Computational and Structural Biotechnology Journal 21 (2023) 2809–2823

Contents lists available at ScienceDirect

Computational and Structural Biotechnology Journal

journal homepage: www.elsevier.com/locate/csbj

Research Article

Network-based drug repurposing for potential stroke therapy

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ARTICLE INFO

Article history:
Received 27 February 2023
Received in revised form 16 April 2023
Accepted 18 April 2023
Available online 20 April 2023

Keywords:
Network-based drug repurposing
Approved drugs
Stroke
In vitro experimental validation

ABSTRACT

Stroke is the leading cause of death and disability worldwide, with a growing number of incidences in developing countries. However, there are currently few medical therapies for this disease. Emerged as an effective drug discovery strategy, drug repurposing which owns lower cost and shorter time, is able to identify new indications from existing drugs. In this study, we aimed at identifying potential drug candidates for stroke *via* computationally repurposing approved drugs from Drugbank database. We first developed a drug-target network of approved drugs, employed network-based approach to repurpose these drugs, and altogether identified 185 drug candidates for stroke. To validate the prediction accuracy of our network-based approach, we next systematically searched for previous literature, and found 68 out of 185 drug candidates (36.8 %) exerted therapeutic effects on stroke. We further selected several potential drug candidates with confirmed neuroprotective effects for testing their anti-stroke activity. Six drugs, including cinnarizine, orphenadrine, phenelzine, ketotifen, diclofenac and omeprazole, have exhibited good activity on oxygen-glucose deprivation/reoxygenation (OGD/R) induced BV2 cells. Finally, we showcased the anti-stroke mechanism of actions of cinnarizine and phenelzine *via* western blot and Olink inflammation panel. Experimental results revealed that they both played anti-stroke effects in the OGD/R induced BV2 cells *via* inhibiting the expressions of IL-6 and COX-2. In summary, this study provides efficient network-based methodologies for *in silico* identification of drug candidates toward stroke.

1. Introduction

Stroke is a leading cause of death and disability globally [1]. This disease can be generally classified into ischemic stroke (IS) and hemorrhagic stroke which result from arterial occlusion or rupture of cerebral arteries. Responsible for about 87 % of all stroke cases [2], IS gives rise to oxygen depletion in the brain, causing inflammation, vascular alterations as well as affecting neuronal and glial function [3]. To date, only intravenous thrombolysis with recombinant tissue plasminogen activator (rt-PA) is approved by the US Food and Drug Administration (FDA) for acute ischemic stroke. Unfortunately, the benefit from rt-PA therapy is limited due to the narrow therapeutic window and reperfusion injury resulting from recanalization of blood flow may also occur [4,5]. Hence, it is urgently needed to develop effective agents for stroke.

Over the last decades, *de novo* drug discovery is considered to be increasingly high cost and time-consuming while the number of novel candidates transformed into therapeutic agents has stagnated [6]. To overcome this problem, drug repurposing has emerged as an effective drug discovery strategy, which is able to identify new indications from existing drugs for complex diseases, such as stroke. For example, a study in 2020 demonstrated that antimycotic ciclopirox olamine (CPX) could be repurposed as a promising anti-IS agent since it effectively alleviated multiple ischemic injuries [7]. CCR5 inhibitors were also considered to be potentially repurposed for stroke recovery [8]. Li et al. has utilized the computational drug repositioning approach for IS and identified 252 drugs with potential neuroprotective effects [9]. Moreover, as a peroxisome proliferator-activated receptor gamma and cannabinoid receptor type 2 dual agonist, VCE-004.8 has shown the possibility of repurposing its use as a delayed treatment option for IS [10]. Since existing drugs may have good post-marketing experience and safety surveillance, drug

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https://doi.org/10.1016/j.csbj.2023.04.018

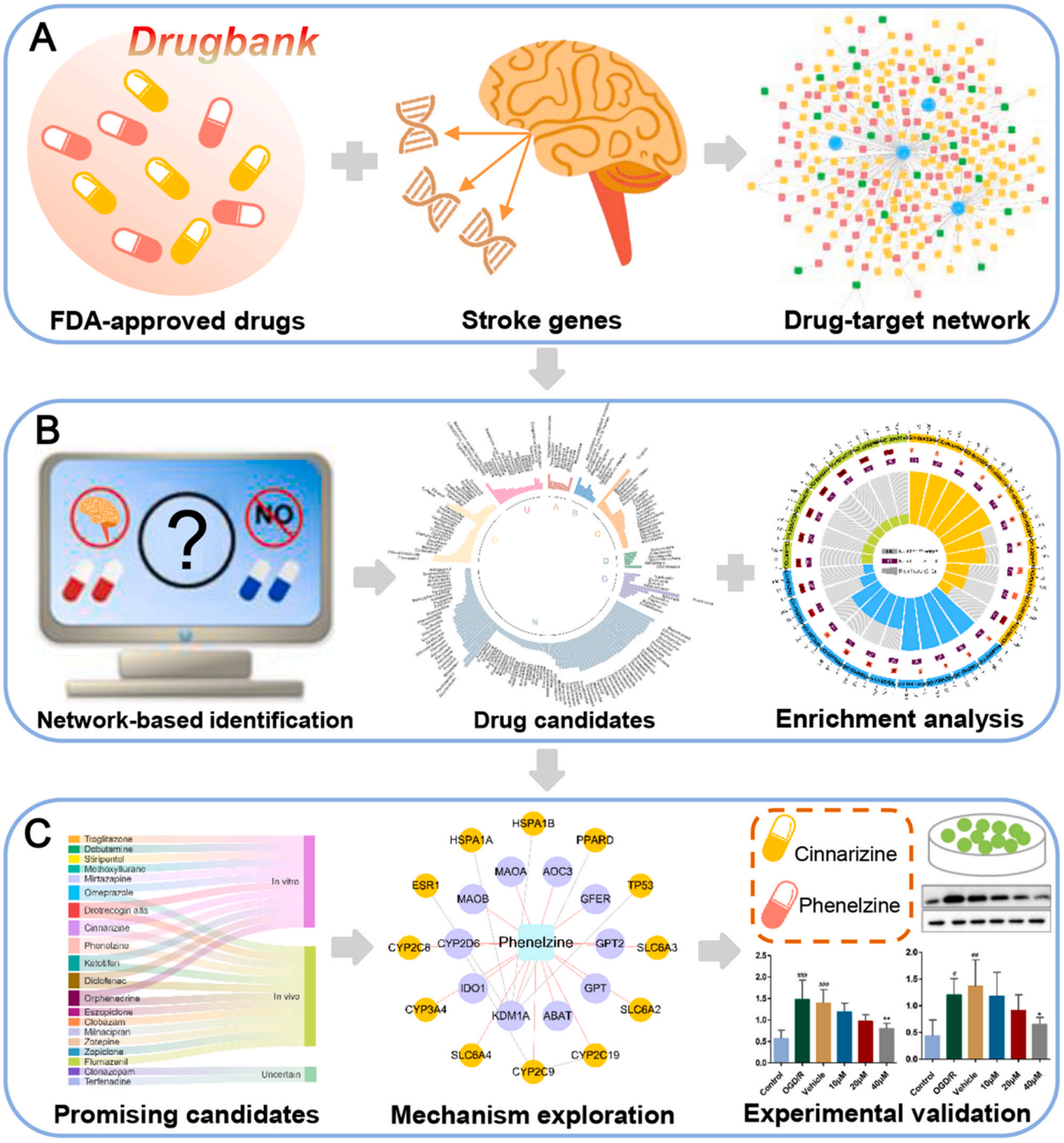


Fig. 1. Network-based framework for identification of stroke therapy. Construction of approved drug-target network (**A**). Network-based identification of potential drug candidates (**B**). *In vitro* experimental validation and mechanisms exploration of promising candidates (**C**).

repurposing can sharply cut down the expense and reduce the time of drug development [11,12].

Several network-based methodologies also provide efficient tools to establish associations between drugs and diseases *via* assembling disease genes and drug-target interactions (DTIs) in human protein–protein interactome. For instance, our previous studies have demonstrated that network-based infrastructure is of great value for prioritizing potential drug candidates toward numerous diseases, such as SARS-CoV-2 [13] and Alzheimer's disease [14,15].

In this study, we proposed network-based framework for drug repurposing for stroke from approved drugs (Fig. 1). We posited that a drug would hold high potential for stroke therapy if its drug targets were more likely to be stroke genes. Specifically, we firstly integrated stroke genes from numerous databases and constructed a drug-target network of all approved drugs. Subsequently, we applied statistical network model to identify promising anti-stroke drug candidates and performed the systems pharmacology analysis. We next selected several drugs without anti-stroke literature evidence for *in vitro* verification on BV2 cells. Finally, mechanism explorations of potential drug candidates (phenelzine and cinnarizine) were performed, which supported the effectiveness of our network-based approach.

