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ORIGINAL RESEARCH

Prediction of Targets of Curculigoside A in Osteoporosis and Rheumatoid Arthritis Using Network Pharmacology and Experimental Verification

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Purpose: Network pharmacology is considered to be the next-generation drug development model that uses bioinformatics to predict and identify multiple drug targets and interactions in diseases. Here, network pharmacology was used to investigate the mechanism by which Curculigoside A (CA) acts in rheumatoid arthritis (RA) and osteoporosis.

Methods: First, TCMSP and SwissADME were applied to predict the druggability of CA. Then, potential targets were identified from overlapping data in SwissTarget and TargetNet, and targets were analyzed using Genemania and DAVID6.8 to obtain information about the GO and KEGG pathways. Ultimately, the drug-target-pathway network was identified after using Cytoscape 3.0 for visualization. Besides, qPCR was used to validate the predicted five major genes targets (*EGFR*, *MAP2K1*, *MMP2*, *FGFR1*, and *MCL1*).

Results: The results of TCMSP and SwissADME demonstrated that CA exhibits good druggability; 26 potential protein targets were classified by SwissTarget and TargetNet. The results of Genemania and DAVID6.8 indicated that CA probably caused anti-osteoporosis and anti-RA effects by regulating some biological pathways, especially nitrogen metabolism, estrogen signaling pathway, Rap1 signaling pathway, and PI3K/Akt signaling pathway. Besides, the result of Cytoscape 3.0 showed that the 26 targets participate in osteoporosis and RA-related pathways, metabolism, and other physiological processes. In vitro induced inflammation cell model experiments, the qPCR results showed that CA pretreatment significantly decreased the expression of *EGFR*, *MAP2K1*, *MMP2*, *FGFR1*, and *MCL1* genes.

Conclusion: These results suggested that network pharmacology may provide possible mechanism of how CA exerts therapeutic effects in osteoporosis and RA.

Keywords: Curculigoside A, network pharmacology, target identity, osteoporosis, rheumatoid arthritis

Introduction

Osteoporosis is a main public health problem that is becoming more and more common in the aging of the population around the world. Osteoporosis is a skeletal disease characterized by impaired bone strength, making individuals susceptible to fractures of the hips, spine, and other bone parts. Osteoporotic fractures are associated with many risk factors, mainly including hormonal factors, the use of certain drugs (such as glucocorticoids), smoking, low physical activity, and low

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intake of calcium and vitamin D.[1] In addition, the clinical consequences and financial burden of the disease require effective measures to provide appropriate and continuous intervention in high-risk populations.

Arthritis is quite prevalent now and has more than 100 types, including rheumatoid arthritis (RA), osteorheumatoid arthritis, psoriatic arthritis, inflammatory arthritis, and so on.[2] Among them, RA is the most common type of immune-mediated arthritis,[3] but the exact cause of RA is still unknown and there is no effective treatment. Anti-inflammatory drugs are often used to treat RA, but they usually accompany significant side effects. Therefore, these patients urgently need new treatments and alternative drugs with known and controllable side effects.

As a traditional Chinese medicinal plant that has been used for the treatment of osteoporosis and RA for a long time, the toxic and side effects of *Curculigo orchioides Gaertn* are known and controllable. Thus, it is undoubtedly that *Curculigo orchioides Gaertn* is an available and effective drug source for osteoporosis and RA.

Traditional Chinese medicine (TCM) is relatively safe and effective, with few side effects, and can prevent and treat various diseases. Over the past several thousand years, TCM has been used widely for clinical treatment in Asian countries. To date, TCM is still the most reliable resource for drug discovery and design.[4–7] At present, TCM uses an accumulated wealth of clinical and practical experience that has been established and gradually improved, to obtain a unique and complex medical system.[8] However, due to the complexity of Chinese herbal medicine and the human body, the mechanisms of most clinically effective Chinese medicines remain unclear. *Curculigo orchioides Gaertn* is a small herb widely distributed in China, India, Malaysia, Japan, and Australia. Its rhizome is known as *Curculigo orchioides* or "Xian Mao" and is a traditional Chinese medicine used in strengthening tendons and bones.[9] Furthermore, Curculigoside A (CA) is the major bioactive compound isolated from the rhizome of *Curculigo orchioides Gaertn*. CA is reported to have potent anti-RA and anti-osteoporotic properties.[10–12] However, relevant molecular mechanisms remain unclear.

