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Article

Pure mechanistic analysis of additive neuroprotective effects between baicalin and jasminoidin in ischemic stroke mice

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Abstract

Both baicalin (BA) and jasminoidin (JA) are active ingredients in Chinese herb medicine *Scutellaria baicalensis* and *Fructus gardeniae*, respectively. They have been shown to exert additive neuroprotective action in ischemic stroke models. In this study we used transcriptome analysis to explore the pure therapeutic mechanisms of BA, JA and their combination (BJ) contributing to phenotype variation and reversal of pathological processes. Mice with middle cerebral artery obstruction were treated with BA, JA, their combination (BJ), or concha margaritifera (CM). Cerebral infarct volume was examined to determine the effect of these compounds on phenotype. Using the hippocampus microarray and ingenuity pathway analysis (IPA) software, we exacted the differentially expressed genes, networks, pathways, and functions in positive-phenotype groups (BA, JA and BJ) by comparing with the negative-phenotype group (CM). In the BA, JA, and BJ groups, a total of 7, 4, and 11 specific target molecules, 1, 1, and 4 networks, 51, 59, and 18 canonical pathways and 70, 53, and 64 biological functions, respectively, were identified. Pure therapeutic mechanisms of BA and JA were mainly overlapped in specific target molecules, functions and pathways, which were related to the nervous system, inflammation and immune response. The specific mechanisms of BA and JA were associated with apoptosis and cancer-related signaling and endocrine and hormone regulation, respectively. In the BJ group, novel target profiles distinct from mono-therapies were revealed, including 11 specific target molecules, 10 functions, and 10 pathways, the majority of which were related to a virus-mediated immune response. The pure additive effects between BA and JA were based on enhanced action in virus-mediated immune response. This pure mechanistic analysis may provide a clearer outline of the target profiles of multi-target compounds and combination therapies.

Keywords: ischemic stroke; traditional Chinese medicine; baicalin; jasminoidin; combination therapy; additive effects; hippocampus microarray; transcriptome; network pharmacology; Fangjiomics

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Introduction

Ischemic stroke (IS) is a common disease with high mortality and disability[1, 2]. Intravenous thrombolysis is the only approved and effective treatment recommended by the FDA[3]. However, thrombolysis can only be performed within a strictly limited time window, and may also lead to fatal complications[3]. Thus, it is of great significance to develop a complementary or alternative therapeutic strategy for the disease, beyond extension of the therapeutic window and indications of thrombolysis. As a complex disease, the pathogenesis of IS features multiple common genetic variations and dysfunctions[4]. The complexity of this disease resists traditional efforts that attempts to seek a "magic bullet" to achieve optimal therapeutic efficacy[5-7]. Therefore, increasing attention has been paid to utilizing one or more multi-target drugs to systematically reverse the disease molecular network to achieve novel homeostasis[8, 9]. It is suggested that multi-target perturbations to the pathological network, especially designed drug arrays (Fangji), may lead to improved effectiveness, reduced adverse effects, and decreased drug resistance[9-12]. Herbal medicine, particularly effective ingredients extracted from Chinese medicinal materials, is characterized by low activity and multi-target action[13]. A combination of several ingredi-

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ents in the holistic treatment of ischemic stroke, also known as "magic shotguns", is expected to shed light on a novel therapeutic solution for this complex disease[5]. Consequently, a series of attempts to identify medicinal herbal ingredients and their underlying pharmacological mechanisms have been made, following conventional methods for drug research and development. However, it remains challenging to clearly elucidate the pharmacological mechanisms of these ingredients due to the complexity of their multiple targets and the potential interrelations between these targets. Polypharmacology, a promising avenue to systemically decipher drug mechanisms of modulating disease molecular networks[14], provides an opportunity to fully understand the activities and pharmacological mechanisms of herbal ingredients[15-17]. Therefore, it is appealing to utilize polypharmacology methods to elucidate the pharmacological mechanisms of herbal medicine on multiple targets.

Qingkailing injection, a complementary and alternative therapy for IS that is widely prescribed across China, has been demonstrated to be effective in reducing ischemia-reperfusion injury[18]. However, its pharmacological mechanism is still far from clear. Baicalin (BA) and jasminoidin (JA), extracted from *Scutellaria baicalensis* and *Fructus gardeniae*, respectively, are the major bioactive ingredients in Qingkailing injection according to fingerprint analysis based on high-performance liquid chromatography (HPLC)[19]. Thus, it is of utmost importance to investigate the biological actions of BA and JA on IS. BA has long been suggested to be a potential therapeutic agent for stroke and a strong candidate for drug discovery[20]. BA has been reported to reduce neuronal damage, brain edema, and blood-brain barrier permeability and to alleviate memory impairment following ischemia by inhibiting MMP-9 expression, MMP-9-mediated occludin degradation[21], and phosphorylation of CaMKII in the hippocampus[22]. BA is also demonstrated to possess notable neuroprotective effects[23], which are mainly attributed to its anti-inflammatory and anti-apoptotic activities, as well as its modulatory effects on multiple pathways such as the TLR2/4 signaling pathway[24, 25] and NF-κB pathway[26-28]. JA has also been shown to exert neuroprotective effects by enhancing growth factor signaling, reducing apoptosis[29], inhibiting oxidative stress, regulating mitochondrial dysfunction[30], and inhibiting inflammatory response[31, 32]. Furthermore, the combination of BA and JA has been suggested to significantly improve their effectiveness in treating cerebral ischemia by promoting neuroprotection and neurogenesis, improving anti-oxidation[33, 34], and regulating apoptosis-related cascades[35]. In our previous studies, the additive effect between JA and BA was demonstrated, and the additive mechanism included pathway cross-talk in both horizontal and vertical patterns[36-38]. The pathways and processes specific to the combination group were mainly related to apoptosis and survival, gamma-secretase activity, neurophysiological processes, development, reproduction, and regulation of lipid metabolism[37].

These prior studies provide systematic "targeting profiles" of BA, JA and their combination on the cerebral ischemia biological network by using polypharmacological methods. Nevertheless, these "targeting profiles" contain all the information about network rewiring responses to perturbations, including not only specific mechanisms contributing to phenotype alterations but also invalid system fluctuations that do not contribute to phenotype variations. What attracted our attention is how to precisely extract the specific pharmacological mechanism that reverses the pathological process. This means that the "most effective shotgun" must be identified from a series of "magic shotguns", a task that has remained challenging until now. In this paper, using a microarray containing 374 stroke-related genes and ingenuity pathway analysis (IPA) software, we compared the networks perturbed by positive-phenotype ingredients (BA, JA and additive combination BJ) to those perturbed by negative-phenotype ingredients (concha margaritifera, CM) to investigate the pure pharmacological mechanism contributing to phenotype variation by eliminating non-specific roles modulating network rewiring[39].

Materials and methods

Animal models

All animals used in our experiments were approved by the Ethics Review Committee for Animal Experimentation, China Academy of Chinese Medical Sciences. The experimental processes and protocols were in compliance with the Prevention of Cruelty to Animals Act 1986 and NIH Guidelines for the Care and Use of Laboratory Animals for Experimental Procedures[40].