2. Materials and methods

2.1. Collection of disease genes associated with stroke

The stroke disease genes were collected from six authoritative databases (access time in October 2019), including the DisGeNET (https://www.disgenet.org/), GWAS Catalog (https://www.ebi.ac.uk/gwas/), the Phenopedia database (https://phgkb.cdc.gov/PHGKB/startPagePhenoPedia.action), the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), the ClinGen database (https://www.clinicalgenome.org/) and the Open Targets Platform (https://www.targetvalidation.org/). We firstly searched the key word "stroke" in these six authoritative databases. For Phenopedia database, only genes with three or more publications were preserved. For DisGeNET database, we only kept the stroke genes with a DisGENET score ≥ 0.3 and an evidence index (EI) > 0. For ClinVar database, we only reserved those genes owning number of golden stars higher than or equal to two. As for the Open Targets Platform, only those stroke genes with score values higher than 0.3 were preserved. After removing the duplicates, 322 stroke genes were finally obtained (Supplementary Table 1).

2.2. Construction of drug-target (D-T) network

The high-quality DTIs were integrated from six recognized databases. The detailed information of integrative process can be found in the previous research [13]. In total, 15367 DTIs which connected 1608 approved drugs to 2251 unique human targets/proteins were assembled. The D-T networks were constructed by Gephi (version 0.9.2) and Cytoscape (version 3.2.1). In each graphical network, drugs or genes or targets were represented by nodes, while interactions were encoded by edges. The degree of each node was also calculated since it characterized the most significant nodes in a network.

2.3. Integration of the human protein-protein interactome

We constructed an extensive human protein interactome *via* integrating six types of protein-protein interactions (PPIs) from multiple databases, including high-throughput Y2H binary (Y2H), protein three-dimensional (3D) interactomes, kinase-substrate interactions, signaling networks, protein complexes and literature. The detailed data source and integration process can be found in previous study [16].

2.4. Identification of potential drug candidates for stroke

In this study, we developed statistical network model to prioritize potential anti-stroke drug candidates *via* integrating D–T network and stroke genes. We assumed that a drug would hold high promise against stroke if its drug targets were more likely to be stroke genes. Then, Fisher's exact test was applied to assess the statistical significance of the enrichment of stroke genes in target profiles of each drug in Drugbank database. The *P*-values were corrected by Benjamini–Hochberg method and a cutoff adjusted *P*-value threshold (q) < 0.001 was set to determine the significant DTIs.

2.5. Experimental validation

2.5.1. Cell cultures and oxygen-glucose deprivation/reoxygenation (OGD/R) model in vitro

Microglial cell line BV2 was purchased from Shanghai institute of life sciences, Chinese academy of sciences. Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), which contained 10 % fetal bovine serum (FBS) were used for BV2 cell culture in a humidified incubator at 37 °C with 5 % CO_2 and 95 % air. Cinnarizine, orphenadrine, diclofenac, phenelzine, ketotifen and omeprazole were purchased from Macklin, China (http://www.macklin.cn/) and dissolved in DMSO. Before establishing OGD/R model, BV2 cells were treated with different concentrations of drugs for 2 h. Then the culture medium was replaced with glucose-free DMEM, and cells were incubated in the incubator of 95 % N_2 and 5 % CO_2 for 3 h. After OGD treatment, BV2 cells were transferred back to the culture medium containing drugs and recovered under normoxic conditions for 5 h.

2.5.2. Cell viability assay

Cell viability was determined by the cell counting Kit-8 (CCK8) (Beyotime, China), according to the manufacturer's instruction. Briefly, BV2 cells cultured in 96-well plate were incubated with CCK8 for 2 h after treatment with different concentrations of drug candidates. The optical density (OD) value was read at 450 nm using a microplate reader (Tecan Austria Gmbh Model-SUNRISE, AT).

2.5.3. Western blot analysis

Cells were lysed in RIPA lysis buffer (Cwbio, China) containing protease inhibitors cocktail (Cwbio, China) on ice. Protein concentrations were next determined using a BCA protein assay kit (Beyotime, China). Proteins were separated by 10–12 % SDS-PAGE, transferred onto PVDF membranes and sealed with 5 % nonfat milk. The membranes were incubated overnight at 4 °C with primary antibodies, including anti-cyclooxygenase-2 (COX-2) (1:1000; CST; #12282), anti-interleukin-6 (IL-6) (1:1000; ABclonal; A2447) and anti-β-actin (1:1000; CST; #3700) followed by incubation with Peroxidase AffiniPure Goat Anti-Rabbit IgG (H + L) (1:5000; Jackson; 111-035-003) or Peroxidase AffiniPure Rabbit Anti-Mouse IgG (H + L) (1:5000; Jackson; 315-035-003) at room temperature for 2 h. Protein bands were captured following adding an enhanced chemiluminescence (ECL) developer and subsequently analyzed by Image J software.

2.5.4. Proteomic profiling with Olink inflammation panel

Proteins were measured using the Olink inflammation panel (Olink Proteomics AB, Uppsala, Sweden), according to the manufacturer's instructions. The Olink panel was able to analyze 92 analytes simultaneously *via* using 1 μL of each sample. Based on the Proximity Extension Assay (PEA) technology, this platform involves a pair of oligonucleotide-labeled antibodies ("probes"), which bind to the target protein. Then, a unique PCR target could be formed *via* a proximity-dependent DNA polymerization event since the probes come in mutual close contact. Consequently, a new target is detected and quantified via using qPCR. The final assay read-out is presented with normalized protein expression (NPX) values. Usually, the larger the value is, the higher the protein expression is.

2.6. Molecular docking

The 2D structures of IL-6 and COX-2 were obtained from the Protein Data Bank database (http://www.rcsb.org/) while the structures of cinnarizine and phenelzine were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Molecular docking analyses were performed *via* using the AutoDock Vina and AutoDock Tools. The AutoDock Tools was utilized to generate the related receptor grid file, and the ligand structures and the receptor grid files were docked with the AutoDock Vina. It was asserted that the lower binding energy represented the better docking effect.

2.7. Statistical analysis

In this study, Gene ontology (GO) terms analysis, including biological process (BP), molecular function (MF) and cellular component (CC) were performed by Omicshare webserver (https://www.

