Variation of pathways and network profiles reveals the differential pharmacological mechanisms of each effective component to treat middle cerebral artery ischemia-reperfusion mice

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Abstract

Using a system pharmacology strategy, this study evaluated the unique pharmacological characteristics of three different neuroprotective compounds for the treatment of cerebral ischemia-reperfusion. A microarray including 374 brain ischemia-related genes was used to identify the differentially expressed genes among five treatment groups: baicalin, jasminoidin, ursodeoxycholic acid, sham, and vehicle, and MetaCore analysis software was applied to identify the significantly altered pathways, processes and interaction network parameters. At pathway level, 46, 25, and 31 pathways were activated in the baicalin, jasminoidin, and ursodeoxycholic acid groups, respectively. Thirteen pathways mainly related with apoptosis and development were commonly altered in the three groups. Additionally, baicalin also targeted pathways related with development, neurophysiologic process and cytoskeleton remodeling, while jasminoidin targeted pathways related with cell cycle and ursodeoxycholic acid targeted those related with apoptosis and development. At process level, three processes were commonly regulated by the three groups in the top 10 processes. Further interaction network analysis revealed that baicalin, jasminoidin, and ursodeoxycholic acid displayed unique features either on network topological parameters or network structure. Additional overlapping analysis demonstrated that compared with ursodeoxycholic acid, the pharmacological mechanism of baicalin was more similar with that of jasminoidin in treating brain ischemia. The data presented in this study may contribute toward the understanding of the common and differential pharmacological mechanisms of these three compounds.

Keywords: Systems pharmacology, baicalin, jasminoidin, ursodeoxycholic acid, brain ischemia

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Introduction

Currently, the only approved measures for the treatment of acute brain ischemia are thrombolysis and antiplatelet therapy.¹ The need to develop an effective treatment for stroke remains paramount. Ongoing researches indicate that combination therapy, especially neuroprotective drugs with different targets, shows promising potential in treating ischemic stroke.^{2,3} However, most existing combination therapies are developed based on clinical experience. We are unfortunately still far from a reliable individualized prediction of synergism and clinical benefit of combination therapy in patients based on mechanism of action information. Thus, the first and most important step toward rationale combination is to fully clarify the pharmacological mechanism of each drug.

Traditional Chinese medicine (TCM) has accumulated a wealth of clinical experiences in treating stroke over the past thousands of years. Combination treatment is always considered as one of the most important characteristics of TCM. There is then a clear need to investigate the molecular mechanism of combination treatment so that it can be assessed and used in a reasonable and consistent manner. Taking baicalin (BA), jasminoidin (JA), and ursodeoxycholic acid (UA) as examples, they are three active ingredients of Qingkailin, which is an herbal formula clinically used to treat stroke in China.^{4,5} BA, a flavonoid compound, is derived from the dried root of *Scutellaria baicalensis* Georgi. Prior studies showed that BA possessed neuroprotective effect against stroke by inhibiting the expression of toll-like receptor 2 and 4 (TLR2/4),⁶ nuclear factor-kappa B (NF- κ B),⁷ inducible nitric oxide synthase (iNOS)

and cyclooxygenase-2 (COX-2),^{6,8} attenuating the serum levels of tumor necrosis factor alpha (TNF- α) and interleukin-1 beta (IL-1 β) as well as increasing the expression of matrix metalloproteinase-9 (MPP-9),⁹ heat shock protein 70 (Hsp70),¹⁰ vascular endothelial growth factor (VEGF) and angiogenesis.¹¹ JA, an iridoid glycoside, is derived from the dried fruit of *Gardenia Jasminoides* Ellis. JA also exhibited preventive effect against ischemic stroke by promoting the expression of brain-derived neurotrophic factor (BDNF) and inhibiting the expression of caspase-3,¹² monocyte chemoattractant protein-1 (MCP-1),¹³ TNF- α and IL- β in ischemic tissue.¹⁴ UA, one of the bile acids, has been reported to inhibit apoptosis by reducing caspase-3 and NF- κ B activities,¹⁵ as well as modulating the expression of certain Bcl-2 family members.¹⁶ However, the existing understanding about the comprehensive and exact pharmacological mechanisms of BA, JA, and UA against cerebral ischemia injury is far from enough.