The concept of network pharmacology was first defined by Hopkins in 2007 to raise awareness about the effects of drugs.[13] It is a new approach toward drug design that takes system biology, network analysis, connectivity, redundancy, and pleiotropy into account.[14] Now, in the new model, TCM, in combination with network pharmacology, provides a new research approach, which can be used as a powerful method to modify Chinese medicines from traditional experience-based medicines to modern evidence-based medicines. This system accelerates the discovery of TCM and improves current drug discovery strategies.[8] In recent years, network pharmacology has greatly promoted the investigation of pharmacological mechanisms to explore Chinese medicine, such as in the use of the Guizhi Fuling pill for treating uterine fibroids,[15] Baihe Dihuang decoction for improving the psychological sub-health state,[16] and Danggui Buxue decoction for its blood-related functions.[17] In summary, network pharmacology may be used as a powerful method to determine the overall pharmacological mechanism of how CA is used to treat osteoporosis and RA.

The network pharmacology approach has been used to determine the active components and potential targets of *Curculigo orchioides* in the treatment of osteoporosis.[18] Using this approach, the mechanisms for the effects of *Curculigo orchioide*s in the prevention and treatment of osteoporosis have been investigated, which inspired us to further explore the mechanism of CA, the main component of *Curculigo orchioides*, in treating osteoporosis and related RA. In our study, we first used TCMSP and SwissADME to predict the druggability of CA. Then, we applied 2D and 3D similarity measurements, using the SwissTarget server and TargetNet, to predict potential targets. These targets were then used for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Ultimately, we established a drug-target-pathway network and visualized the molecular mechanisms and pathways by which the CA plays its anti-osteoporosis and anti-RA role; this might provide guidance for the clinical application of CA. In addition, we verified five key target genes associated with anti-osteoporosis and anti-RA effects predicted in the network through qPCR analysis. The chemical structure of CA and the flow chart for this study are shown in Figure 1.

Materials and Methods

Evaluation of the Druggability the TCMSP Server and SwissADME

In drug discovery and development, early ADME screening is critical. The proper use of ADME results can give preference to drug candidates that are more likely to have good pharmacokinetic properties, minimize potential drug–drug interactions, and avoid costly late-stage failures in drug development.[19]