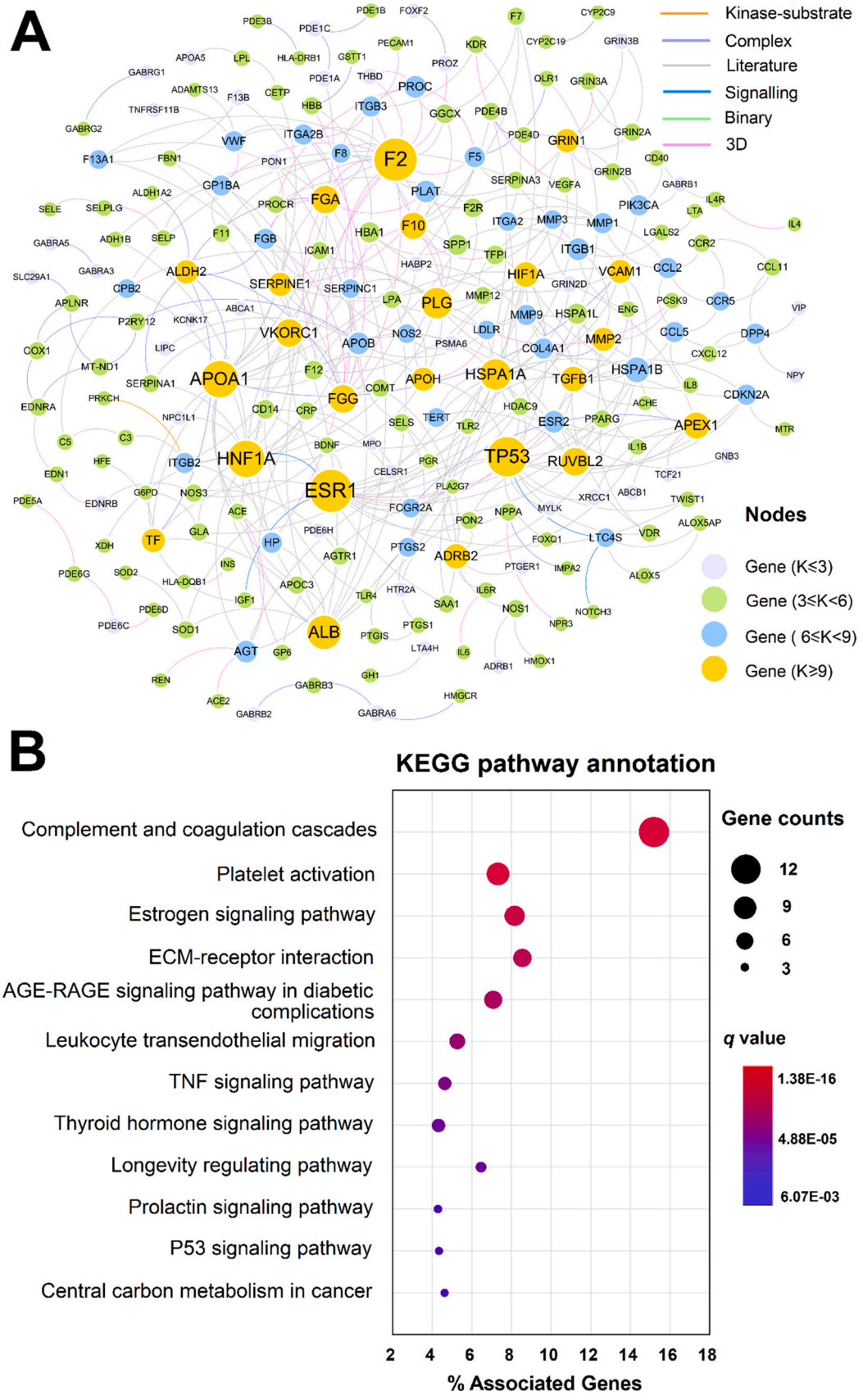


Fig. 2. Protein-protein interaction (PPI) network and enrichment analysis of stroke genes. PPI network consisting of 261 stroke genes and 937 edges (PPIs) (**A**) and KEGG pathway enrichment result of the top 20 stroke genes in PPI network (**B**). The sizes of node and label are proportional to degree. Edge colors represent six types of the PPI evidence, including binary, 3D, kinase-substrate, signaling, complexes and literature.

omicshare.com/tools) while KEGG pathway enrichment results were obtained *via* DAVID (The Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov) database. All experiments in this study were repeated at least three times. Data were represented as mean ± standard deprivation (SD) and analyzed using GraphPad Prism 5.01 (GraphPad Software). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was utilized to compare the data in multiple groups. *P* value smaller than 0.05 was considered as statistically significant ($^{\#}P$ *vs.* control group, $^{*}P$ *vs.* vehicle group).

3. Results

3.1. PPI network and enrichment analysis of stroke genes

To investigate the effects of different stroke genes on an interactome network, we first constructed a comprehensive human PPI network by assembling six types of PPIs mentioned above. This PPI network comprises 261 stroke genes and 937 edges (Fig. 2**A**). Among these genes, 14 out of them have degree higher than 10: F2, ESR1, TP53, APOA1, HNF1A, ALB, HSPA1A, PLG, FGA, FGG, RUVBL2, VKORC1, APEX1 and F10, suggesting their important roles in stroke. For example, it is demonstrated that TP53-induced glycolysis and apoptosis regulator is able to exert neuroprotection against ischemic injury *via* increasing the flow of pentose phosphate pathway [17]. Moreover, HNF1A polymorphisms are likely to be the genetic risk factors for IS [18]. To explore the potential pathways they may function on, we further performed KEGG pathway analysis (Fig. 2**B**) on the top 20 stroke genes in PPI network. The enrichment results indicated that these genes might be involved with multiple pathways which exerted significant effects on stroke, such as platelet activation [19] and estrogen signaling pathway [20]. The inappropriate activation of platelets might result in the formation of occlusive thrombi within the circulation which would finally induce the IS under several pathological circumstances [19]. Recent literature also reported that inhibiting platelet activation could attenuate experimental IS [21]. As for the estrogen signaling pathway, emerging evidence has revealed that the selective estrogen receptor modulator could serve as an effective and safer alternative which benefits for diabetic IS outcome after successful reperfusion [20]. Overall, these genes mentioned above do play vital roles in stroke-related KEGG pathways.

3.2. D-T network analysis

We next constructed a D-T network for FDA approved drugs through assembling curated physical DTIs that connected to stroke genes. The D-T network covers 4568 DTIs interacting 1514 drug candidates with 236 stroke genes (Supplementary Table 2**,** Fig. 3). Among these genes, the top 10 are HTR2A ($D = 183$), SLC6A2 ($D = 150$), SLC6A4 ($D = 146$), SLC6A3 ($D = 131$), DRD3 ($D = 131$), ESR1 ($D = 115$), ABCB1 ($D = 105$), ADRB2 ($D = 99$), ADRB1 ($D = 97$) and PTGS1 ($D = 95$). Meta-analysis indicates that ESR1 rs2234693 polymorphism is highly associated with an increased risk of stroke, especially IS [22]. Also, ESR1 genetic polymorphisms may accelerate the development of cerebral infarction, which more easily happened in the female population [23]. Meanwhile, network analysis shows that 10 drugs have more network connections to stroke genes than other drugs in the D-T network, including caffeine ($K = 22$), pentobarbital ($K = 23$), olanzapine ($K = 21$), amoxapine ($K = 22$), acamprosate ($K = 23$), prasterone ($K = 27$), trapidil ($K = 20$), propofol ($K = 19$), halothane ($K = 19$) and midazolam ($K = 18$), indicating their high promise for stroke therapies. In fact, multiple literature has confirmed the possibilities. For instance, *in vivo* study deciphered that olanzapine attenuated brain damage after focal cerebral ischemia [24] and case report showed that an adolescent with abulia from left middle cerebral artery stroke was treated successfully by using short duration olanzapine [25]. Recent literature has verified that propofol plays a protective role on regulatory T cells, suppresses neurotoxic astrogliosis, and enhances neurological recovery after IS [26]. Propofol is also reported to protect against cerebral ischemia/reperfusion injury though inhibiting long noncoding RNA SNHG14 [27]. To sum up, the D-T network reveals that these approved drugs interact with multiple stroke genes, which may own higher possibilities against stroke.

3.3. Network-based drug repurposing for stroke

In this study, we applied the state-of-the-art network-based approach to prioritize US FDA-approved drug candidates for stroke. In total, statistical network model identified 185 drug candidates ($q < 0.001$) (Fig. 4). It is obvious that most of the drug candidates belong to nervous system ($n = 85$), followed by cardiovascular system ($n = 21$) and genito urinary system and sex hormones ($n = 10$), except "unknown & others". Since stroke is deemed to a neurological deficit caused by an acute focal injury of the central nervous system (CNS) by a vascular cause [28], nervous system drugs are naturally designed as stroke candidates. Cardiac causes of stroke, such as permanent and paroxysmal atrial fibrillation, both increase the risk of cardioembolic IS [29]. Thus, cardiovascular system drug may hold high promise against stroke. Moreover, drug candidates belonging to genito urinary system and sex hormones are likely to be repurposed since gonadal hormones are reported to regulate stroke risk and severity [30], showing their potentials in stroke.

To identify the most promising ones, we next developed a D-T network of these drug candidates (Fig. 5**A**) mentioned above. This network comprises 1888 edges interacting 185 drugs with 160 stroke genes. Most drug candidates are connected to multiple genes with the average degree of 10.2 for each drug. Among them, prasterone has the largest gene interactions ($D = 27$), followed by pentobarbital ($D = 23$) and acamprosate ($D = 23$), indicating their synergistic effects against stroke. Taking pentobarbital for example, it is claimed that delayed pentobarbital administration in gerbils could limit ischemic brain damage [31]. Pentobarbital also reduces infarct size and attenuates the expected time course of ischemic edema in cats [32]. Besides, acamprosate was reported to significantly reduce the neurological deficits following transient hemispheric ischemia [33] and another *in vivo* study has demonstrated that acamprosate is an intriguing candidate for adjuvant future stroke treatment [34]. To further decipher the biological process (BP) they may function on, we performed enrichment analysis on the top 20 stroke genes (Fig. 5**B**). These drug candidates may be involved in lots of BPs, such as gamma-aminobutyric acid (GABA) signaling pathway and dopamine transport. Recent study implies that environmental enrichment could reduce GABA inhibition and facilitate phasic GABA inhibition in the peri-infarct cortex, thus promoting the recovery of stroke [35]. GABA signaling was demonstrated to mediate the protective effects of continuous theta burst stimulation against cerebral ischemia in mice [36]. Moreover, molecular function (MF) results suggested that these potential candidates could participant in neurotransmitter receptor activity and transmitter-gated channel activity *etc.* Sigma-1 receptor activation could alleviate blood brain barrier (BBB) disruption post cerebral IS *via* the stimulation of the GDNF-GFRα1-RET pathway [37]. Indeed, Sigma-1 receptor agonists are regarded as potential treatment for stroke [38]. As for the cellular component (CC), seven items (*ie*, GABA receptor complex and synaptic membrane) were obtained. Intriguing, we found that BP, MF and CC items were simultaneously involved with GABA, indicating its significance in stroke.