According to our previous reports, BA, JA, and UA were all effective in reducing infarction volume, and no significant difference was observed among the three groups.¹⁷ A microarray including 16,463 genes showed that the numbers of significantly altered genes were 361, 414, and 470 in the BA, JA, and UA groups, respectively, and only three genes (namely, *Gnb5*, *Il1rap*, and *Wdr38*) were commonly altered in all the three groups.¹⁷ Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis mapped on corresponding genes indicated that no common pathways were found among these three treatments.¹⁷ Moreover, previous research mainly focused on the synergism or additive effects of BA, JA, and UA,^{18,19} whereas the unique molecular mechanism of each individual drug was neglected.

Systems biology analysis methods combined with high-throughput data, such as at pathway level, network level or even a system network dynamics level, will help to improve the understanding of the complex molecular mechanisms underlying stroke and other treatments. Therefore, in this study, we aimed to explore the unique pharmacological mechanisms of each component drug in the combination treatment, i.e. BA, JA, and UA, at systems level using pathway, process and network analysis tools provided in the Metacore software suite developed by GeneGo (www.genego.com).

Materials and methods

Animal handling procedures

Male KunMing mice, weighing 45 ± 5 g, were purchased from the Laboratory Animal Center of Peking University (Certificate no. 2005-0004). Animals were housed five per cage under a normal 12 h light/dark cycle with lights on at 08.00 a.m., and had free access to tap water and food. Ambient temperature and relative humidity were maintained at $22\text{--}25^\circ\text{C}$ and $55 \pm 10\%$, respectively. The mice were allowed one week to adapt to the laboratory environment before experiment.

All animal handling procedures were performed according to the "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) and Chinese legislation for the use and care of laboratory animals. Every effort was made to minimize the number and suffering of mice throughout the study.

Grouping

A total of 20 were randomly selected and allocated to the sham-operated group (N). Eighty mice survived from the middle cerebral artery occlusion reperfusion model (MCAO) operation were randomly divided into four groups, i.e. MCAO model group (vehicle), baicalin-treated group (BA), jasminoidin-treated group (JA), and ursodeoxycholic acid-treated group (UA) ($n = 20$ for each group). In each group, nine mice received infarction volume test, five for microarray analysis and six for Western blotting test.

Preparation of MCAO

MCAO was prepared according to the method provided by Hara et al.²⁰ Firstly, the mice were anesthetized with 2% sodium pentobarbiturate solution (4 mg/kg weight, i.p.), and a middle incision was made on the neck to expose the surgical field. The left common carotid artery, external carotid artery, and inner carotid artery were dissected. After a ligation in the distal part, a small incision was made in the left external carotid near the carotid bifurcation. A 4 to 0 monofilament nylon suture coated with silicon was inserted through this incision and gently advanced into the internal carotid artery. The filament was sent into the intracranial portion of the internal carotid and positioned 11 ± 0.5 mm from the carotid bifurcation for 1.5 h. By this method, a large infarct in the territory of the middle cerebral artery is typically formed. Body temperature measured in the temporal muscle was maintained at $37 \pm 0.5^\circ\text{C}$ with a heated water-blanket under feedback control during the period of surgery. Reperfusion was performed by withdrawal of the inserted filament for 24 h. The above procedure was also applied to the sham-operated group with the exception of the insertion of the nylon filament into the inner carotid.

Drug administration

BA, JA, and UA were kindly supplied by the pharmaceutical factory affiliated with Beijing University of Chinese Medicine. The active ingredients in refined Qingkailing included BA 6.25 g/L, JA 12.5 g/L, and UA 7 g/L.²¹ According to the results from previous research on the dose selection of Qingkailing (data not shown), the doses for mice were as follows: BA (20 mg/kg) for BA group, JA (100 mg/kg) for JA group, and UA (28 mg/kg) for UA group. The drugs were dissolved in saline (4 ml/kg) and injected intravenously into the tail vein just before reperfusion. Mice in the sham-operated group and the vehicle group were given an injection of 4 ml/kg saline with the same method as drug-treated groups.