As described in previous studies[41-45], all mice except those in the sham group were subjected to middle cerebral artery obstruction (MCAO) to induce ischemic stroke. Briefly, after anesthesia, the left middle cerebral artery was occluded with an intraluminal filament for 1.5 h and then reperfused to established cerebral ischemia models. Mice in the sham group were subjected to the same surgical procedures, but no filament was inserted. Then, the mice were treated with various compounds, and after the 24-h treatment, the mice were sacrificed for further experiments.

Drug administration and group

All compounds used in this study, including BA, JA, and CM, were prepared according to the standards issued by the National Institutes for Food and Drug Control. Quality control was performed using fingerprint chromatographic methodologies. The three compounds were dissolved in 0.9% NaCl to reach the required concentrations: BA (5 mg/mL), JA (25 mg/mL), and CM (50 mg/mL).

A total of 84 male mice (Kunming strain, 12 weeks old, 38–48 g) were randomly divided into 6 groups: sham, vehicle, BA-treated, JA-treated, CM-treated, and BJ-treated (combination of BA and JA) groups. Mice in the drug-treated groups were injected with corresponding drug solutions (2 mL/kg body weight) via the tail vein 1.5 h after MCAO modeling, and mice in the BJ-treated group were injected with a combination of BA and JA at a 1:1 volume ratio. Mice in the sham and vehicle groups were injected with 0.9% NaCl (2 mL/kg body weight).

To determine infarct volume, 9 mice in each group were employed to explore the effect of these compounds on the cerebral ischemia-related phenotype. The remaining 5 mice in each group were used for subsequent microarray analysis to investigate the underlying pharmacological mechanisms.

Cerebral infarct volume examination for phenotype-related effect analysis

Twenty-four hours after MCAO modeling, 9 mice in each group were sacrificed to calculate the infarct ratio. The brain was removed and sectioned into five slices in the coronal plane 1, 3, 5, and 7 mm from the prefrontal cortex. The slices were stained with 1% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37 °C for 30 min. Images of these slices were captured with a digital camera (Color CCD camera TP-6001A, Topica Inc, Tokyo, Japan). The area of the infarct region was analyzed using a Pathology Image Analysis System (Topica Inc). The cerebral infarct ratio was the ratio of infarct volume to the total volume[43].

RNA extraction and microarray

The hippocampi of 5 mice per group were separated 24 h after MCAO modeling and used for RNA isolation and microarray analysis. The hippocampus was chosen because it should sustain some cell injury or cell death in most models without including any areas of infarction. After the hippocampi were homogenized in TRIzol Reagent, the total RNA was isolated and purified for further experiments, as described in our previous studies[41-47]. A total of 374 ischemia-related genes were selected from the Science STKE database to comprise the cDNA microarray in this study. The details of the gene screening process are reported in our previous studies[43, 46, 47].

All microarray analyses were performed based on the Array-Track system (US Food and Drug Administration, Silver Spring, ML, USA). Microarray analysis was performed in accordance with the Minimum Information in a Microarray Experiment (MIAME) guidelines and the Microarray Quality Control (MAQC) project. We submitted the results to the Array Express database. The robust multiarray analysis (RMA) was employed to preprocess the raw cell intensity files (CEL), which contained the fluorescence intensity values for each probe. The data were analyzed and normalized as described in our previous studies[43]. To investigate the altered genes related to phenotype, we compared the mean expression of genes in the positive-phenotype compound-treated groups (BA, JA, and BJ) with those in the negative-phenotype compound-treated group (CM) using one-way analysis of variance and significance analysis. Genes with a P value <0.05 and >1.5-fold increase or <0.67-fold decrease in expression level were defined as significantly differentially expressed genes for further analysis.

A list of these differentially expressed genes was uploaded to the IPA (ingenuity pathway analysis) system (http://www.ingenuity.com/) to identify the significantly differentially regulated molecules, which were defined as network-eligible molecules. Based on these network-eligible molecules and their connectivity, the regulated target networks were generated algorithmically. Then, the biological functions of these networks were enriched using a right-tailed Fisher's exact test. The significance of canonical pathways based on these genes was measured in two ways: (1) the ratio of the number of genes mapping to the pathway divided by the total number of genes in this canonical pathway and (2) Fisher's exact test, which was used to calculate a P value to determine the probability that these genes are associated with the canonical pathways outside of chance alone. Finally, canonical pathways with a P value < 0.05 were screened for and analyzed[36, 45].

Results

Phenotype variation on ischemia-induced infarction

In a cerebral ischemia reperfusion injury experiment, BA and JA significantly reduced the infarct volume compared to the vehicle group, whereas CM showed no significant therapeutic effect on the infarct volume. Details of the infarct volume and its significance in each group were reported in our previous studies[38, 43]. Therefore, the BA, JA, and BJ groups were defined as phenotype-positive groups, and the CM group was defined as phenotype-negative group. BJ reduced the infarct volume to a greater extent than BA or JA monotherapies. Further combination effect analysis revealed additive effects between BA and JA with a combination index value close to 1.0[36].

Divergence and similarity of pure and non-pure pharmacological analysis outcomes

In our previous studies, by comparing the perturbed networks in the compound-treated groups to those in the vehicle group, we investigated the pharmacological mechanisms of BA, JA and BJ, which were named non-pure mechanisms[35-38]. Nevertheless, these non-pure mechanisms contain all the information about network rewiring response to perturbations, including not only on-target actions contributing to phenotype variation but also invalid system fluctuations not contributing to phenotype variations. What attracts our attention is how to precisely extract the specific pharmacological mechanism that reverses the pathological process. For the phenotype-negative group (CM group), although the biological networks were perturbed by CM, no significant phenotype variation was found between CM and the vehicle group. Therefore, we defined the perturbation of CM as invalid effects. In this study, we compared the networks perturbed by positive-phenotype compounds to those perturbed by phenotype-negative compound (CM) to eliminate invalid effects (effects of CM) from the holistic actions of positive-phenotype compounds, in order to screen out the on-target actions and corresponding targets, which were defined as pure pharmacological mechanisms, providing more precise effects that contribute to phenotype variation.

As a result, pure and non-pure pharmacological analysis showed definite distinctions in specific target molecules (genes with EXP fold-change values above 1.0 or below -1.0 were defined as specific target molecules of corresponding com-

pounds). As shown in Figure 1, BA targeted 7 molecules in both pure and non-pure analysis, but only 2 target molecules, ATF3 and BCL2L1, overlapped between pure and non-pure analysis. This indicated that although the other 5 molecules (CAMK2B, CSF1, F5, HTR3A, and JUND) were identified as BA targets in previous non-pure analysis, they did not contribute to phenotype reversal; equally, the other 5 molecules (ARF1, FGF12, GRIN1, MAP2K6, and PRKAR1E) identified only in pure analysis might be considered potential targets of BA, although they were not discovered in non-pure analysis. In the BJ group, 4 overlapping target molecules, *ie*, IKBKG, IL6R, PGAM2, and TCF12, were detected between the 11 pure and 13 non-pure target molecules. There were 7 and 9 specific target molecules of pure and non-pure analysis in the BJ group, respectively (details shown in Figure 1). In the JA group, none of the target molecules overlapped between the 4 pure and 4 non-pure target molecules.