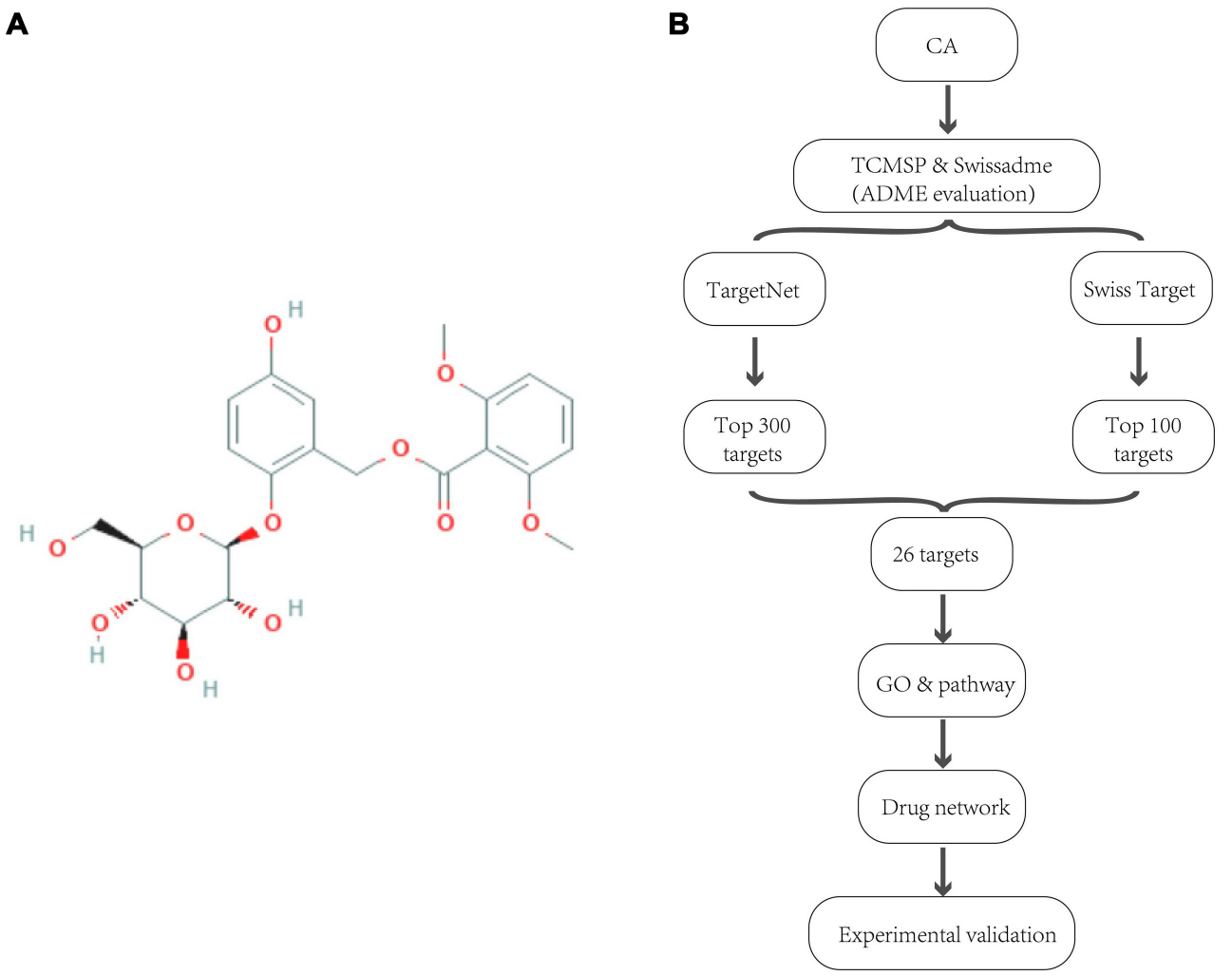

Figure 1 (A) Chemical structure of CA provided by the PubChem database (CID: 158845). **(B)** Flow chart for CA target prediction that includes ADME assessment, reverse docking, CPI evaluation, GO and pathway analyses, and interaction network construction in order.

The TCMSP server (https://tcmspw.com/tcmsp.php) is a drug screening and evaluation platform established by the Wang research team, based on the pharmacology of herbal systems. It provides information about important features related to ADME, such as human oral bioavailability, half-life, Caco-2 permeability, blood-brain barrier, and Lipinski's rule of five.[20]

By analyzing the physicochemical properties and structural characteristics of drug candidates, the researchers summarized the concept of drug-like properties, which has been widely used to identify drug candidates for the screening of poor absorption, distribution, metabolism, excretion, and toxicity properties. Thus, as an established concept for drug design, drug-likeness (DL) can estimate which compounds have "drug-like" features. A model that can filter out drug candidates is formulated using the molecular descriptors and Tanimoto coefficient (displayed below), as follows:[20]

$$T(A,B)=(A\times B)/(||A||2+||B||2-A\times B)$$

In this formula, A represents the molecular descriptor of the drug candidate and B represents the average molecular properties of all molecules in the Drug-Bank database. When investigating oral drugs, oral bioavailability (OB) and Caco-2 permeability are the critical pharmacokinetic properties. They are critical for evaluating the efficacy of the distributed delivery of oral drugs that enter the systemic circulation. Their value was, respectively, estimated using the OB prediction model OBioavail1.1 and the Caco-2 permeability prediction model preCaco2 in the TCMSP database.[20]

The SwissADME server (http://www.swissadme.ch/) is another network tool that provides a set of mature and efficient predictive models to users for calculating physicochemical properties, pharmacokinetics, ADME, DL, and

medicinal chemistry friendliness with a focus on parameters such as BOILED-Egg, iLOGP, and Bioavailability Radar.[21]

In this study, "Curculigoside A" and the Canonical Simplified Molecular-Input Line-Entry System (SMILES) of "Curculigoside A" (PubChem CID: 158845) were, respectively, entered as keywords in the search box and its druggability was evaluated at the molecular level.

Computational Target Fishing Using TargetNet and SwissTarget

TargetNet is a user-friendly server that is widely used at present to connect or integrate connections between multiple targets of molecules that need to be detected. TargetNet has built a large number of QSAR models based on proven chemogenomic data for prediction.[22] Furthermore, the SwissTarget server can also be used to evaluate the most probable macromolecular targets of a small molecule, which are considered to be bioactive compounds. This prediction is based on the 2D and 3D similarity alignment in the established active substance library.[23] Both are effective tools that are commonly used to predict targets.