TTC staining and measurement of cerebral infarct volume

The mice's brains were cut into 2 mm thick coronal sections and stained with 1% 2, 3, 5-triphenyl tetrazolium chloride (TTC) in phosphate buffer (0.1 M, pH 7.4) for 30 min at 37°C . These sections were then cryopreserved. The images of these slices were captured by a digital camera (Color CCD camera TP-6001 A, Topica Inc., Japan). The areas of the

infarction region were calculated with a Pathology Image Analysis System (Topica Inc., Japan) and the ratio of the infarction volume to the total slice was recorded.

Microarray experiments and data analysis

A cDNA microarray was manufactured by mechanical spotting on glass slides using Array Spotter Generation III (Molecular Dynamics Inc., Sunnyvale, CA, USA). This microarray included 374 ischemia-related cDNA derived from a cDNA library (Cat.1065-025; Invitrogen, Carlsbad, CA, USA). Each clone was verified by DNA sequencing, whereas each cDNA represented one gene.

Five mice in each group were randomly selected to extract RNA. At 24 h after reperfusion, mice were decapitated, and the brains were removed under RNase-free conditions. The left cerebral hemisphere was carefully dissected out, placed into Trizol reagent (Life Technologies, Gibco, Rockville, MD, USA), and subsequently homogenized according to the Chomczynski and Sacchi's method.²² Total RNA was extracted with chloroform and isopropyl alcohol and purified with a RNeasy kit (Qiagen, Valencia, CA, USA). The quality of RNA was ensured with electrophoresis in 1% agarose gel. For microarray analysis, 50 mg total RNA of each sample was used. RNA from the vehicle group as a pool was labeled with Cy3, while RNA from the other groups was labeled with Cy5. cDNA was reverse transcribed from RNA using an ampliscribe T7 transcription kit (Epicenter, Madison, WI, USA). cDNA was then hybridized to the microarray at 42°C in a buffer containing 50% formamide, 5 × saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 0.25 mg/ml human cot1 DNA and 0.125 mg/ml poly-dA, for 16 h. The hybridized chip was washed with a series of SSC/SDS solutions. After washing, slides were immediately scanned on an Axon Gene Pix 4000B and quantified using Axon GenePix Pro (Axon, Union city, CA, USA). Data were analyzed using GeneSpring (Silicon Genetics, Redwood City, CA, USA), and normalized with housekeeping gene correction and LOWESS method (parameters, smoothing factor: 0.2, robustness iterations: 3). At length, there were five independent microarray results in each group. The difference of gene expression between drug-treated group and vehicle group as well as among drug-treated groups was then analyzed.

Pathways and networks profile analysis

Data were analyzed using Arraytrack software (<http://www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack>). Differential genes ($P < 0.05$, fold change > 1.5) were presented to GeneGo (MetaCore Version 5.2, www.genego.com). Enrichment analysis consisted of mapping gene IDs of the dataset onto IDs in the entities of built-in functional ontologies represented in MetaCore by pathway map and process. Moreover, in order to compare the potential interaction of differentially expressed genes, the differential genes, as the input list, were used to reconstruct the biological networks using analyze networks (AN) algorithm with default settings. The differential genes were only included in the network. At last, the overlapping

proportion of pathways, edges, and nodes was calculated between any two drug datasets. For each paired datasets, for example, we defined the number of significant pathways, nodes or edges in dataset-1 as g_1 , in dataset-2 as g_2 . The overlapping percentage between two datasets was designated as the number of overlapping pathways/nodes/edges (m) divided by the number of pathways/nodes/edges in the union of g_1 and g_2 . It could be calculated as follows

$$\text{Overlapping percentage} = \frac{m}{g_1 + g_2 - m} \times 100\%$$

Western blotting

The hippocampus of remaining six mice from each group was dissected and homogenized. The proteins (40 µg per lane) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Hybond-C, Amersham, UK) by electroblotting. Membranes were incubated in 5% non-fat milk for 1 h and incubated with antibodies to VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and developed using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). Band density was calculated with a GS-700 densitometer (Bio-Rad, Hercules, CA, USA). For each measurement, three replicates were performed. Data were expressed as the mean ± SD for the indicated analyses.