Pure network and pathway alterations were analogous in BA and JA groups

In the pure mechanistic analysis, BA and JA showed similar regulating patterns in specific target molecules, canonical pathways, and biological functions. According to pure mechanistic analysis, 41 and 22 differentially expressed genes were identified in the BA- and JA-treated groups, respectively. The results revealed that BA and JA targeted 7 and 4 specific molecules, respectively (Figure 2A, Supplementary Table S1), and all 4 specific target molecules in JA group overlapped with those in the BA group (Figure 2B). Based on these target genes, statistically significant networks were constructed in the BA and JA groups as target networks (Figure 2A). The functions of the target network in the BA group included cell death, genetic disorder, and immunological disease, while the functions of the target network in the JA group included carbohydrate metabolism, lipid metabolism, and small molecule biochemistry.

According to pure mechanistic analysis, a total of 51 and 59 statistically significant pathways involving differentially expressed genes were identified as canonical pathways in the BA and JA groups, respectively (Supplementary Table S2). The P-values of the top 20 canonical pathways in the BA and JA groups were compared and listed in Figure 3A. As shown in Figure 3B, 39 canonical pathways overlapped between the BA and JA groups, accounting for 76.47% and 66.10% of all canonical pathways in the BA and JA groups, respectively. We roughly classified these pathways based on prior knowledge, published literature and relevant databases. As a result, the overlapping canonical pathways between BA and JA were closely related to the nervous system, the immune system and inflammation. The non-overlapping canonical pathways in the BA group tended to contribute to inflammation and apoptosis, while those in JA group mainly involved hormone and endocrine regulation (Table 1). Among the top 20 canonical pathways (canonical pathways with minimum *P*-values), such

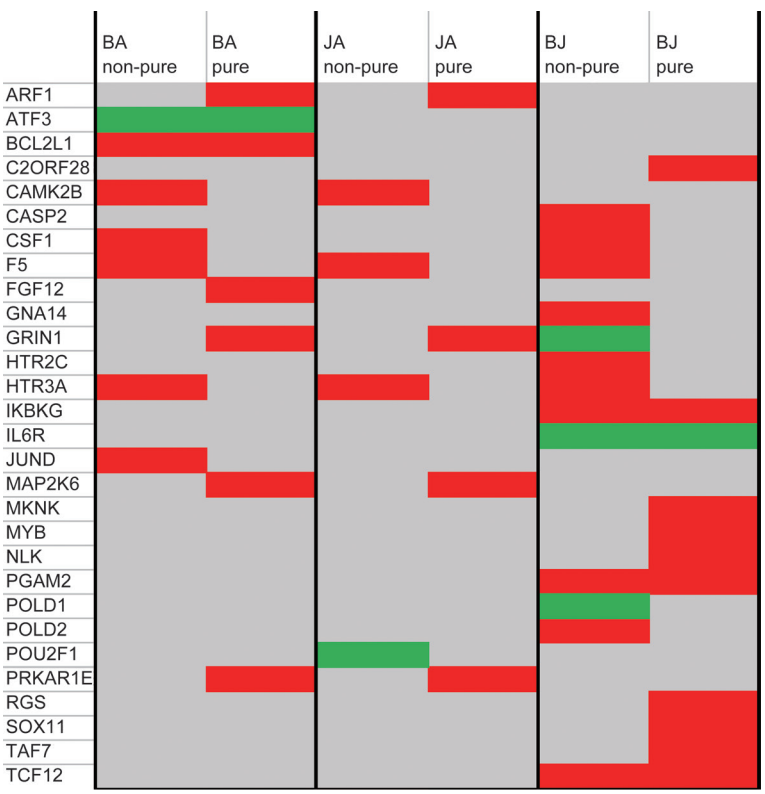

Figure 1. Specific target molecules in each group identified by pure and non-pure analyses. Red and green represent up- and down-regulated genes, respectively. Gray represents genes that were not differentially expressed.

