ not only inhibits the expression of phospho-IKK α and IKK α but also suppresses the subsequent phosphorylation and degradation of I κ B α , thereby preventing NF- κ B activation (Figure 7B). Consistently, both NF- κ B and phospho-NF- κ B were significantly down-regulated by GDJ in a dosage dependent manner (Figure 7B), indicating that the neuroprotection of GDJ might be through inhibiting the NF- κ B signaling pathway. Additionally, to explore the effects of GDJ on the inflammatory process of an ischemic brain, COX-2 and c-myc in the ischemic cortex were measured, and the expressions of TNF- α and IL-6 in serum were also investigated. As expected, rats treated with GDJ showed an obvious blocked I/R-induced increase and inhibited expression

of COX-2 and c-myc (Figure 7B) and affected TNF- α and IL-6 levels (Figure 7C and D).

Moreover, we further investigated whether GDJ could regulate the PI3K-Akt pathway after ischemia. Protein levels of phospho-PI3K, PI3K, and its downstream proteins were measured. Clearly, I/R induced a decrease of phospho-PI3K, phospho-Akt, and phospho-GSK3 β levels, and rats post-treated with GDJ showed a significantly improved expression of these proteins (Figure 7E), indicating that activating the PI3K-Akt pathway is vital for GDJ neuroprotection. Furthermore, to explore the antiapoptotic effects of GDJ in an ischemia brain, the expression changes of ¹³⁶p-Bad and Bcl-2 were detected. It is found that in areas of ischemic injury, the levels of ¹³⁶p-Bad and Bcl-2 are decreased, and GDJ administration acutely blocks these decreases (Figure 7F), demonstrating the antiapoptotic effects of GDJ. Moreover, GDJ treatment significantly prevents the injury-induced increase in cytochrome c and cleaved caspase-3 levels, thereby blocking the apoptosis and contributing to the neuroprotective activity of GDJ (Figure 7F).

CONCLUSIONS

Currently, an integrative systems pharmacology approach was proposed to study the effective substances, therapeutic targets, and pharmacological mechanisms of GBLs for treating CCVDs. By incorporating ADME screening, drug targeting and network analysis, and the bioactive compounds, multiple targets/pathways were obtained, and the multiscale pharmacological mechanisms of GBLs in treating CCVDs were systematically interpreted. Moreover, we experimentally present the protective effects of GDJ from GBLs on ischemic injury in an animal model and validate the molecular mechanism of GDJ, confirming the reliability of our method. However, further studies are needed to test the effects of other ingredients on other pathways and their interactions. Also, this work is expected to be useful for carrying out a systematic study of herbal medicine as well as for making the TCM drug discovery predictable.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00762.

(Figure S1) C-T-P network of GBLs on treating CCVDs, (Table S1) 31 bioactive compounds obtained from Ginkgo biloba leaves and their corresponding ADME parameters, (Table S2) 80 potential drug targets and their corresponding diseases, (Table S3) 40 KEGG pathways associated with CCVDs, and materials and methods (PDF)

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Notes

The authors declare no competing financial interest.

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