Results

Pharmacodynamic results

BA, JA, and UA were all effective in reducing infarction volume compared with the vehicle group in our previous study, and no significant difference was observed among these three groups.¹⁷ Further Genego pathway analysis showed that, compared with the sham group, the vehicle group activated 72 pathways ($P < 0.05$), and in comparison with the vehicle group, 46, 25, and 31 pathways were activated in the BA, JA, and UA groups, respectively ($P < 0.05$). In all of these pathways, 18, 14, and 16 overlapping pathways were shared between BA and vehicle, JA and vehicle, as well as UA and vehicle groups, respectively. Meanwhile, apart from the known pathways, some new pathways were also identified in our study. In the vehicle group, nine pathways (Supplementary Table 1) were found for the first time to be related with stroke, as well as five pathways in the BA group and one pathway in the JA group were also firstly identified in brain ischemia model.

Common and differential pathways activated among different treatment groups

Only 13 pathways were commonly altered in all three drug-treated groups. Among them, nine pathways were observed in the vehicle group, including neurophysiological process N-methyl-D-aspartate receptor (NMDA)-dependent postsynaptic long-term potentiation in CA1 hippocampal neurons, G-protein signaling_G-Protein alpha-i

signaling cascades, signal transductionErk Interactions: inhibition of Erk, development_G-Proteins mediated regulation MARK-ERK signaling, development_A2B receptor: action via G-protein alpha s, apoptosis and survival_tumor necrosis factor receptor (TNFR1) signaling pathway, Development_Delta-type opioid receptor signaling via G-protein alpha-14, Development_activation of Erk by Alpha-1 adrenergic receptors, as well as

development_activation of Erk by ACM1, ACM3 and ACM5. Moreover, 4, 3, and 1-altered pathways overlapped between BA and JA, JA and UA, as well as BA and UA groups, respectively (Figure 1(a)).

Overlapping analysis showed that the overlapping percentage at pathway level among the BA, JA, and UA groups was only 14%. The overlapping percentage between JA and UA (40%) was higher than that between BA and JA (30.9%)

Figure 1 Comparative analysis of stroke-related pathway profiles among BA, JA, and UA group. (a) Three-way Venn diagram of BA, JA, and UA, which shows that (1) 13 pathways overlapped among the three groups; (2) four pathways overlapped between BA and JA, three pathways overlapped between UA and JA, and only one pathway overlapped between BA and UA; (3) 29, 5, 14 pathways were only regulated in BA, JA or UA, respectively. (b) The top 10 significant common pathway maps of BA, JA, and UA. Green indicates BA, yellow indicates JA and blue indicates UA. (A color version of this figure is available in the online journal.)
BA: baicalin; JA: jasminoidin; UA: ursodeoxycholic acid

Figure 2 The histogram of overlapping percentage of pathways, edges, and nodes of interaction network among BA, JA, and UA. BA: baicalin; JA: jasminoidin; UA: ursodeoxycholic acid

as well as that between BA and UA (21.9%) (Figure 2). Meanwhile, according to GeneGo pathway analysis, 29, 5, and 14 non-overlapping pathways were noted in the BA, JA, and UA groups, respectively. All unique pathways for BA, JA, and UA were listed in Supplementary Table 1. This suggested that BA, JA, and UA exerted their beneficial effects against brain ischemia by targeting different molecular targets. The top-ranked pathway in BA group was G-protein_G-Protein alpha-i signaling cascades, and that in both JA and UA groups was apoptosis and survival_TNFR1 signaling pathway.

Moreover, even in the commonly activated pathways, BA, JA, and UA might have different target proteins/molecules. For example, in the TNFR1 pathway (Figure 4), in addition to the common targets of Bid, tBid, jBid, and IKK- γ , BA, JA and UA also had their individual unique target proteins. BA uniquely targeted FADD and Flash, while UA targeted caspase-2 to prevent neuron apoptosis. Still, even in the common target signaling molecular of the TNFR1 pathway, BA, JA, and UA might have different potencies. The effect of BA and JA appeared to be stronger than that of UA.

Common and differential GeneGo processes among different treatment groups

The top 10 process networks of each drug are shown in Figure 5(a) to (c). In these top 10 process networks, signal transduction_Wnt signaling, proliferation_positive regulation cell proliferation, and transcription_nuclear receptors transcriptional regulation were commonly regulated by BA, JA, and UA. Additionally, BA specifically regulated TREM1 signaling, NOTCH signaling, relaxing signaling and ESR1-membrane pathway; JA uniquely regulated cholecystokinin signaling and DNA damage_checkpoint; and transmission of nerve impulse, anti-apoptosis mediated by external signals via MAPK and JAK/signal transducer and activator of transcription (STAT), G1-S growth factor regulation, male sex differentiation and leptin signaling were only regulated by UA. Moreover, in the top 10 common process networks

Figure 3 Differences in VEGF expression between BA, JA, and UA in Western blotting validation.