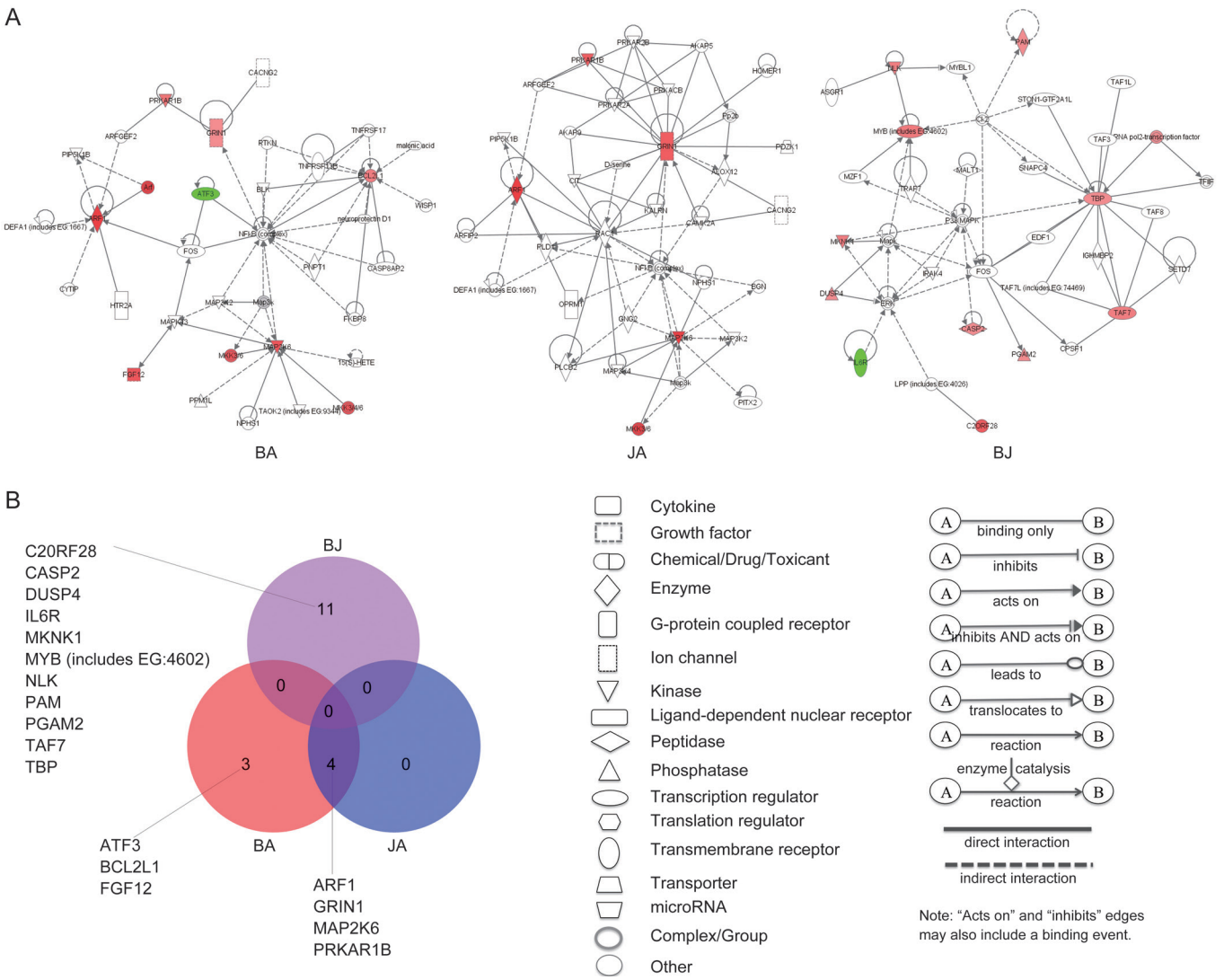

Figure 2. Representative significant networks and overlapping patterns of specific target molecules from these networks in each group. (A) Representative significant network in the BA, JA, and BJ groups. Red and green nodes represent up- and down-regulated genes, respectively. Color intensity indicates the fold-change of up- or down-regulation. Uncolored nodes denote genes that were not differentially expressed in our experiment but were integrated into networks based on IPA knowledge. (B) Overlapping patterns of specific target molecules (genes with an EXP fold-change more than 1.0 or less than -1.0) in each group.

an overlapping phenomenon was also observed; up to 75% of the top 20 canonical pathways in both BA and JA groups overlapped between the two groups (Figure 3C).

Based on the differentially expressed genes in the BA and JA groups compared to the CM group, IPA also identified 70 and 53 significant biological functions in the BA and JA groups (Supplementary Table S3), respectively. The *P*-values of the top 10 functions in the BA and JA groups were compared and are listed in Figure 4A. As shown in Figure 4B, all 53 functions in JA group were included in the BA group, accounting for 75.71% of BA functions. These 53 overlapping functions were divided into three categories: 16 (30.19%) were classified as diseases and disorders, 22 (41.51%) as physiological system development and function, and the other 15 (28.30%) as molecular and cellular functions. Additionally, among the 17 non-overlapping functions of the BA group, 10 functions (58.82%) were associated with physiological system development and function, 6 (35.29%) belonged to diseases and disorders, and the remaining 1 (5.88%) was related to molecular and cellular functions (Figure 4B). No unique functions were identified in the JA group.

Combination group produced novel variations based on pure mechanism analysis

To investigate the phenotype-related additive effects between BA and JA on improving cerebral ischemia, we also compared the BJ group to the phenotype-negative CM group. According to pure mechanistic analysis, the combination group had novel targets entirely different from those of BA or JA. A total of 11 genes were detected as specific target molecules of BJ (Figure

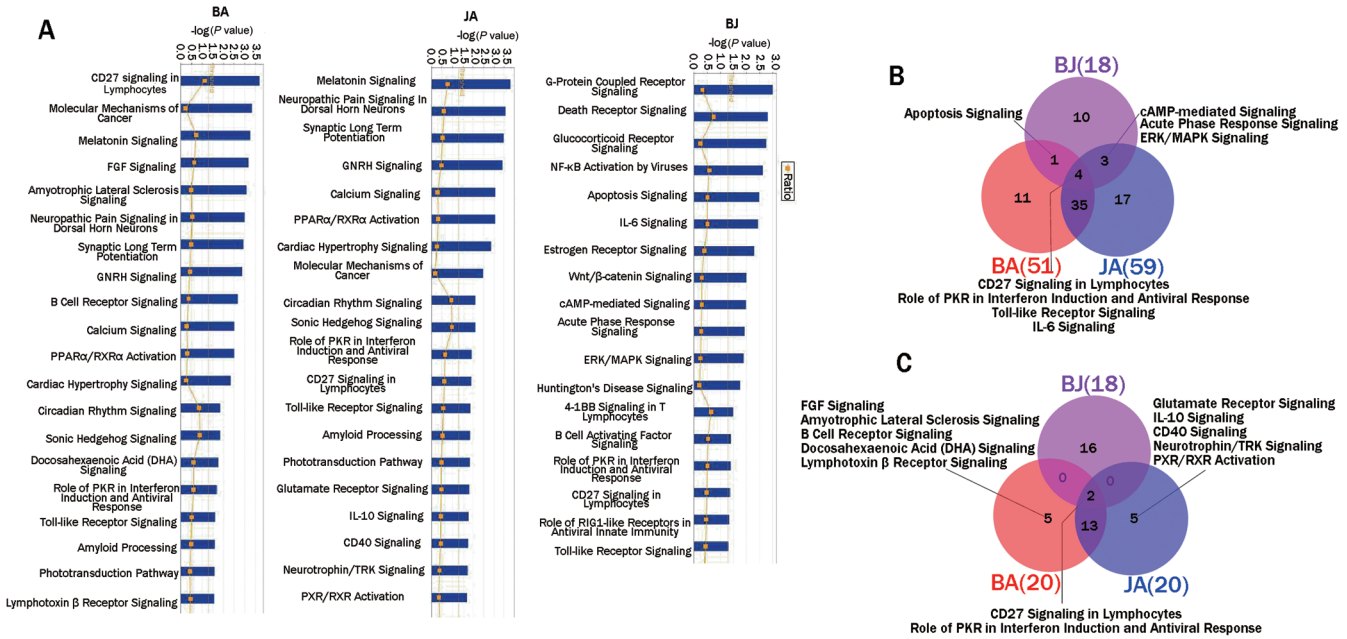

Figure 3. Top 20 canonical pathways and overlapping patterns of canonical pathways in each group. (A) Top 20 canonical pathways in each group; (B) overlapping patterns for all canonical pathways in each group; (C) overlapping patterns for top 20 canonical pathways in each group.

2A, Supplementary Table S1). Notably, none of these 11 target molecules overlapped with those of the BA or JA groups (Figure 2B). Four networks including these target genes were constructed for the BJ group. The functions of the 4 target networks included gene expression, DNA replication, recombination, and repair, cellular development; gene expression, cell cycle, tissue morphology; cell death, skeletal and muscular disorders, neurological disease; cancer, gastrointestinal disease; and hepatic system disease.

Compared with the CM group, 18 significant pathways were identified in the BJ group (Supplementary Table S2). Among these pathways, more than half (10 pathways, accounting for 55.56%) were included exclusively in the BJ group. Four pathways overlapped with both the BA and JA groups, including CD27 signaling in lymphocytes, the role of PKR in interferon induction and antiviral response, Toll-like receptor signaling, and IL-6 signaling. The target molecules of BA, JA, and BJ in these pathways are shown in Supplementary Table 4, and the CD27 signaling pathway was set as an example to show the target molecules in each group (Figure 5). One overlapping pathway, apoptosis signaling, was found between BA and BJ but not JA, and three overlapping pathways, *ie*, cAMP-mediated signaling, acute phase response signaling, and ERK/MAPK signaling, were detected between JA and BJ but not BA (Figure 3B). Additionally, we compared the top 20 canonical pathways in the BJ group with those in the BA and JA groups. Only 2 overlapping pathways were detected among the three groups, and the remaining 16 pathways in the BJ group did not overlap with those in either the BA or JA groups (Figure 3C).