We downloaded the SDF file of CA (PubChem CID: 158845) from the PubChem database and input it into the TargetNet and SwissTarget servers. Default values were set for all parameter settings and the protein targets identified by the two servers were allowed to overlap. Thus, they were considered as pre-selected targets for further studies.

Analysis by GeneMANIA

GeneMANIA (http://www.genemania.org) is an open-source, highly usable web server, used for predicting and identifying gene functions, investigating gene lists, and determining gene priorities for further functional analysis. After entering a list of genes, GeneMANIA expands the gene clusters that display similar functions based on known and trusted genomics and proteomics information. GeneMANIA will also show the predictive value of each selected data set for the query.[24] In our study, after selecting *Homo sapiens* from the nine options for the study species, we entered the name of the genes to be tested into the search box and obtained the results of the prediction process.

GO and Pathway Analyses, and Network Construction

The DAVID database includes an exhaustive biological knowledgebase; the accompanying analytics tools can be used to explore biological information from complex gene/protein lists.[25] The first step in the use of this database is to upload or enter a list of genes for any type of identifier that needs to be studied. Next, one or more mining methods is selected from the gene functional classification, functional annotation chart or clustering chart, and functional annotation table. This allows the researcher to gain an in-depth biological understanding of the list of the genes to be tested. In our study, in the first step, we entered the gene list into the search box, subsequently selected identifier "OFFICIAL GENE SYMBOL" and chose list type "Gene List", then submitted list. In the second step, we selected "Homo sapiens" to limit annotations and selected "List 1". In the third step, we selected the background "Homo sapiens". In the last step, we chose four parameters "GOTERM-BP-DIRECT", "GOTERM-CC-DIRECT", "GOTERM-MF-DIRECT", and "KEGG-PATHWAY" below the Annotation Summary Results, then selected "Functional Annotation Chart" to obtain GO and KEGG Pathway Analysis results.

Finally, we used Cytoscape 3.0 to build and explore a network to get a more accurate and in-depth insight into the internal complexities of the drugs to be tested, corresponding targets, and related diseases.

Pharmacological Verification of Network Analysis

Materials

Curculigoside A (purity >99%, as determined using high-performance liquid chromatography), which was obtained from Tokyo Chemical Industry (Tokyo Japan), was prepared as a 10 mM stock solution in dimethyl sulfoxide and stored in small aliquot at −80°C.

Cells and Cell Culture

The RAW 264.7 murine macrophage and human monocyte THP1 cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI1640 medium (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, U.S.A.), glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL) and were maintained at 37°C in humidified 5% CO2 incubators. Cells in the mid-log phase were used for further experiments.

Determination of Pro-Inflammatory Cytokines

The cells were plated at an appropriate density and allowed to grow to anastomosis for drug treatment. CA

was dissolved in complete culture medium to different concentrations (0, 1, 5, 10, 20, 40, 80 μg/mL) and then filtered through a 0.22 μm membrane filter. Firstly, RAW264.7 cells were pretreated with CA (0, 1, 5, 10, 20, 40, 80 μg/mL) for 4 hours, and the solution was changed and stimulated with LPS 1 μg/mL (Sigma, Burlington, Massachusetts, USA) for 24 hours. All the experiments were repeated three times. Real-time PCR (qPCR) assay was performed for the expression of pro-inflammatory cytokines (IL-1β and IL-6) in RAW264.7 cells. Total RNA was harvested using Trizol reagent (Yeason, ShangHai, China). The RNA quality was checked using the ratio A260/A280, and pure RNA samples were converted to cDNA using PrimeScript RT Reagent Kit (Takara Bio, Kusatsu, Japan) with gDNA Eraser in accordance with the manufacturer's instructions. The polymerase chain reaction was performed using SYBR Green based on the instruction manual (Takara Bio, Kusatsu, Japan). The primer sequences (Comate Bioscience, Changchun, China) of pro-inflammatory cytokines (IL-1β and IL-6) are presented in Table 1. The relative mRNA expression of different genes was quantified using the ΔΔCt method. β-actin was used as housekeeping gene.