* $P < 0.05$ vs. the vehicle group.

BA: baicalin; JA: jasminoidin; UA: ursodeoxycholic acid

regulated by BA, JA, and UA, the effect of each individual drug still varied. For example, anti-apoptosis mediated by external signals via NF- κ B was the top ranked process network. The inverse log of the P value of BA was nearly three times that of UA (Figure 5(d)).

Differential interaction networks among different treatment groups

Genes individually specific for BA, JA, and UA constituted a direct interaction network. Overall, as shown in Figure 6(a) to (d), the network structure of BA was more similar with that of JA as compared with UA. For example, BA and JA had more identical edges, leading to several same modules, such as a module consisting of five nodes and five edges (Oct-1, JunD, Bcl-XL, tBid, and Bid). But in UA group, the nodes and edges changed obviously, and more novel interactions were observed. Particularly, a big subnetwork containing 16 nodes and 21 edges appeared in UA group. In this subnetwork, STAT5 with four edges

Figure 4 Differentially expressed genes in BA, JA and UA groups mapped on the TNFR signaling pathway (1 denotes JA, 2 denotes UA, and 3 denotes BA). The thermometer represents differentially expressed level (red indicates up-regulation and blue indicates down-regulation). Significant common nodes are encircled in red. Unique nodes for BA are encircled in blue square, and unique nodes for UA are encircled in red square. (A color version of this figure is available in the online journal.) BA: baicalin; JA: jasminoidin; UA: ursodeoxycholic acid

exhibited the highest node degree. Besides, at edge level, the overlapping percentage between BA and JA groups (57.14%) was higher than that between BA and UA (14.29%) as well as that between UA and JA groups (20%). At node level, the overlapping percentage between BA and JA groups (55.77%) was also higher than that between BA and UA (34.92%) as well as that between UA and JA groups (45.16%) (Figure 2). Finally, we quantitatively characterized the BA, JA, and UA networks using the measures of number of nodes, number of edges, shortest path length, characteristic path length, and average number of neighbors. Similarly, these five parameters of BA and JA groups were distinct from those of UA group. The details are shown in Table 1.

Western blotting validation

Data were expressed as the mean $\pm$ SD for the indicated analyses. The results of Western blot analysis showed that the expression level of VEGF was significantly decreased in JA group as compared with vehicle group as shown in Figure 3 ($P < 0.05$). This finding was consistent with the

results shown in Figure 6(a), (c) and (d). In addition, although the level of VEGF in UA group was not completely consistent with the results in Figure 6(b), the same trend was observed, which may be explained by the use of different analytical methods.

Discussions

Based on Metacore analysis with BA, JA, and UA gene data, our systems pharmacology strategy provides a rationale for explaining both common and differential mechanisms of BA, JA, and UA in the treatment of cerebral ischemia. The three drug-treated groups commonly influenced molecular targets related with excitotoxicity, apoptosis, and inflammation process following cerebral ischemia to improve somatosensory and cognitive functions, such as NMDA-dependent postsynaptic long-term potentiation in CA1 hippocampal neurons, Erk-related signaling pathways and TNFR1 signaling. These overlapping pathway, process or network attributes were composed of their basic pharmacological effects against cerebral ischemia. Meanwhile, each drug also had its own unique pharmacological

Figure 5 GeneGO Biological Processes in BA, JA, and UA groups. Horizontal bar indicates the negative log of the P from the hypergeometric distribution calculation. Figure 4(a) to (c) is the top 10 processes of BA, UA, and JA (red dot indicates the unique process networks for each group. Green square indicates the common process networks in the top ten process networks of each group). Figure 4(d) is the top 10 significant common process networks of BA, JA, and UA (green bar indicates BA, blue indicates JA and yellow indicates UA). (A color version of this figure is available in the online journal.)