To determine the prediction accuracy of our statistical network model, we next systematically searched the previous literature for these 185 drug candidates. Totally, 68 out of them (36.8 %) have been

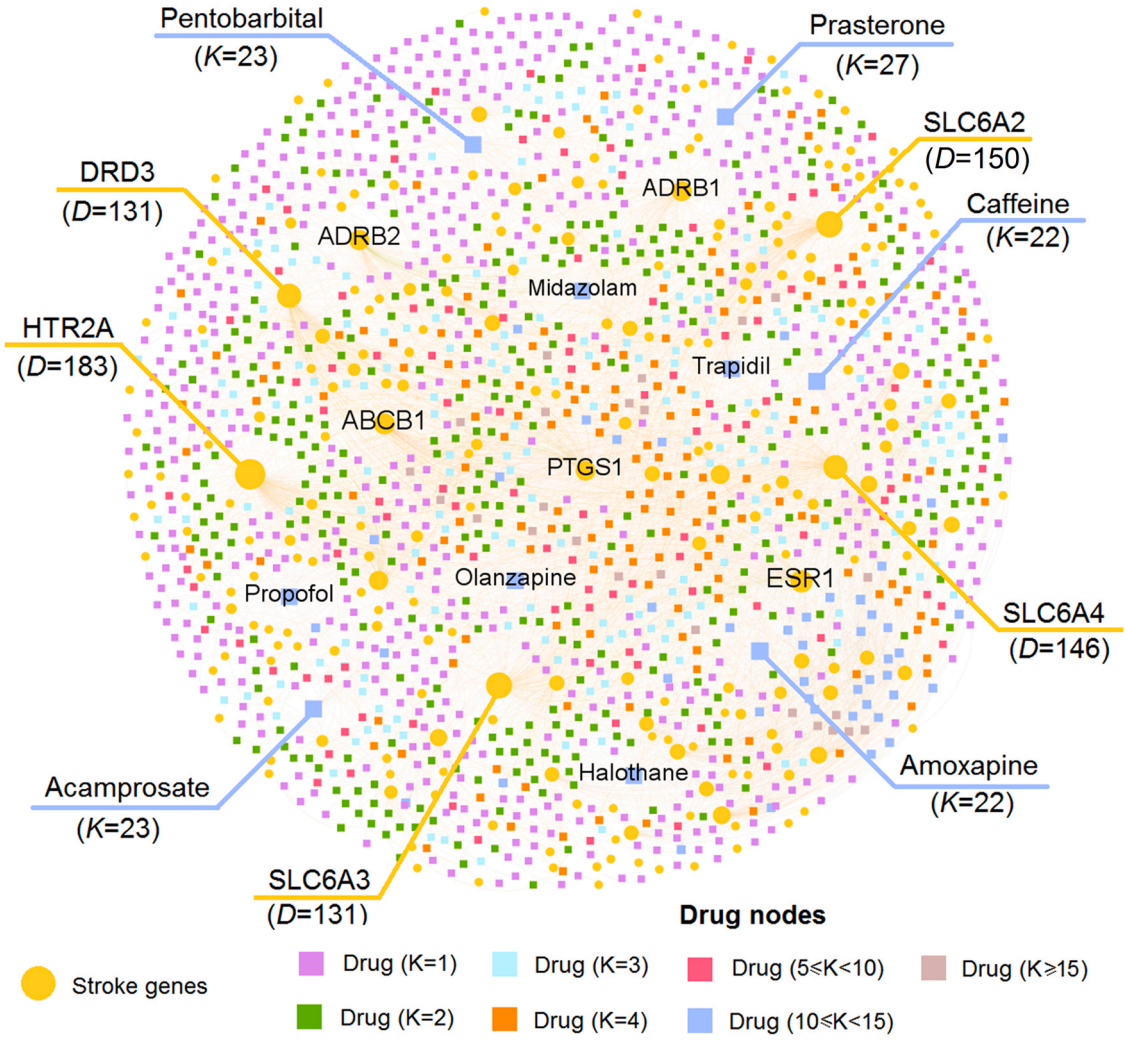


Fig. 3. Drug-target network for approved drugs. This network contains 4568 DTIs interacting 1514 drug candidates with 236 stroke genes. Drug nodes were classified by the degree (K). Labels of the top 10 drug candidates and stroke genes with the highest degree were displayed. The network was generated by the Gephi (version 0.9.2). The square node represents the drug while the dot denotes the stroke gene. The sizes of node and label are proportional to degree.

confirmed with anti-stroke effects by *in vivo*, *in vitro* or other literature evidence (Supplementary Table 3). Taking midazolam for example, abundant research indicated that midazolam exerted beneficial effects on focal cerebral ischemia in rats *via* anti-apoptotic mechanisms [39]. Diazepam was also demonstrated to reduce brain lesion size in a photothrombotic rat model of focal ischemia [40]. Moreover, it is proposed that sildenafil treatment could induce microglial modulation which acts as a potential strategy for neonatal IS treatment/recovery [41]. As for the other 117 drugs without stroke evidence, we interestingly found that 20 out of them were reported to show neuroprotection, indicating their potentials against stroke (Fig. 6). For instance, stiripentol (*adj-p* = 2.28E-26), significantly promoted the number of surviving neurons relative to controls, suggesting its neuroprotective activity [42]. To further narrow up the scope of drug candidates and improve the verification efficiency, we preserved those drugs satisfied with the following factors: (i) confirmed neuroprotective effects *in vivo* and *in vitro*; (ii) availability of drugs; and (iii) strength of the network-based prediction. As shown in Fig. 6, 7 drug candidates owned neuroprotective effects both *in vivo* and *in vitro*. However, drotrecogin alfa was excluded since this drug could not be obtained, which was not satisfied with the availability of drugs. Thus, we prioritized 6 drugs, including ketotifen (*adj-p* = 1.95E-10), cinnarizine (*adj-p* = 1.19E-06), diclofenac (*adj-p* = 3.08E-06), phenelzine (*adj-p* = 5.76E-06), orphenadrine (*adj-p* = 3.02E-04) and omeprazole (*adj-p* = 6.06E-04), to test their anti-stroke effects.

3.4. Experimental validation and mechanism exploration of drug candidates for stroke

3.4.1. Evaluation of drug candidates on cell viability

Based on the encouraging findings discussed above, we next validated the anti-stroke activity of 6 drug candidates, including cinnarizine, orphenadrine phenelzine, ketotifen, diclofenac and omeprazole. Fig. 7 revealed that OGD/R insult group and vehicle group extremely lowered the BV2 cells viability. Compared to the vehicle group, treatment with cinnarizine (10, 20, and 40 μM), orphenadrine(25 μM), phenelzine (20, 40 and 60 μM), ketotifen (40 μM), diclofenac (20 and 40 μM) significantly increased the BV2 cells viability productively (Fig. 7**A–E**, $P < 0.05$ and $P < 0.01$, respectively). Intriguingly, we found that omeprazole, a proton pump inhibitor commonly used for gastric acid hypersecretion, also showed good activity on BV2 cells (Fig. 7**F**, $P < 0.05$), suggesting its positive role in stroke. Especially, the survival rates of BV2 cells were significantly increased after treatment with three different concentrations of cinnarizine (10, 20, and 40 μM), and phenelzine (20, 40 and 60 μM) (Fig. 7, $P < 0.05$, $P < 0.01$), indicating their excellent neuroprotective activity on stroke.