Determination of Predicted Five Major Gene Targets

The cells were plated at an appropriate density and allowed to grow to anastomosis for drug treatment. CA was dissolved in complete culture medium to a concentration of 40 μg/mL and then filtered through a 0.22 μm membrane filter. In RAW264.7 cell culture, first, cells were pretreated with CA (40 μg/mL) for 4 hours (control group did not add CA), and the solution was changed and stimulated with LPS 1 μg/mL (Sigma, Burlington, Massachusetts, USA) for 24 hours. In THP1 cell culture, first, pre-treated the cells with CA (40 μg/mL) for 4 hours (control group treat without CA), changed the solution with PMA 60 ng/mL (Sigma, Burlington, Massachusetts, USA) and incubated for 48 hours, then in the presence of LPS (0.5 μg/mL), the differentiated cells were incubated for an additional 24 hours. All the experiments were repeated three times. Real-time PCR (qPCR) assay was performed for the expression of predicted five major gene targets in RAW264.7 cells and THP1 cells pretreated or not pretreated at concentrations of CA (40 μg/mL). Total RNA was harvested using Trizol reagent (Yeason, ShangHai, China). The RNA quality was checked using the ratio A260/A280, and pure RNA samples were converted to cDNA using PrimeScript RT Reagent Kit with gDNA Eraser in accordance with the manufacturer's instructions (Takara Bio, Kusatsu, Japan). The polymerase chain reaction was performed using SYBR Green based on the instruction manual (Takara Bio, Kusatsu, Japan). The primer sequences of these predicted five major gene targets enriched on these putative osteoporosis and RA-related KEGG pathways are presented in Table 1 (Comate Bioscience, Changchun, China). The relative mRNA expression of different genes was quantified using the ΔΔCt method. β-actin was used as housekeeping gene.

Statistical Analysis

All data represent at least three independent experiments, and statistical analyses were performed with Prism (version 7.0, GraphPad Software, San Diego California, U.S.A.). All results are shown as mean ± SD. Statistical comparisons were performed using one-way analysis of variance. $P<0.05$ were considered to denote statistical significance.

Table 1 Oligonucleotide and Primer Sequence

Oligonucleotide	Primer Sequence
Human *EGFR*	5′-AGGCACGAGTAACAAGCTCAC-3′ (forward), 5′-ATGAGGACATAACCAGCCACC-3′ (reverse)
Mouse *EGFR*	5′-GCCATCTGGGCCAAAGATACC-3′ (forward), 5′-GTCTTCGCATGAATAGGCCAAT-3′ (reverse)
Human *MAP2K1*	5′-CAATGGCGGTGTGGTGTTC-3′ (forward), 5′-GATTGCGGGTTTGATCTCCAG-3′ (reverse)
Mouse *MAP2K1*	5′-AAGGTGGGGGAACTGAAGGAT-3′ (forward), 5′-CGGATTGCGGGTTTGATCTC-3′ (reverse)
Human *MMP2*	5′-TACAGGATCATTGGCTACACACC-3′ (forward), 5′-GGTCACATCGCTCCAGACT-3′(reverse)
Mouse *MMP2*	5′-CAAGTTCCCCGGCGATGTC-3′ (forward), 5′-TTCTGGTCAAGGTCACCTGTC-3′ (reverse)
Human *FGFR1*	5′-CCCGTAGCTCCATATTGGACA-3′ (forward), 5′-TTTGCCATTTTTCAACCAGCG-3′ (reverse)
Mouse *FGFR1*	5′-TAATACCACCGACAAGGAAATGG-3′ (forward), 5′-TGATGGGAGAGTCCGATAGAGT-3′ (reverse)
Human *MCL1*	5′-TGCTTCGGAAACTGGACATCA-3′ (forward), 5′-TAGCCACAAAGGCACCAAAAG-3′ (reverse)
Mouse *MCL1*	5′-AAAGGCGGCTGCATAAGTC-3′ (forward), 5′-TGGCGGTATAGGTCGTCCTC-3′ (reverse)
Mouse *IL-1β*	5′-GAAATGCCACCTTTTGACAGTG-3′ (forward), 5′-TGGATGCTCTCATCAGGACAG-3′ (reverse)
Mouse *IL-6*	5′-CTGCAAGAGACTTCCATCCAG-3′ (forward), 5′-AGTGGTATAGACAGGTCTGTTGG-3′ (reverse)

Results

The Druggability of Curculigoside A

To predict the druggability of CA, we analyzed the parameters related to Lipinski's rule of five, provided by TCMSP and SwissADME servers. The data obtained are, respectively, shown in Table 2 and Figure 2. TCMSP showed that CA was basically in line with the Lipinski's rule of five (MW≤500, AlogP≤5, Hdon≤5, Hacc≤10, RBN≤10).[26] Consistently, SwissADME server also showed that CA generally fulfilled Lipinski's rule of five. The results of TCMSP and SwissADME analysis enabled us to conclude that CA exhibited good druggability.