BA: baicalin; JA: jasminoidin; UA: ursodeoxycholic acid

characteristics. BA preferred to influence the upstream molecular target to regulate cytoskeleton remodeling, cell proliferation as well as some neurophysiological processes; JA targeted cell cycle regulation; and UA mainly influenced angiogenesis and apoptosis. Additionally, our results indicated that network-level analysis might be more appropriate to clarify drug diversities than pathway-level analysis.

At the pathway level, BA, JA, and UA targeted distinct pathways and induced their own unique pharmacological effects. The top-ranked target pathway of BA was G-Protein α -i signaling cascades. BA may activate the G-protein α -i signaling cascade by regulating SOS and RGS18 to trigger the ERK signaling, leading to cell proliferation and activation of transcription factor STAT3. Moreover, BA can activate the PKA signaling by up-regulating the PKA-reg expression to inhibit a number of inflammatory signaling pathways including NF- κ B and MAPK (see supplementary Figure 1). It can be concluded that stress-related signaling is the most important therapeutic mechanism of BA. Apoptosis and survival_TNFR1 signaling pathway was the top-scored pathway of both JA and UA, revealing that preventing neuron cell apoptosis constituted the basic pharmacological mechanism of JA and UA.

Even in their common pathways, BA, JA, and UA might exhibit different effects. Taking the TNFR1 signaling pathway as example, although all of the three compounds targeted Bid, jBid, tBid, and IKK- γ to inhibit apoptosis,

inflammation, and proliferation,²³ each drug also had their own specific therapeutic targets. JA targeted FADD and FLASH to bind with the death-effector-domain (DED) of caspase-8, thereby inducing apoptosis,²⁴ while UA targeted caspase-2, an initiator caspase responding to cellular stress or damage and translocating the death protein, Bax, to the mitochondria, as well as facilitating the release of proapoptotic factors from the mitochondria.²⁵ Caspase-2 might also use mitochondria as an amplification mechanism to induce cell death.²⁶ Thus, our results indicated that UA might also function via the mitochondrial apoptotic death pathway to treat brain ischemia through TNFR1 signaling.

To further enhance our understanding on the molecular targets of BA, JA, and UA, we incorporated the common and differential protein targets involved in BA, JA, and UA into one diagram (Figure 6(e)) to compare the differences among the three drugs. The diagram revealed that BA, JA as well as UA were multiple target drugs, with significant differences in their target sites. BA mainly targeted upstream sites, indicating that BA had an upstream mechanism action at the level of neuron activation. On the contrary, JA and UA targeted many downstream transcription factors. JA regulated the expression of C-myb, STAT5, and HP1 to modulate cell proliferation, apoptosis, development, inflammation, and cell cycle to treat brain ischemia^{27–29}, and UA uniquely targeted XPG and DP1, by which directly regulated gene transcription and gene repair.^{30,31}

Figure 6 Interaction network diagram of differentially expressed genes of BA (a), JA (b), UA (c) and vehicle group (d), and signaling diagram of both common and differential target proteins of BA, JA, and UA (e). Node module in the green square indicates the common module of BA, UA and JA signature network (a–d). Blue represents the common target and cellular process, yellow indicates BA, pink indicates UA and green indicates JA (e). The number from 1 to 12 represents the different biological processes, 1: cell cycle regulation; 2: apoptosis; 3: differentiation; 4: proliferation; 5: inflammation; 6: development; 7: gene transcription; 8: DNA repair; 9: chromosome condensation; 10: cell survival; 11: cytoskeleton remodeling; and 12: long-term potentiation. (A color version of this figure is available in the online journal.) BA: baicalin; JA: jasminoidin; UA: ursodeoxycholic acid

Table 1 Network topological parameters of BA, JA, and UA

Parameters	BA	JA	UA
Number of nodes	38	43	47
Number of edges	9	13	13
Shortest paths	19(1%)	22(3%)	81(16%)
Characteristic path length	1.737	1.636	2.593
Average number of neighbors	0.476	0.963	2.0

BA: baicalin; JA: jasminoidin; UA: ursodeoxycholic acid.