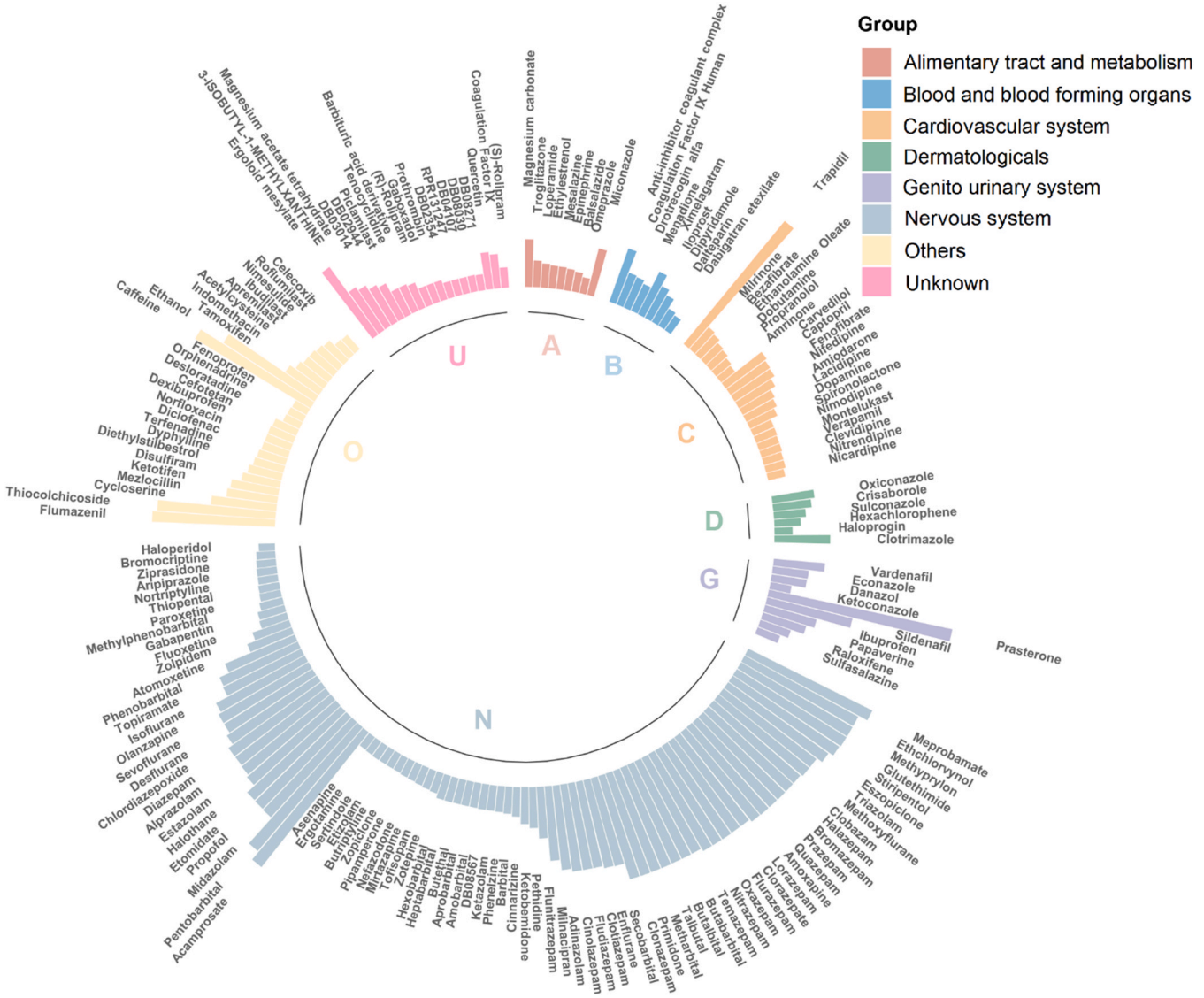


Fig. 4. Network-based drug repurposing for stroke. A total of 185 drugs were considered as candidates for stroke. Drug candidates were classified by the first-level of Anatomical Therapeutic Chemical Classification (ATC) code. Note: A: alimentary tract and metabolism; B: blood and blood forming organs; C: cardiovascular system; D: dermatologicals; G: genito urinary system and sex hormones; N: nervous system; O: others; U: unknown. The lengths of the line bars are consistent with the absolute values of Z-score.

3.4.2. Mechanisms exploration of cinnarizine and phenelzine for stroke

Since cinnarizine and phenelzine showed good anti-stroke effects, we next performed mechanisms exploration for cinnarizine and phenelzine *via* constructing a D-T and PPI network. As a specific calcium channel blocker, cinnarizine can be used for the management of labyrinthine disorder symptoms, including vertigo, tinnitus, nystagmus, nausea, and vomiting [43]. It also has been found to act as an alternative recommendation for migraine prophylaxis and has a potential antipsychotic effect with an atypical profile [44]. Interestingly, both *in vivo* and *in vitro* studies uncovered that cinnarizine could protect dorsal root ganglion neurons [45], while the molecular mechanism still remains unknown. Fig. 8**A** displayed that cinnarizine connected to 9 stroke genes and 12 PPI partners (*e.g.*, IL-6, COX-2). Recent literature has revealed that polymorphism of IL-6 receptor gene is related with IS in patients with metabolic syndrome [46]. Moreover, intravenously administered IL-6 was reported to show beneficial effect in experimental stroke of C57BL/6 mice [47], indicating the potential anti-stroke mechanism of cinnarizine. As for COX-2, evidence indicated that additional pharmacological properties of individual COX-2 inhibitors might have an influence on an increased risk of IS [48].

It is well-known that brain ischemia releases several damage-associated molecular pattern molecules, which can trigger a sterile inflammatory response [49]. Indeed, multiple evidence has confirmed a causal role for inflammation in the pathogenesis of stroke [50], in which IL-6 and COX-2 are important markers of inflammation [51]. To assess whether cinnarizine was involved in inflammation for treatment of stroke, we first performed the molecular docking to explore the possible binding between cinnarizine and these two targets. Fig. 8**B** suggested that cinnarizine could bind to IL-6 and COX-2, with the docking scores of − 7.0 and − 9.2, respectively. We next measured the expression of inflammatory related protein through western blot. As shown in Fig. 8**C–D**, both the protein levels of COX-2 and IL-6 in OGD/R and vehicle groups were significantly increased compared with the control group. 40 μM cinnarizine treatment effectively reduced the protein expression of COX-2 and IL-6 **(**Fig. 8**C–D**).

Similarly, phenelzine, a monoamine oxidase inhibitor (MAO inhibitor), is mainly utilized to treat moderate-to-severe depression, while its FDA-approved indications also include the management of treatment-resistant depression, panic disorder, and social anxiety

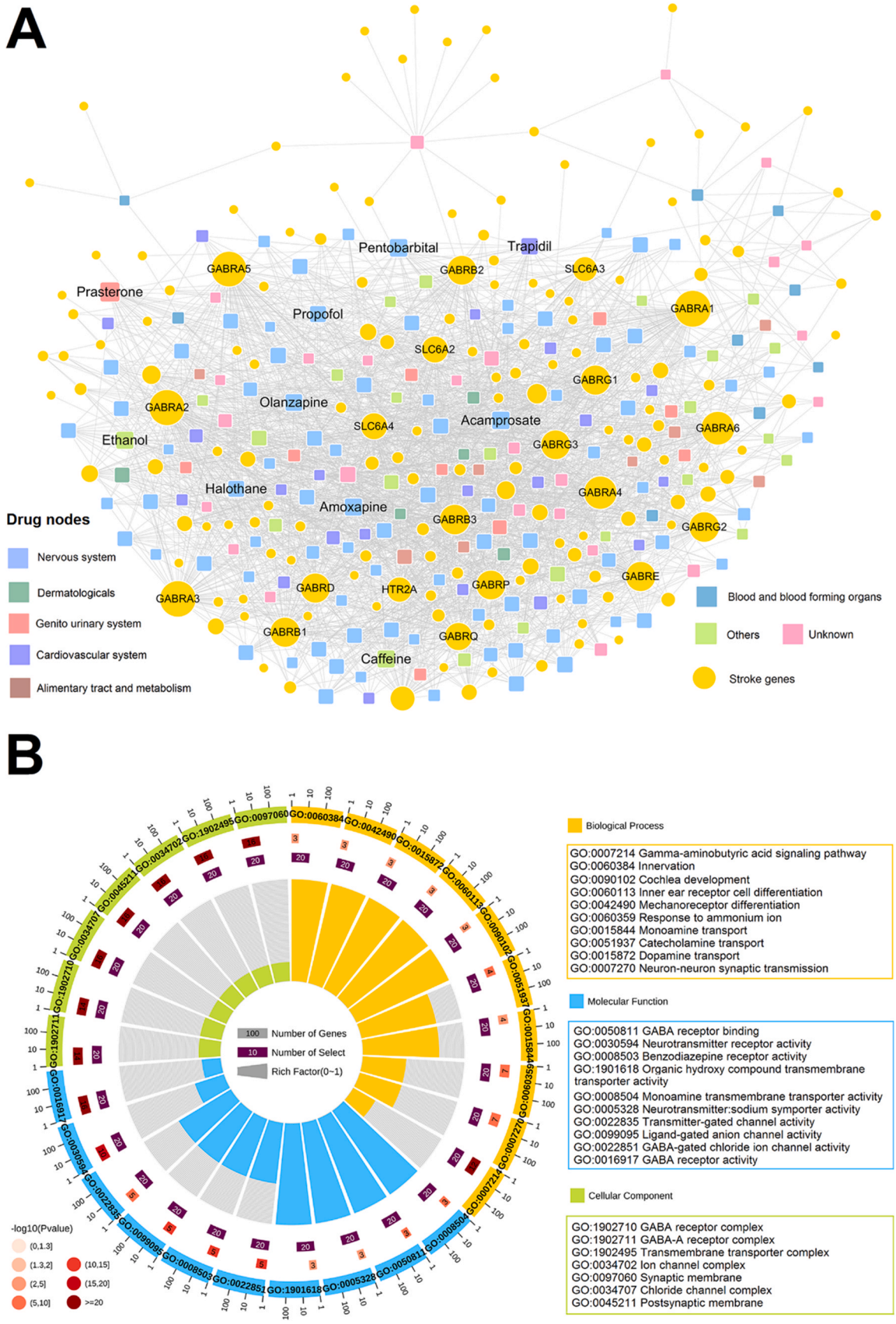


Fig. 5. Drug-target network of 185 drug candidates and gene enrichment analysis. A drug-target network comprising 185 drug candidates and 160 stroke genes. Drug nodes were classified according to the first-level of ATC code. The sizes of node and label are proportional to degree (**A**). BP, MF and CC enrichment results of the top 20 genes in the network are displayed (**B**).