Based on the differentially expressed genes, IPA also identified 64 significant functions in the BJ group, most of which (54 functions, accounting for 84.38%) overlapped with those in the BA and/or JA groups. Among these 54 overlapping functions, 18 (33.33%) were classified as diseases and disorders, 19 (35.19%) were classified as physiological system development and function, and the other 17 (31.48%) were classified as molecular and cellular functions. Among the 10 non-overlapping functions in the BJ group, the majority (8/10, accounting for 80%) were related to physiological system development and function (Figure 4C). Notably, 4 of these 8 functions were related to immune reaction, including hypersensitivity response, immune cell trafficking, infection mechanism, and antigen presentation.

Discussion

Herbal medicines, especially ingredients extracted from traditional Chinese medicine, feature multiple targets, which constitute target networks (on-modules) of herbal medicines in cellular network rewiring[48]. In our previous studies, the pharmacological mechanisms of BA, JA, and their combination BJ were explored, and the target pathways of these compounds were partially investigated. However, these target pathways included not only specific mechanisms contributing to phenotype alteration but also invalid system fluctuations that do not contribute to phenotype variations. What deserves more investigation is the specific pharmacological mechanism that actually reverses the pathological process. In our previous studies we proposed that the target pathways of BA, JA and BJ could be classified into three categories, *ie*, immune-related, neuron-related, and ERK/MAPK signaling cascade-related pathways[36]. Further investigation by GeneGo MetaCore anal-

Table 1. Pathways and their categories in each group and overlapping patterns of these pathways.

BA (11)	Pathway category
Docosahexaenoic Acid (DHA) Signaling	nervous system
Lymphotoxin β Receptor Signaling	inflammation and immune
Induction of Apoptosis by HIV1	inflammation and immune
viral infection immunity	
IL-15 Signaling	inflammation and immune
GM-CSF Signaling	inflammation and immune
Small Cell Lung Cancer Signaling	apoptosis and cancer
VEGF Signaling	others
Bladder Cancer Signaling	apoptosis and cancer
p53 Signaling	apoptosis and cancer
PTEN Signaling	apoptosis and cancer
Chronic Myeloid Leukemia Signaling	apoptosis and cancer
JA (17)	**Pathway category**
Type I Diabetes Mellitus Signaling	endocrine and hormone
Renin-Angiotensin Signaling	endocrine and hormone
Corticotropin Releasing Hormone Signaling	endocrine and hormone
Androgen Signaling	endocrine and hormone
Cardiac β-adrenergic Signaling	endocrine and hormone
Relaxin Signaling	endocrine and hormone
Insulin Receptor Signaling	endocrine and hormone
Cellular Effects of Sildenafil (Viagra)	endocrine and hormone
Inositol Phosphate Metabolism	others
Hepatic Cholestasis	others
Germ Cell-Sertoli Cell Junction Signaling	endocrine and hormone
NF-κB Signaling	inflammation and immune
Tight Junction Signaling	others
RAR Activation	others
CREB Signaling in Neurons	nervous system
Ephrin Receptor Signaling	nervous system
NRF2-mediated Oxidative Stress Response	others
BJ (10)	**Pathway category**
G-Protein Coupled Receptor Signaling	inflammation and immune
	viral infection immunity
Wnt/β-catenin Signaling	others
Death Receptor Signaling	apoptosis and cancer
Glucocorticoid Receptor Signaling	endocrine and hormone
NF-κB Activation by Viruses	inflammation and immune
	viral infection immunity
Estrogen Receptor Signaling	endocrine and hormone
Huntington's Disease Signaling	nervous system
4-1BB Signaling in T Lymphocytes	inflammation and immune
	viral infection immunity
B Cell Activating Factor Signaling	inflammation and immune
	viral infection immunity
Role of RIG1-like Receptors in	inflammation and immune
Antiviral Innate Immunity	viral infection immunity
BA and JA (35)	**Pathway category**
Molecular Mechanisms of Cancer	apoptosis and cancer
Melatonin Signaling	nervous system

continued

Table 1. Continued.

BA and JA (35)	Pathway category
FGF Signaling	others
Amyotrophic Lateral Sclerosis Signaling	nervous system
Neuropathic Pain Signaling in	nervous system
Dorsal Horn Neurons	
Synaptic Long Term Potentiation	nervous system
GNRH Signaling	nervous system
B Cell Receptor Signaling	inflammation and immune
Calcium Signaling	others
PPARα/RXRα Activation	others
Cardiac Hypertrophy Signaling	others
Circadian Rhythm Signaling	nervous system
Sonic Hedgehog Signaling	nervous system
Amyloid Processing	nervous system
Phototransduction Pathway	nervous system
Glutamate Receptor Signaling	nervous system
IL-10 Signaling	inflammation and immune
CD40 Signaling	inflammation and immune
Neurotrophin/TRK Signaling	nervous system
PXR/RXR Activation	others
IL-17 Signaling	inflammation and immune
Acute Myeloid Leukemia Signaling	nervous system
LPS-stimulated MAPK Signaling	inflammation and immune
Nitric Oxide Signaling in the	others
Cardiovascular System	
Dopamine Receptor Signaling	nervous system
BMP signaling pathway	others
Melanocyte Development and	nervous system
Pigmentation Signaling	
CDK5 Signaling	nervous system
IGF-1 Signaling	others
α-Adrenergic Signaling	nervous system
HMGB1 Signaling	inflammation and immune
p38 MAPK Signaling	inflammation and immune
Nicotinate and Nicotinamide Metabolism	others
Fc Epsilon RI Signaling	inflammation and immune
Cholecystokinin/Gastrin-mediated Signaling	others
BA and BJ (1)	**Pathway category**
Apoptosis Signaling	apoptosis and cancer
JA and BJ (3)	**Pathway category**
cAMP-mediated Signaling	inflammation and immune
	endocrine and hormone
Acute Phase Response Signaling	others
ERK/MAPK Signaling	inflammation and immune
BA, JA and BJ (4)	**Pathway category**
CD27 Signaling in Lymphocytes	inflammation and immune
Role of PKR in Interferon Induction	inflammation and immune
and Antiviral Response	viral infection immunity
Toll-like Receptor Signaling	inflammation and immune
IL-6 Signaling	inflammation and immune