At the process level, the top-ranked process of BA was apoptosis_anti-apoptosis mediated by external signals by NF- κ B, which also confirmed previous reports that BA inhibited the expression of NF- κ B to inhibit neuron apoptosis.⁷ As for UA, the top-ranked process was long-term potentiation, one of the major cellular mechanism underlying learning and memory.³² Prior research showed that bear acid could improve learning and memory deficit induced by ethanal, CCl₄, and scopolamine.³³ Our results indicated that UA had potential effects on vascular dementia and might be regarded as the main active substrate of bear acid. For JA, signaling transduction_cholecystokinin signaling was the top process. CCK is one of the most abundant neuropeptides and a potent excitatory neurotransmitter in the brain.^{34,35} Previous observations have demonstrated that CCK signaling is involved in many symptoms complicated with brain ischemia including gastric mucosal damage,³⁶ mood and cognitive disorders.^{37,38} This suggested that regulating brain-gut axis to treat brain ischemia via the CCK system was an important characteristic of JA.

At the level of interaction network, dramatic differences were also observed among BA, JA, and UA. Firstly, the network structure was different. In UA group, a closely interactive subnetwork appeared. Within this subnetwork, we identified C-myb and STAT5 as the hub nodes to regulate other protein expressions. C-myb is a DNA-binding transcription factor implicated in the regulation of multiple biological processes, including apoptosis, proliferation, differentiation and inflammation, and it has been found to be associated with delayed neuronal death after ischemic damage.^{39,40} STAT5, also known to play a role in transcriptional regulation of Bcl-xl gene, regulates cell specification, proliferation, differentiation, and survival.⁴¹

Secondly, an interesting finding was that the module of VEGF A-Cyclin A was formed. VEGF A plays an important role in mediating endothelial cell proliferation,⁴² while Cyclin A interacting with CDK1 and CDK2 triggers the S and G2 phase of cell cycle to regulate cell proliferation, differentiation, and cell death.^{43,44} Although this module was found in the BA, JA, and UA networks to regulate angiogenesis, its expression level varied among different treatment groups. In the vehicle network, the expressions of both VEGF A and Cyclin A were up-regulated. Conversely, the VEGF-A expression in JA and UA networks was down-regulated; and the Cyclin A expression in BA network was also down-regulated. This phenomenon suggested that different active components exerted different

effects on the same module, but the ultimate result was to keep a normal balanced state.

Additionally, overlapping analysis was also performed to assess the differences among BA, JA, and UA. Interesting results appeared; at the pathway level, the overlapping percentage between JA and UA was higher than that between BA and JA or that between BA and UA. However, at the direct interaction network level, the overlapping percentage between BA and JA at the node and edge level became the highest. Moreover, our previous PCA analysis results⁴⁵ combined with network parameters including shortest path, characteristic path and neighbor's edge also supported that BA was more similar to JA in terms of pharmacological targets.

Derived from different Chinese medicinal herbs, BA, JA, and UA work differently in the treatment of stroke. Both Scutellaria and Gardenia belong to the same category of Chinese herbs, and have similar indications in clinical practice, which is consistent with the above mentioned findings from the overlapping analysis at the network level. Thus, we conclude that analysis at the network level may be more appropriate and reliable in exploring the molecular mechanism of therapeutic compounds.

More importantly, except for known established pathways, nine novel pathways were firstly enriched in brain ischemia (see supplementary Table 1). Among of them, five pathways were also affected by BA, JA and/or UA. Especially, G-Protein alpha-i signaling cascades was simultaneously enriched in vehicle, BA, JA and UA group by regulating B-RAF, RGS18, and PKA-reg expression to regulate cell proliferation. It is suggested that this pathway and related genes may be potential targets involved in brain ischemia.

In summary, our study provides an overall picture of the pharmacological information about BA, JA, and UA at the level of pathways, processes and interaction networks, which may contribute to rational clinical use and render a basis for future experimental and clinical research on individual drug or combinations. Moreover, our results also indicate that analysis of both similarities and differences among different drugs at the network level may be more robust and stable than that at the pathway or gene level. In addition, it should be pointed out that our comparison was based on 374 brain ischemia-related gene chips, which might not fully reflect the pharmacological mechanism of each compound. Therefore, further investigation into the genome-wide data regulated by BA, JA, and UA should be carried out to deepen the understanding of pharmacological mechanism of each drug.

Authors' contributions: All authors participated in the design, acquisition of data, interpretation of the studies and analysis of the data and review of the manuscript; Dang and Wang wrote the manuscript.

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