Identification of Potential Targets

Potential targets were obtained by overlapping TargetNet and SwissTarget servers, using the aforementioned method. Figure 1B shows the top 300 potential protein targets of CA from all 623 QSAR models that were obtained using TargetNet, as well as the potential 100 targets identified using the SwissTarget server. In TargetNet, the fingerprints model was set to the default value "ECFP4 fingerprints" and an area under the curve threshold of 0.7 indicated the presence of favorable targets.[22] In SwissTarget, the threshold value of the similarity value was set as 0.85 and the value of 2D was 0.65.[23] Finally, to increase the accuracy of the target prediction process, the overlapping 26 predicted targets obtained using these two methods were selected for further studies (Table 3).

Analysis by GeneMANIA

GeneMANIA generated a list of genes with similar functions to the query gene and constructs an interactive functional-association network to illustrate relationships between genes and datasets. GeneMANIA also assigned weights to data sets based on how useful they are for each query. Weights indicated the predictive value of each selected data set for the query. Individual data sets were represented as networks. The network weights were non-negative, sum to 100%. The interaction analysis of the 26 genes and their 20 interacting genes at the gene level was performed by GeneMANIA to clarify the correlations among colocalization, shared protein domains, co-expression, prediction and pathways. Among the 26 targets and their 20 interacting genes, the forecast results showed the portion that were predicted to interact with each other occupy 37.72% of the weight. Among all genes, the portion that shared the same protein domains occupy 28.28% of the weight, whereas the portion that displayed similar co-expression characteristics occupy 20.10% of the weight. The weights of these three portions add up to account for 86.1%. These results indicated that the 26 genes may comprehensively interact with each other and may function through alliance mechanisms. Other prediction outcomes, such as physical interactions, pathways, and co-localization are shown in Figure 3.

GO and Pathway Analyses, and Network Construction

We used DAVID 6.8 to analyze the internal interaction networks of the 26 potential targets. We used -log10 (P value) for clarity to compare the P value and the top five functions were GO:0004089~carbonate dehydratase activity, GO:0015701~bicarbonate transport, GO:0006730~one-carbon metabolic process, GO:0005886~plasma membrane, and GO:0001609~G-protein-coupled adenosine receptor activity (Figure 4). The count, which represents the number of genes enriched in this pathway among the 26 target genes, showed that the 26 targets mainly participated in 17 KEGG pathways, including hsa00910: nitrogen metabolism, hsa04915: estrogen signaling pathway, and hsa04015: Rap1 signaling pathway (Table 4). More detailed pathway analysis data are provided in Supplementary Table S1. On the basis of target identification and pathway analysis, we then constructed the entire interaction network profile of how CA treats osteoporosis and RA using Cytoscape 3.0. The interaction network has 44 nodes and 95 edges (Figure 5).

Experimental Validation

IL-1β and IL-6 are pro-inflammatory cytokines that play a key role in osteoporosis and RA. We chose them to represent the severity of inflammation in vitro inflammation cell models. RAW264.7 cells were pretreated with CA (0, 1, 5, 10, 20, 40, 80 μg/mL) for 4 hours. RAW264.7 cells displayed a basal IL-1β and IL-6 expression in absence of CA, while RAW264.7 cells pretreated with CA (1, 5, 10, 20, 40, 80 μg/mL) for 4 hours displayed a reduced pro-inflammatory

Table 2 Pharmacological and Molecular Properties of CA by TCMSP

Name	MV	AlogP	Hdon	Hacc	OB(%)	Cac0-2	BBB	DL	FASA	TPSA	RBN
CA	466.44	0.80	5	11	14.89	−1.05	−1.69	0.71	0.27	164.37	9