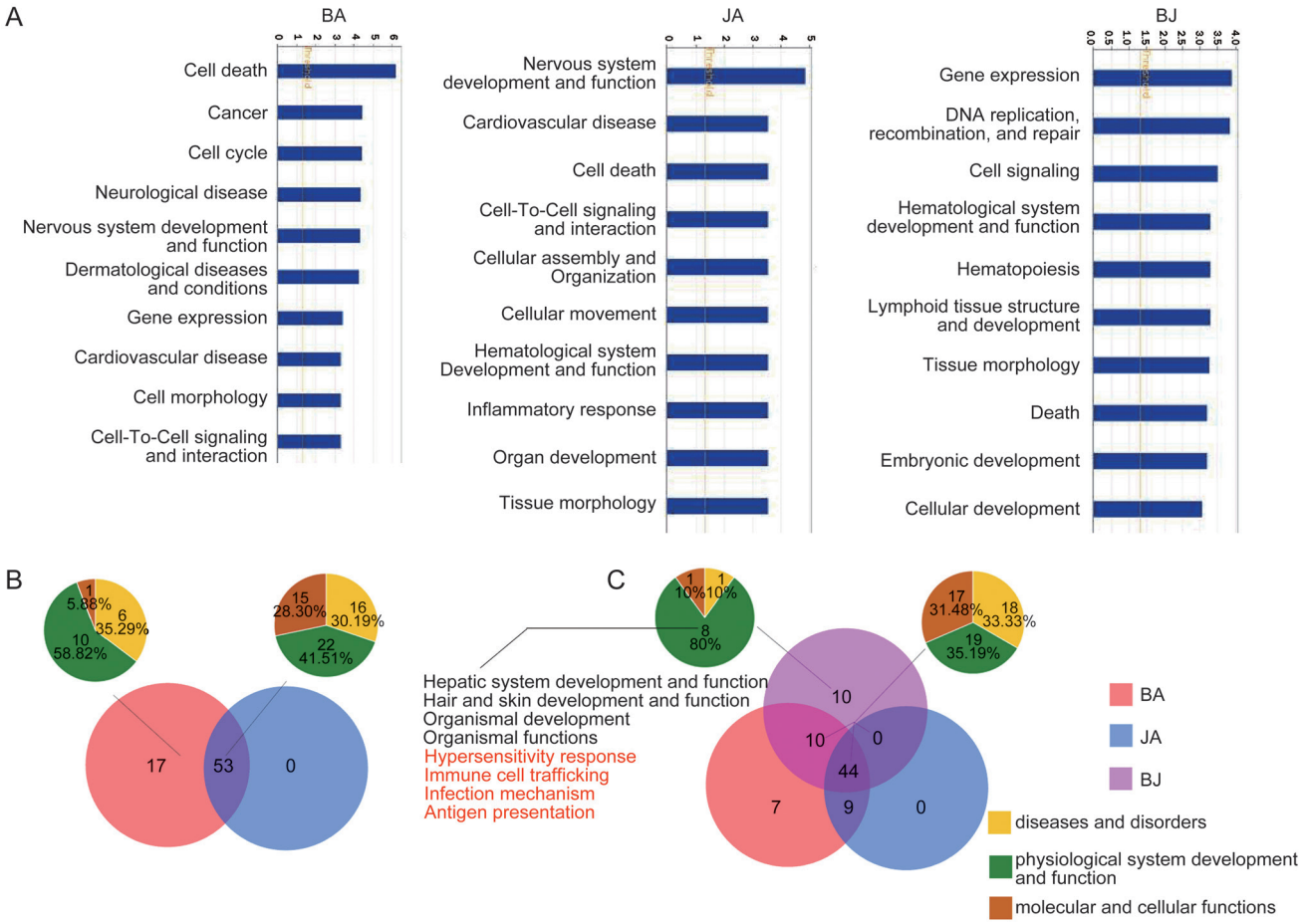

Figure 4. Top 10 biological functions and overlapping patterns of biological functions in each group. (A) Top 10 biological functions in each group; (B) overlapping patterns of all biological functions between the BA and JA groups; and (C) overlapping patterns of all biological functions among the BA, JA, and BJ groups. The small pie charts represent the constitutive categories of corresponding sections of biological functions. Yellow, green, and brown represent diseases and disorders, physiological systems development and function, and molecular and cellular functions, respectively. The biological functions in red are related to immunity.

yses showed that the specific pathways of BJ mainly involved apoptosis and survival, gamma-secretase, neurophysiological processes, development, reproduction, and regulation of lipid metabolism[37]. In this study, we compared the networks perturbed by positive-phenotype ingredients to those perturbed by negative-phenotype ingredients to precisely extract the pure pharmacological mechanism of phenotype variation[39].

Pure pharmacological analysis provides a clearer outline of target profiles than non-pure analysis

First, the pure target analysis provides us an opportunity to distinguish ineffective targets from targets contributing to phenotype alterations, which may help to narrow the scope of potential action targets. By comparing the target molecules found by pure and non-pure analyses, three molecules, F5, HTR3A, and HTR2C, were identified in the BA, JA or BJ groups in non-pure analysis but not in pure analysis. Thus, we speculate that regulation of the three molecules is not specific to phenotype variation. Noticeably, all three molecules were exactly the target molecules of CM. This also supported the hypothesis that regulation of the 3 molecules might be insufficient to reverse the pathological phenotype. Similar findings were also observed in canonical pathway analysis. For example, in the JA group, the G-protein coupled receptor (GPCR) signaling pathway was identified in non-pure analysis[35] but not in pure analysis. The GPCR signaling pathway was also detected in the CM group[39]. This indicates that the GPCR signaling pathway is not the key pathway leading to phenotype alteration; in other words, only regulation of GPCR signaling is insufficient to alter the pathological phenotype. Therefore, peeling off ineffective target molecules or pathways from "target profiles" may facilitate more precise on-target action of multi-target compounds.

Second, pure target analysis provides an opportunity to discover new therapeutic targets. In this study, pure analysis revealed a set of potential targets that were not identified in non-pure analysis. For example, a total of 5 molecules, namely, ARF1, FGF12, GRIN1, MAP2K6 and PRKAR1B, were detected as specific targets of BA in pure analysis but not in non-pure analysis. More remarkably, all 4 specific target mol-