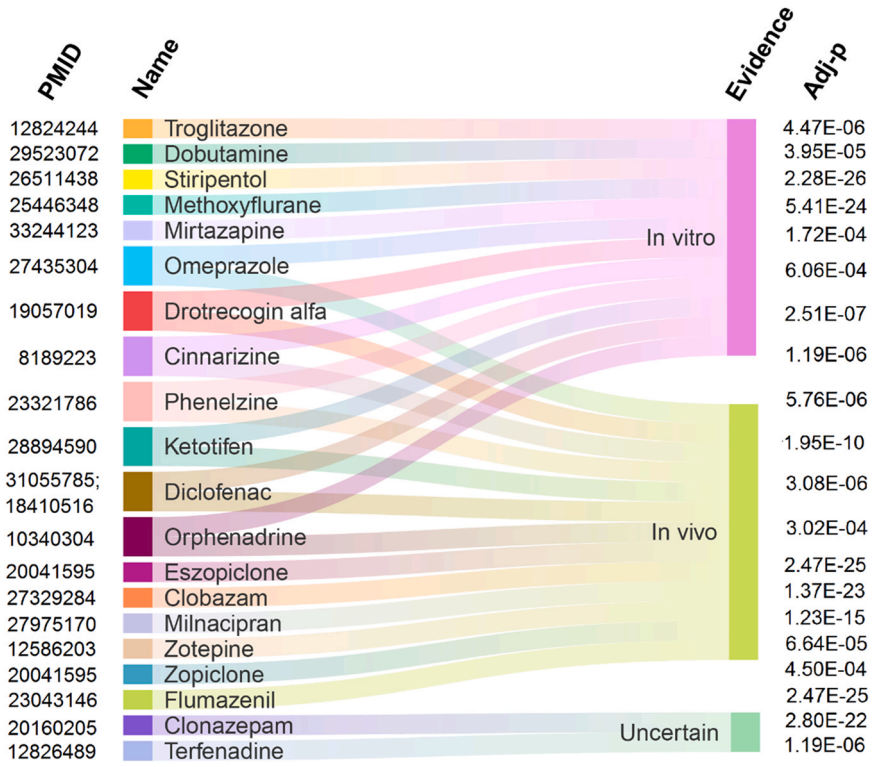


Fig. 6. Twenty drug candidates with neuroprotective effect. PMID denotes the specific previous references. Evidence represents the literature evidence with confirmed *in vitro* or *in vivo* neuroprotective effects. If the previous literature had no specific information of *in vitro* or *in vivo*, we considered it uncertain. Adj-p corresponds to the adj-p values of drug candidates. A cutoff adjusted *P*-value < 0.001 was considered as statistically significant.

disorder. Moreover, clinical trial shows that phenelzine can exert efficacy on patients with biochemical recurrent castrate-sensitive prostate cancer, representing a new avenue for recurrent prostate cancer [52,53]. Additionally, phenelzine has been confirmed to be a strong inhibitor of primary amine oxidase (PrAO) [54,55], indicating its promising role in stroke, since inhibition of PrAO could attenuate ischemia–reperfusion injury in a mouse model of stroke [56]. Intriguingly, Fig. 9**A** displays that phenelzine interacts with 7 stroke genes and 5 PPI partners, suggesting the potential molecular mechanism of phenelzine on stroke. We next confirmed whether phenelzine also played anti-stroke role *via* anti-inflammation. Molecular docking results indicated that phenelzine showed good binding energy with IL-6 and COX-2 (Fig. 9**B**). *In vitro* experiment revealed that 40 or 60 μM phenelzine treatments dramatically decreased protein expression of IL-6 and COX-2 **(**Fig. 9**C–D).** Taken together, results aforementioned demonstrated that cinnarizine and phenelzine might protect OGD/R-induced BV2 cells injury *via* anti-inflammation.

3.4.3. Cinnarizine and phenelzine regulated inflammatory cytokines

In this study, Olink inflammation panel was applied to determine the protein levels of related inflammatory markers. Fig. 10 revealed that the expression level of inflammation related proteins including CCL3, CXCL1, CXCL9, IL-17A, IL-17F, IL-1α,IL-1β, IL-5, IL-6, and TNF-α were significantly decreased after cinnarizine or phenelzine administration compared to the vehicle group. Moreover, results also showed that apoptosis-related proteins (CASP-3, PARP-1) were inhibited by cinnarizine or phenelzine (Fig. 10**A–B**). Surprisingly, we found that the expression levels of protective proteins, including neurotrophic factor NTF-3, IL-10, HGF, TGF-β1 and VEGF-D were increased after drug treatments. Taken together, these results suggested that cinnarizine or phenelzine might have potential therapeutic effects on stroke *via* acting on these proteins.

4. Discussion and conclusion

Despite that stroke is the second leading cause of death and third leading cause of disability globally [1], there are still few therapeutic agents for this disease. Fortunately, drug repurposing provides significant advantages (*e.g.*, low cost and reduced risk) in comparison with traditional drug discovery. In this study, we presented a network-based drug repurposing approach to prioritize potential drug candidates for stroke, which integrated the D-T network, systems pharmacology analysis, literature evidence, experimental validation and mechanism exploration. Specifically, we constructed a comprehensive D-T network of approved drugs and identified potential drug candidates for stroke *via* network-based approach. In total, we prioritized 185 candidates to be associated with stroke, including 85 nervous system drugs, 21 cardiovascular system drugs, 9 alimentary tract and metabolism drugs, 9 blood and blood forming organs drugs, 6 dermatologicals drugs and other drugs. After in-depth literature mining, we intriguingly found that 68 out of them were reported with anti-stroke effects. We next sought literature evidence for the other 117 drug candidates and found that 20 out of them showed neuroprotective effect. Subsequently, we selected 6 drug candidates with *in vivo* and *in vitro* neuroprotective effect for further experimental validation.

We next established an OGD/R model to simulate cerebral ischemic injury and determined the anti-stroke effects of potential drug candidates. All the six candidates showed good activities on BV2 cell. we further showcased the mechanism of action (MOA) of cinnarizine and phenelzine *via* network analysis, molecular docking,