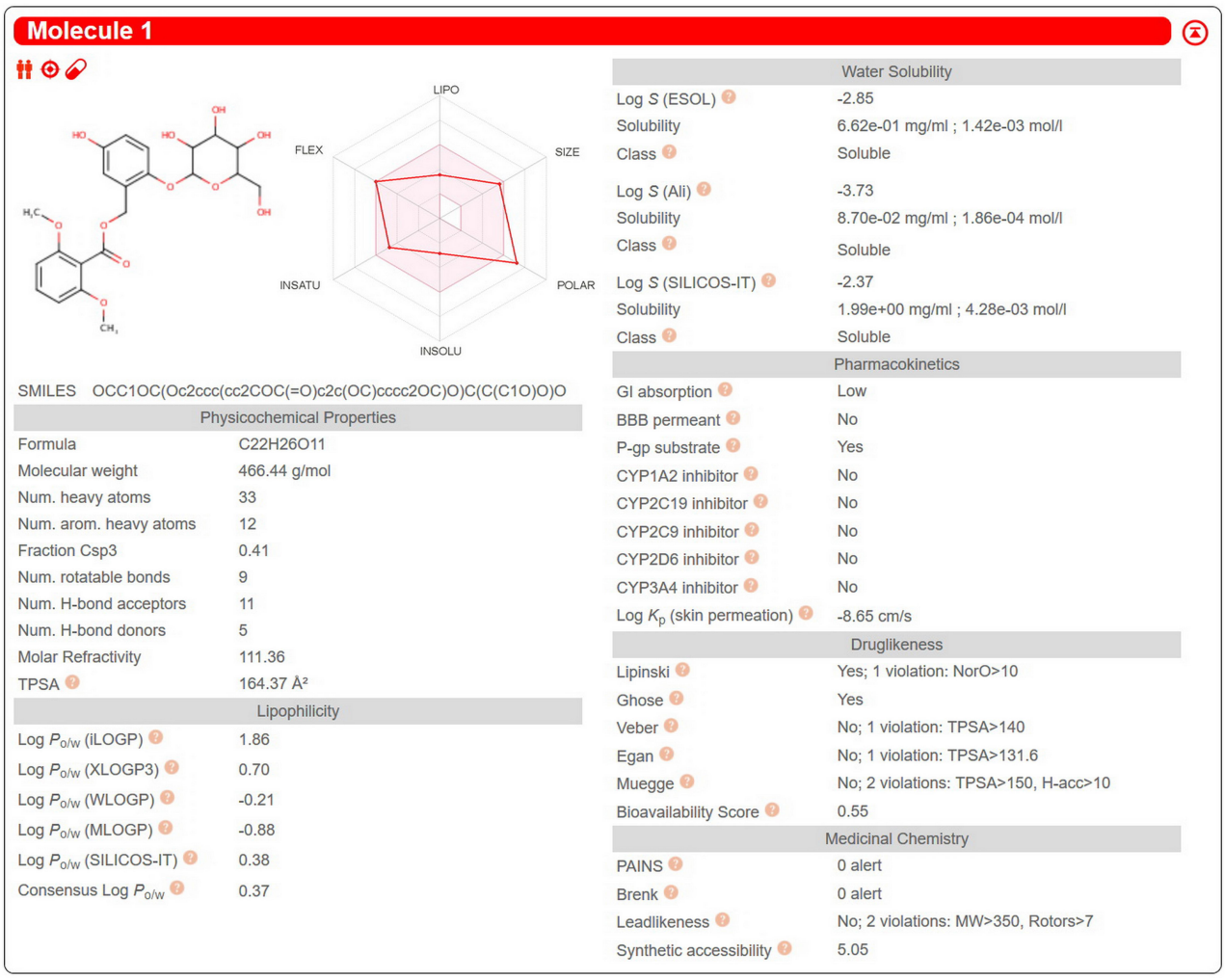

Figure 2 Pharmacological and molecular properties of CA by SwissADME.