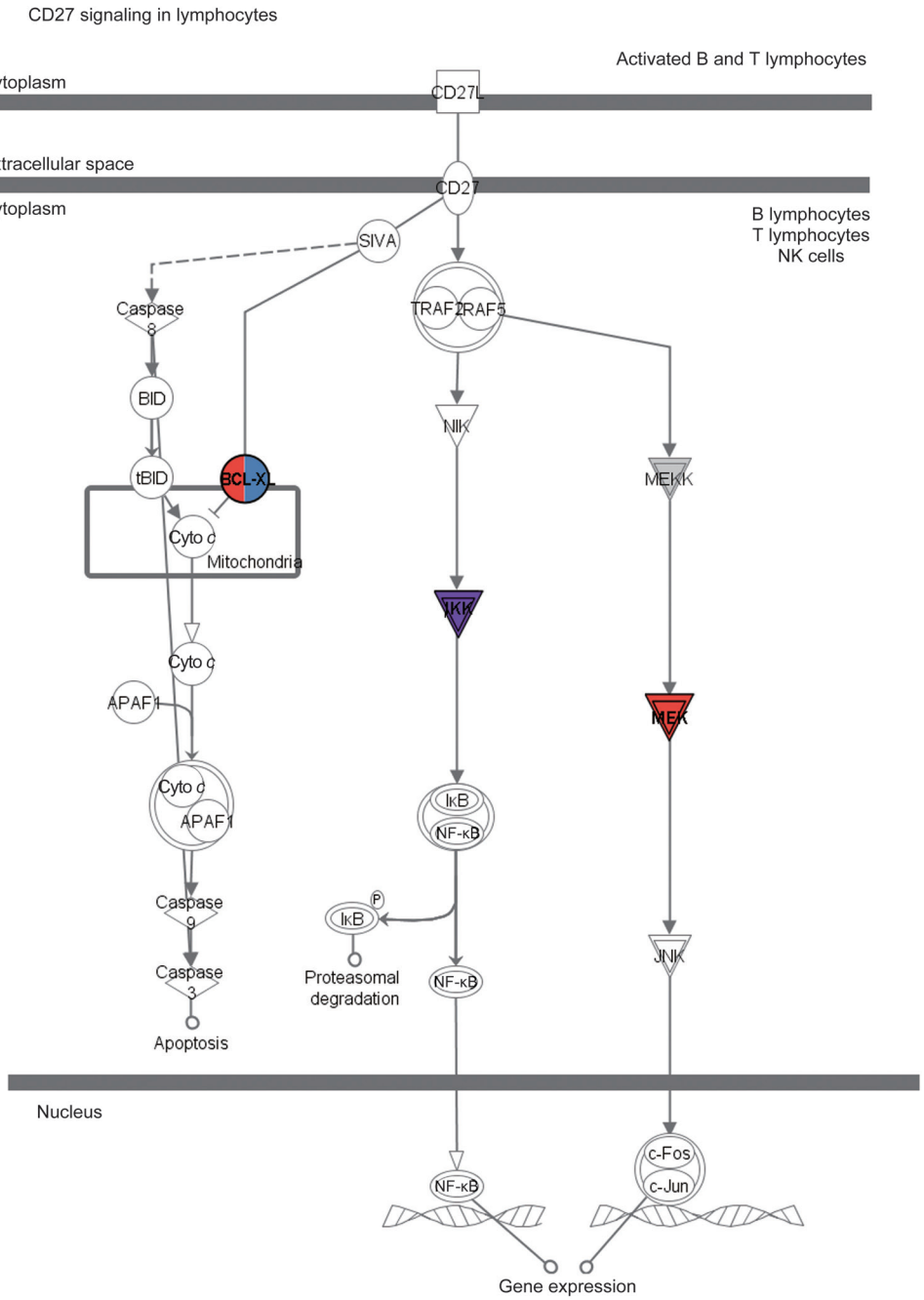

Figure 5. Targets of BA, JA, and BJ in the CD27 signaling pathway in lymphocytes. The target molecules of BA, JA, and BJ are red, blue and purple, respectively.

ecules of JA identified by pure analysis were entirely different from those identified by non-pure analysis, and 7 specific target molecules of BJ were identified by pure analysis but not by non-pure analysis. All specific targets can be regarded as potential targets of these compounds for further pharmacological analysis. Additionally, multiple pathways were also identified as potential targets of these compounds. For example, the CD40 signaling and IL-17 signaling pathways, which were not discovered in non-pure analysis[35], were identified as potential target pathways of JA in pure pharmacological analysis. These pathways may add to the "target profiles" of these compounds.

Third, the pure target analysis validates target molecules and pathways detected by non-pure analysis. Several molecules overlapped between pure and non-pure analyses, indicating that these molecules contributed not only to differences between perturbed and unperturbed biological systems but also to differences between phenotype-positive and phenotype-negative perturbed systems. As a result, we infer that these molecules definitely account for some phenotypic varia-

tions. For instance, both pure and non-pure analyses identified BCL2L1 and IKBKG as the target molecule in BA and BJ groups, respectively. We propose that the two molecules might be part of the primary causes of phenotype alteration. In addition, several pathways, such as ERK/MAPK signaling and GNRH signaling, were identified as specific target pathways in the JA group by both pure and non-pure analyses[35]. Therefore, we hypothesize that phenotype alteration can be partially attributed to these pathways. In fact, as shown in the diagram of target pathways (Figure 6), the two pathways were in the core position among all pathway cross-talk, which also supported the hypothesis above.

Pure pharmacological mechanisms of analogous ingredients: similarities and diversities

It was demonstrated in our previous study[37] that BA and JA have many similar molecular mechanisms. The similarities of regulatory mechanisms between BA and JA were more obvious in pure mechanistic analysis. All 4 specific target molecules of JA, *ie*, ARF1, GRIN1, MAP2K6, and PRKAR1B, overlapped with those of BA (Figure 2). MAP2K6 is a component in the MAPK pathway, which is a stress and inflammation-related pathway[49, 50], and can be activated by ARF1[51]. GRIN1 is believed to play a key role in neuron function[51-53]. Mutations of GRIN1 or PRKAR1B lead to nervous system disease or neurodegenerative disorder[54, 55]. These overlapping target molecules, which are associated with the nervous system and inflammation, might be the cellular basis of similar pharmacological actions between BA and JA.

Notably, among the 39 overlapping pathways between BA and JA, 16 pathways were associated with development and signal propagation of the nervous system, and 12 pathways were related to inflammation and immune response. For example, suppression of synaptic long-term potentiation can be induced by cerebral ischemia[56, 57], and up-regulation of sonic hedgehog signaling underlies neurogenesis after cerebral ischemia[58]. For inflammatory and immune pathways, the Toll-like receptor signaling pathway mediates the inflammatory reaction in cerebral ischemia and is involved in the extension of cerebral infarction and the aggravation of ischemic brain damage[59, 60], which is also a critical component of the innate immune system that has been shown recently to mediate ischemic injury[61]. The pathway of CD27 signaling in lymphocytes has an impact on the balance between adaptive responses and immunopathology[62], and the role of PKR in interferon induction and antiviral response and Toll-like receptors also affects the innate immune response[63, 64]. Therefore, we infer that the similar molecular mechanisms between BA and JA in treating cerebral ischemia may be attributed to their effects on nervous system as well as inflammation and immune response. Furthermore, all of the biological functions in JA group overlapped with those in BA group, which also demonstrated the similarities between BA and JA.

However, diverse regulatory mechanisms between BA and JA were also observed. Among the 12 non-overlapping canonical pathways of BA, in addition to those related to the nervous system and inflammation response, the remaining 7 pathways were closely related to cancer, including small cell lung cancer signaling, VEGF signaling, bladder cancer signaling, p53 signaling, PTEN signaling, chronic myeloid leukemia signaling, and apoptosis signaling. Based on published reports, the VEGF signaling pathway is suggested to mediate central nervous system involvement in acute lymphoblastic leukemia[65], and regulation of VEGF may improve neurobehavioral recovery after cerebral ischemic stroke[66]. The PTEN signaling pathway is involved in osteosarcoma[67], T-cell acute