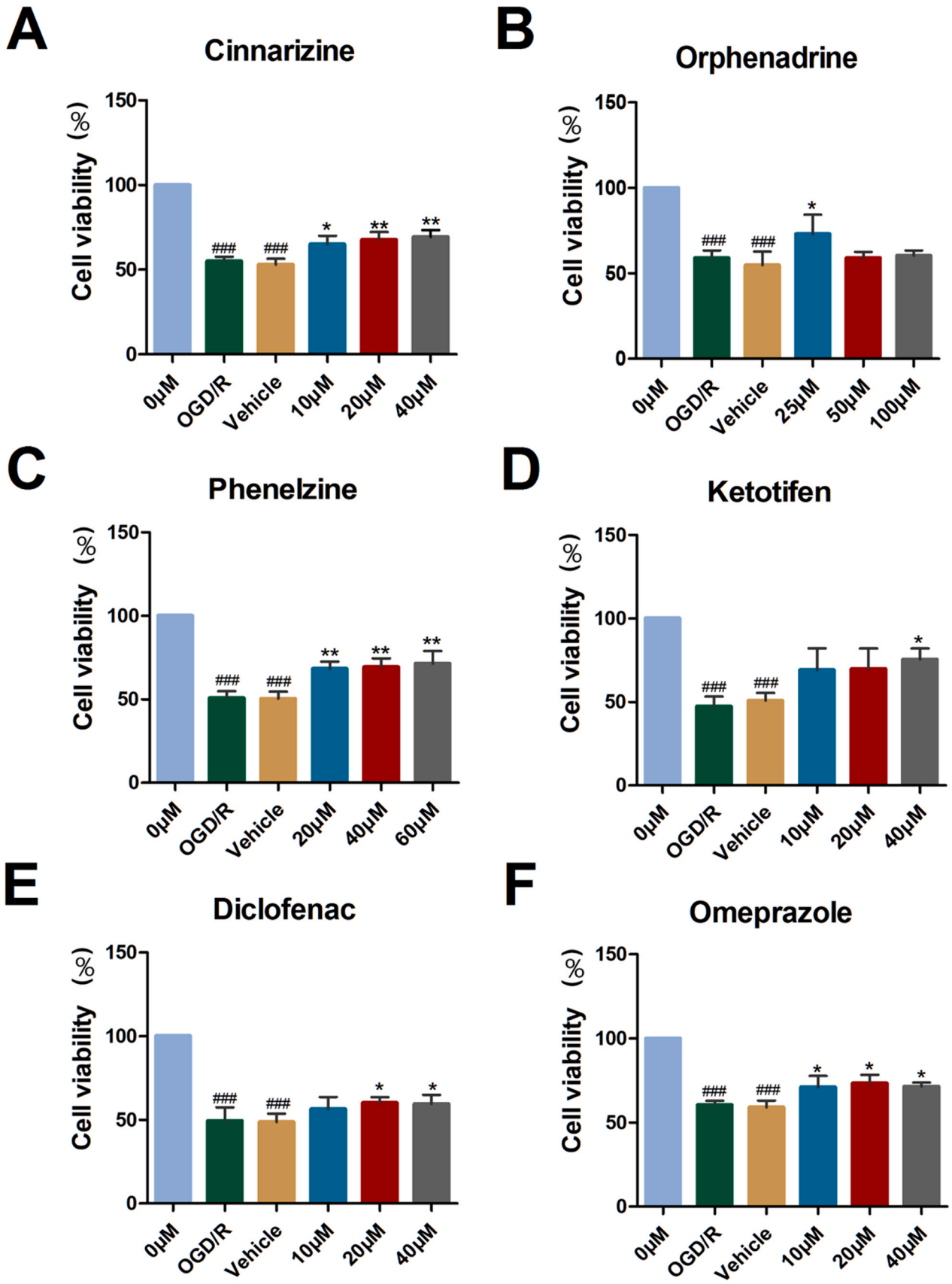


Fig. 7. Effects of drug candidates on BV2 cells viability. BV2 cells viability was tested *via* CCK8 after treatment with cinnarizine (**A**), orphenadrine (**B**), phenelzine (**C**), ketotifen (**D**), diclofenac (**E**), omeprazole (**F**). Data from three times independent experiments were expressed as means ± SD. n = 3. $^{###}P < 0.001$ *vs.* control group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* vehicle group.

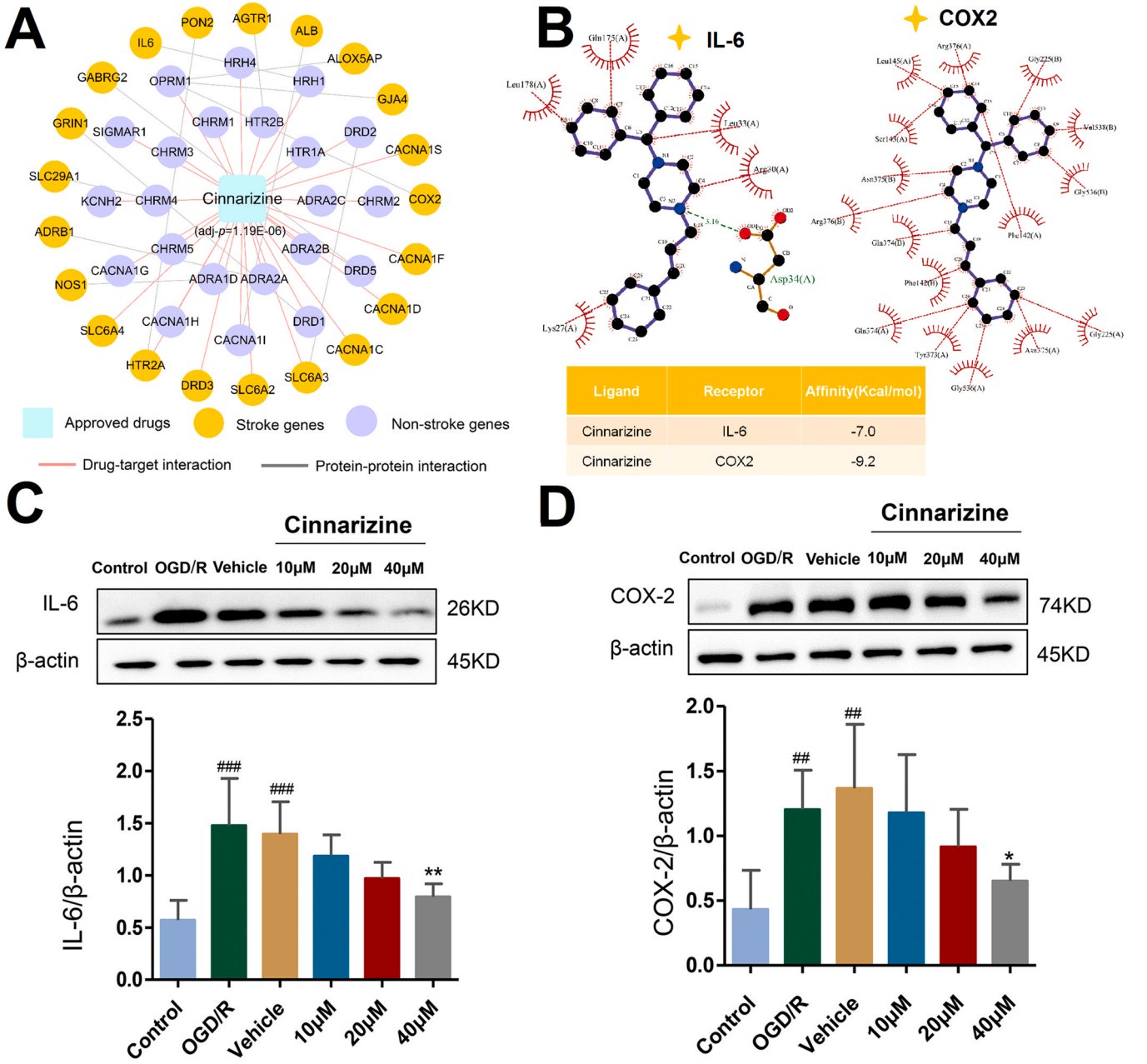
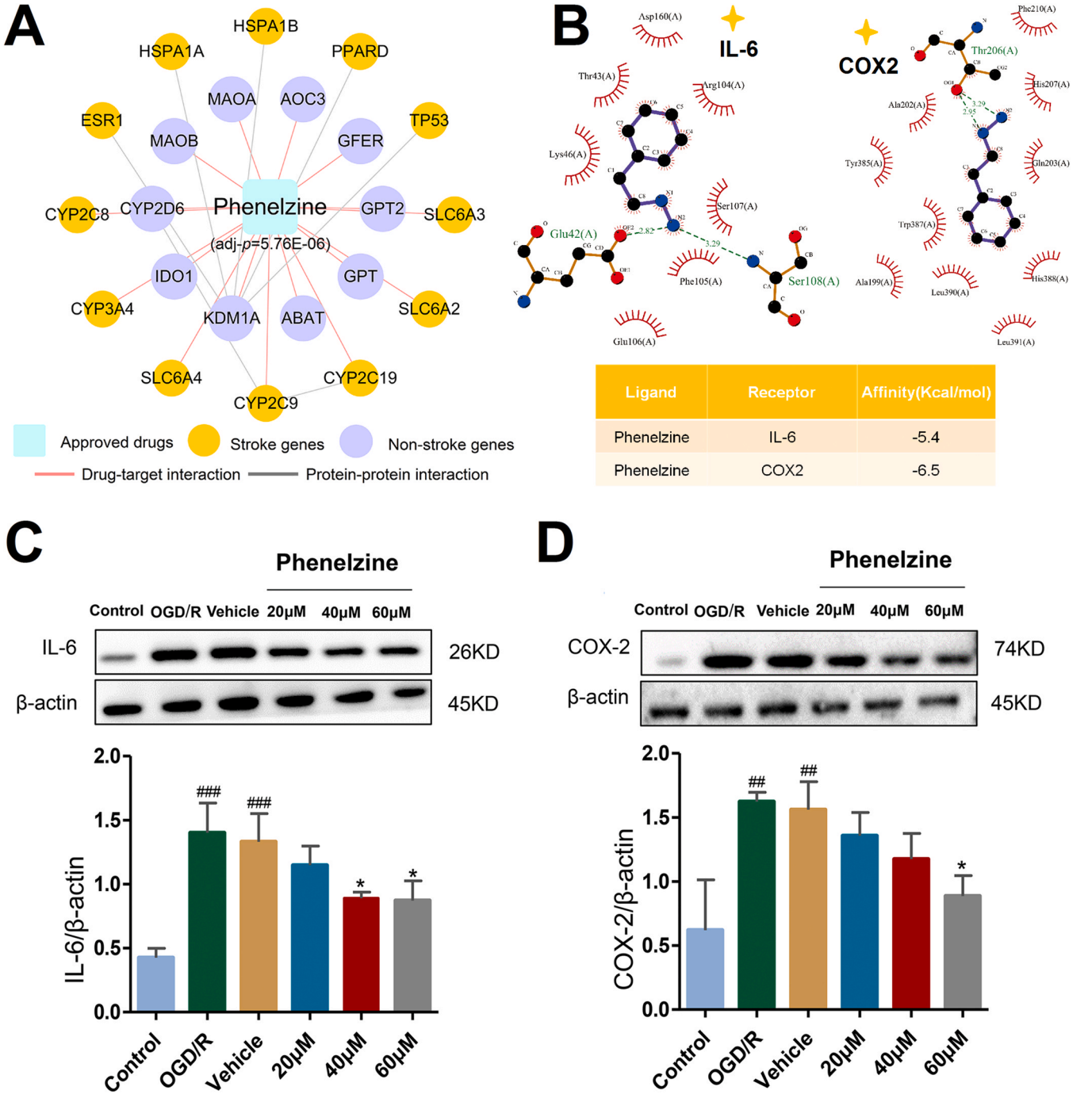