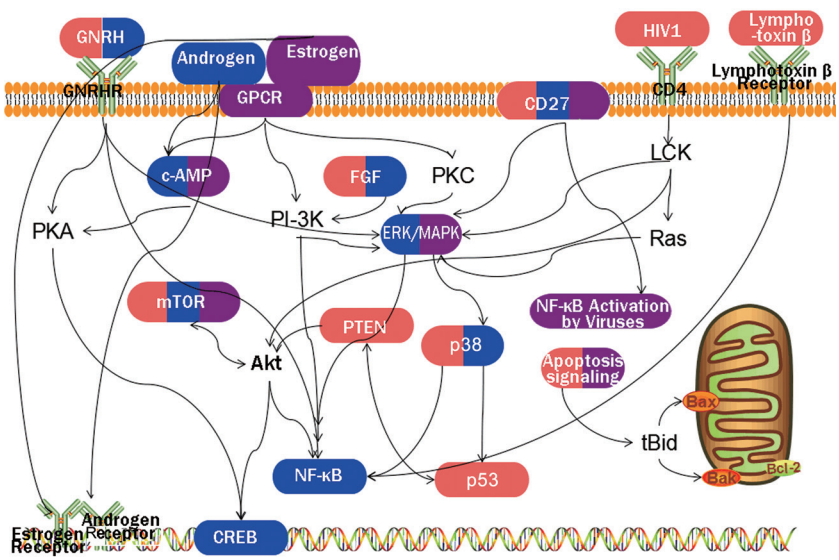

Figure 6. A diagram of target pathways of BA, JA, and BJ. Pathways activated by BA, JA, and BJ are labeled red, blue and purple, respectively. The solid line represents the association between pathways. It should be noted that the diagram only displays some of the classic pathways as an example to illustrate the pure additive mechanism between BA and JA and does not include all target pathways for these compounds.

lymphoblastic leukemia[68], and glioblastomas[69, 70]; PTEN is also reported to be related to nervous regeneration and repair[71-73], and promotion of PTEN degradation may prevent hippocampal neuronal loss and memory impairment[74]. Thus, we speculate that apoptosis and cancer-related signaling pathways are unique targets of BA in treating cerebral ischemia, which are quite different from those of JA.

Among the 20 non-overlapping canonical pathways of JA, 9 pathways, *ie*, type I diabetes mellitus signaling, renin-angiotensin signaling, corticotropin-releasing hormone signaling, androgen signaling, cardiac β-adrenergic signaling, relaxin signaling, insulin receptor signaling, and cellular effects of sildenafil (Viagra), and germ cell-sertoli cell junction signaling, were associated with endocrine regulation, such as adrenocortical hormone, insulin, and gonadal hormone. Activation of the renin-angiotensin system exaggerates ischemic brain damage[75]. Androgens are suggested to be an important factor in cerebrovascular disease[76, 77]. Cerebral ischemia induces insulin synthesis and secretion in the pancreas[78]. Therefore, we infer that endocrine and hormone regulation may be a mechanism of JA in improving cerebral ischemia.

Pure additive mechanisms: combination of paralleled compounds enhances the effect on viral infection immunity

The molecular mechanisms underlying the BJ combination in treating cerebral ischemia were remarkably distinct from those of BA or JA monotherapies. We found that all specific target molecules of BJ were completely different from those of BA or JA. Moreover, a total of 10 canonical pathways were identified exclusively in the BJ group. We noted that 5 of the 10 non-overlapping pathways were related to immune response and signaling, including the role of RIG1-like receptors in antiviral innate immunity, 4-1BB signaling in T lymphocytes, B cell activating factor signaling, NF-κB activation by viruses, and G-protein coupled receptor signaling. It is reported that cerebral ischemia induced interference in the function of the innate and adaptive immune cells, resulting in systemic immunosuppression. This post-stroke immunodeficiency could potentially protect the brain by reducing autoimmune reactions to brain antigens. However, any potential brain protective effect of stroke-induced immunosuppression might be confounded by an increased incidence of infection[79-81]. Regulation of the immune response will contribute to a long-term prognosis of stroke. In our studies, the above mentioned 5 immune response related non-overlapping pathways were related to either innate or adaptive immunity. Notably, these 5 pathways were also suggested to be related to the viral infection-mediated immune response and signaling. For instance, RIG1-like receptors play a critical role in antiviral immunity[82], both 4-1BB signaling and B cell activating factor signaling are involved in viral infection processes[83, 84], and a constitutively active viral G protein-coupled receptor (vGPCR) contributes to the pathogenesis of viral oncogenesis[85]. This may play a regulatory role in the post-stroke infection pathological process. Thus, we hypothesize that immune response, especially viral infection immunity, may be a specific target mechanism of BJ, which is fairly different from BA or JA. This hypothesis is also supported by the biological functions of BJ: in the 10 total non-overlapping biological functions of BJ, 4 functions (immune cell trafficking, infection mechanism, antigen presentation, hypersensitivity response) were associated with immunity.

The other 5 non-overlapping canonical pathways of BJ were related to apoptosis and cancer, endocrine and hormone systems, and the nervous system (Table 1), similar to those of BA or JA. Therefore, we proposed that the additive effects between BA and JA were generated not only by inheriting the targets of each monotherapy but also by integrating the mechanisms of single ingredients to produce novel pharmacological actions, which are indicated as enhanced effects on viral infection immunity.

Based on pure mechanistic analysis, we concluded that the underlying pharmacological mechanisms of BA and JA in treating cerebral ischemia were similar, and mainly involved the nervous system, inflammation and immune response. In addition, the unique targets of BA were related to apoptosis and cancer-related signaling, while the unique mechanisms of JA were associated with endocrine and hormone regulation. When BA was combined with JA, their impacts on infection immunity were enhanced and additive effects were produced.

Conclusion

In this study, the pure therapeutic mechanisms of BA, JA and BJ in treating cerebral ischemia were explored by comparing differentially expressed microarrays to those with a negative phenotype. Our methodologies for the pure mechanistic analysis of multi-target compounds may contribute substantially to the interpretation of the precise pharmacological actions of multi-target compounds. Further investigation with a larger number of genes and higher precision will be attempted in future studies.

Abbreviation

BA, Baicalin; JA, Jasminoidin; CM, Concha margaritifera; BJ, Baicalin combined with jasminoidin; MCAO, Middle cerebral artery obstruction; IPA, Ingenuity pathway analysis; IS, Ischemic stroke; FDA, U S Food and Drug Administration; HPLC, High-performance liquid chromatography; MMP, Matrix metalloproteinase; CaMKII, Calmodulin-dependent protein kinase II; TLR, Toll-like receptors; NF-κB, Nuclear factor-κB; STKE, Science signal transduction knowledge environment; PKR, Protein kinase R; IL, Interleukin; cAMP, Cyclic adenosine monophosphate; ERK, Extracellular signal-regulated kinase; MAPK, Mitogen-activated protein kinases; GPCR, G-protein coupled receptor; GnRH, Gonadotropin releasing; VEGF, Vascular endothelial growth factor; PTEN, Phosphatase and tensin homolog deleted on chromosome ten; HIV, Human Immunodeficiency Virus; mTOR, Mammalian target of rapamycin; FGF, Fibroblast growth factor; CREB, cAMP-response element binding protein.

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Author contribution

Zhong WANG designed and directed the research; Peng-qian WANG performed the research and drafted the manuscript; Jun LIU, Qiong LIU, Ying-ying ZHANG, Wen-juan XU, and Bing LI performed the histological experiment and revised the manuscript; Ya-nan YU analyzed the experimental data.

Supplementary information

Supplementary files are available at the website of Acta Pharmacologica Sinica.

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