Fig. 8. Mechanism exploration of cinnarizine against stroke. The drug-target and protein-protein interaction network of cinnarizine (**A**) and molecular docking result (**B**). Representative western blot images of IL-6 (**C**) and COX-2 (**D**) protein expression in BV2 cells after OGD/R injury treated by cinnarizine. The data in the figures were presented as mean ± SD. n = 3 per group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* control group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* vehicle group.

western blot, and Olink inflammation panel. We found that cinnarizine and phenelzine could significantly inhibit the release of inflammatory factors (IL-6 and COX-2). Overall, cinnarizine and phenelzine could significantly inhibit inflammation to exert anti-stroke effects, which further supported our network-based prediction on the potential MOA of cinnarizine and phenelzine for stroke.

Several advantages of this study can be highlighted. Firstly, we proposed network-based drug repurposing approach to prioritize promising candidates, which might be applied to identify repurposable drugs for other diseases, such as AD and cardiovascular disease. Xu et al. proposed a network-based, multimodal methodology for drug discovery and prioritized fluticasone and mometasone as the potential treatments against AD [57]. In 2021, Fang et al. also developed an integrated, network-based artificial intelligence methodology for therapeutic discovery in AD, which found three drugs (pioglitazone, febuxostat, and atenolol) were highly related with decreased risk of AD [58]. As for the cardiovascular disease, Cheng et al. presented network-based approach for prediction of *in silico* drug repurposing, and identified that carbamazepine was associated with an increased risk of coronary artery disease (CAD) while hydroxychloroquine was associated with a decreased risk of CAD [16]. Moreover, this study performed experimental validation for pharmaceutical effect and MOA of potential drug candidates that was an advance compared with our previous studies [13,59] and other drug repurposing researches [60]. Furthermore, our study integrated D–T network, molecular mechanism exploration and *in*



Ligand	Receptor	Affinity(Kcal/mol)
Phenelzine	IL-6	-5.4
Phenelzine	COX2	-6.5

Fig. 9. Mechanism exploration of phenelzine against stroke. The drug-target and protein-protein interaction network of phenelzine (**A**) and molecular docking result (**B**). Representative western blot images of IL-6 (**C**) and COX-2 (**D**) protein expression in BV2 cells after OGD/R injury treated by phenelzine. All data was presented as mean ± SD. n = 3 per group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* the control group. $^{*}P < 0.05$ *vs.* the vehicle group.

vitro verification, which to some extent provides case study for other scholars.

However, several limitations of this work should be acknowledged. First, the D-T network data may still be incomplete although we have tried to assemble large scale, experimentally reported DTIs from multiple publicly available databases. To further expand D–T network data, we may apply the computational approaches, such as network-based inference method to systematically predict the DTIs [61]. Moreover, considering the limited cost and time, we just validated several promising drugs in this study whereas other predicted drug candidates are also worth verification. Finally, we performed *in vitro* experiments on candidates at the cellular level while *in vivo* studies and randomized controlled clinical trials are still needed to further confirm the anti-stroke efficiency, since a gap may exist between the anti-stroke effects and the true effect in stroke patients.

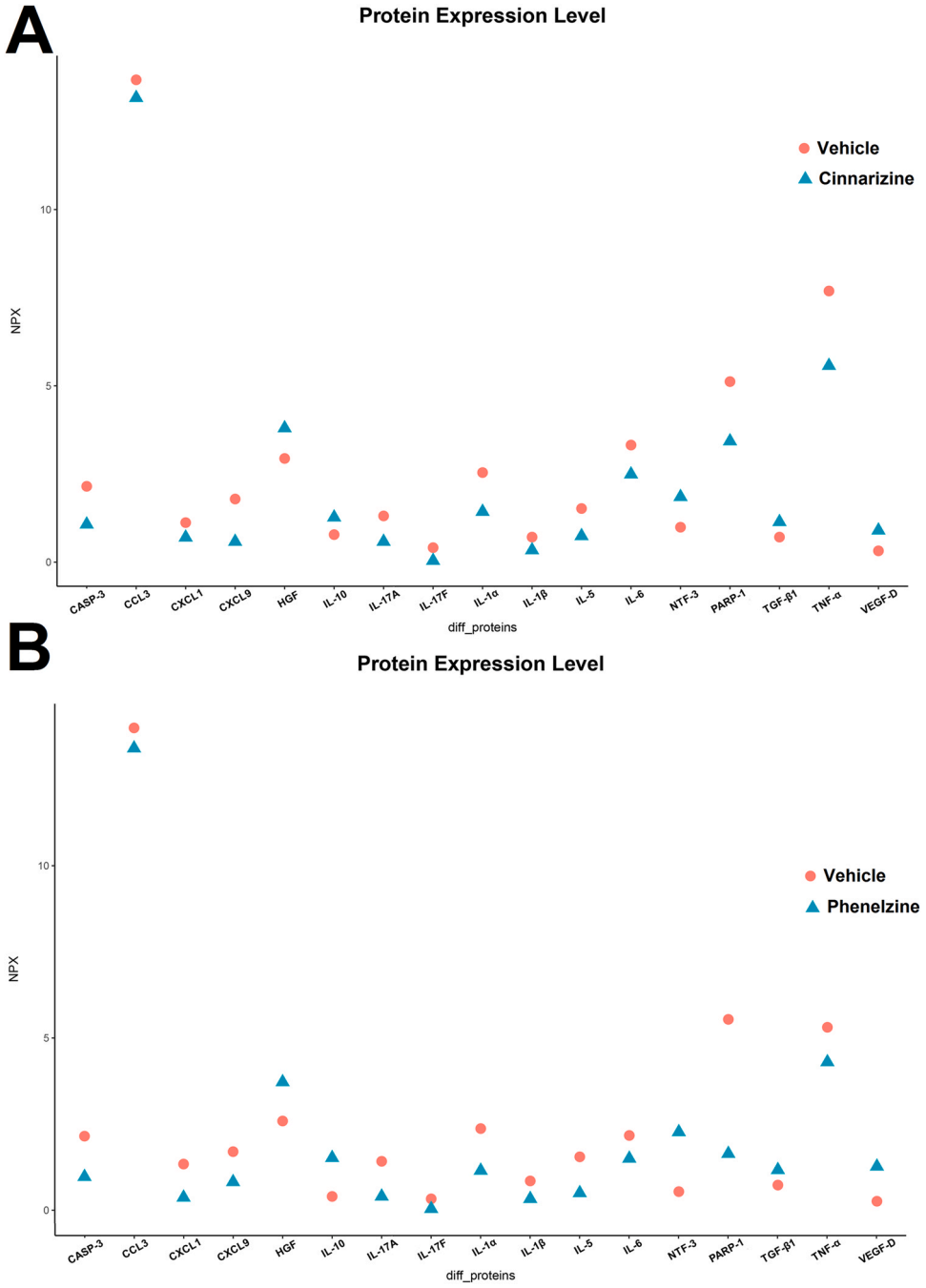


Fig. 10. 17 differential expressed proteins between the drug groups compare with the vehicle group. Abscissa represents differential expressed proteins while ordinate represents the expression level of protein.

In summary, we demonstrated that the network-based drug repurposing could provide efficient strategies for uncovering the potential therapeutic medications for stroke through exploiting the great wealth of existing drugs.

CRediT authorship contribution statement

YG and GW: the conception and design of the study; **WL and YZ:** acquisition of data; **QW and CC:** drafting the article. All authors contributed to the production of this manuscript and had approved the final version.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos. 82160956, 81960227, 82260923), Research and training funds of Hainan Medical University (No. HYPY2020040), Hainan Provincial Natural Science Foundation of China (Nos. 821QN0992, 821RC1129), Hainan Province Science and Technology Special Fund (No. ZDKJ2021034), and Hainan Provincial

Clinical Research Center Program (No. LCYX202104). We acknowledged that Maozhong Yao and Jiansong Fang helped to revise the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.04.018.

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