cytokines (IL-1β and IL-6) expression in a concentration-dependent manner, as shown in Figure 6. In addition, we compared the effects of CA at 40 μg/mL and 80 μg/mL. The results showed that CA at 80 μg/mL was slightly better than 40 μg/mL in value, but had no significant difference. So we selected CA at 40 μg/mL to investigate its effects on the expression of predicted five major gene targets. Based on the above predictions, to verify the results derived using the target–pathway interaction network, we chose the predicted five major gene targets (*EGFR*, *MAP2K1*, *MMP2*, *FGFR1*, *MCL1*) for experimental validation in vitro inflammation cell model induced by RAW264.7 cells and THP1 cells. These predicted five gene targets (*EGFR*, *MAP2K1*, *MMP2*, *FGFR1*, *MCL1*) have been shown to participate in pathogenesis, pathological process and related treatment of RA and osteoporosis in patients or induced RA and osteoporosis cell models and animal models.[27–34] Therefore, we speculated that CA may exert anti-RA and anti-osteoporosis effects by regulating these five genes. Thus, we chose these five genes for further experimental verification, to explore if CA exerts anti-RA and anti-osteoporosis effects by regulating these five genes. Real-time polymerase chain reaction analysis results showed that compared with the control group (no CA pre-treatment), *EGFR*, *MAP2K1*, *MMP2*, *FGFR1*, and *MCL1* were down-regulated by treatment of 40 μg/mL CA in both RAW 264.7 cells and THP1 cells ($P<0.05$). (Figure 7)

Discussion

The concept of network pharmacology is relatively new and first defined by Hopkins in 2007.[13] It is a new paradigm in drug discovery that takes system biology, network analysis, connectivity, redundancy, and pleiotropy into account. Besides, it can use bioinformatics to predict and identify multiple drug targets and interactions in disease.[14] In recent

Table 3 Putative Targets of CA Identified by TargetNet and SwissTarget

RANK	Uniprot ID	Name	Target Gene
1	P30542	Adenosine A1 receptor	*ADORA1*
2	P29274	Adenosine A2a receptor	*ADORA2A*
3	P13866	Sodium/glucose cotransporter 1	*SLC5A1*
4	P29275	Adenosine A2b receptor	*ADORA2B*
5	O43570	Carbonic anhydrase XII	*CA12*
6	Q9ULX7	Carbonic anhydrase XIV	*CA14*
7	Q16790	Carbonic anhydrase IX	*CA9*
8	P08253	Matrix metalloproteinase 2	*MMP2*
9	P31639	Sodium/glucose co-transporter 2	*SLC5A2*
10	P00533	Epidermal growth factor receptor erbB1	*EGFR*
11	P11387	DNA topoisomerase 1	*TOP1*
12	P12268	Inosine-5′-monophosphate dehydrogenase 2	*IMPDH2*
13	P34913	Epoxide hydratase	*EPHX2*
14	P35354	Cyclooxygenase-2	*PTGS2*
15	P25101	Endothelin receptor ET-A	*EDNRA*
16	P07900	Heat shock protein HSP 90-alpha	*HSP90AA1*
17	Q07820	Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*
18	P26358	DNA (cytosine-5)-methyltransferase 1	*DNMT1*
19	P43166	Carbonic anhydrase VII	*CA7*
20	Q8N1Q1	Carbonic anhydrase XIII	*CA13*
21	P23219	Cyclooxygenase-1	*PTGS1*
22	P08473	Neprilysin	*MME*
23	P11362	Fibroblast growth factor receptor 1	*FGFR1*
24	Q06187	Tyrosine-protein kinase BTK	*BTK*
25	Q02750	Dual specificity mitogen-activated protein kinase kinase 1	*MAP2K1*
26	P22748	Carbonic anhydrase IV	*CA4*

years, with the importance of network pharmacology in drug development research and analysis, network pharmacology tools have been introduced to meet more needs. Especially in the scope of TCM network pharmacology, new tools such as BATMAN-TCM, ETCM, TCMSP, TCM-Mesh have provided great benefit for drug development and research.[20,35–37] Moreover, the application of network pharmacology is more and more extensive and in-depth. For example, network pharmacology can be used to identify the combinations of clinically effective drugs for specific diseases.[38] It can be used to estimate the untapped target space and therapeutic potential of natural products.[39] Additionally, it is also used to guide and assist in drug repositioning to overcome the low drug productivity that has been an important issue in the pharmaceutical industry.[40] In the field of TCM research, network pharmacology promotes the research on the synergistic effect of TCM in their formulas and clarifies the molecular mechanism of the formulas.[41] Active drug ingredients for specific difficult diseases such as stroke and cancer from drug Banks can be screened by network pharmacology approach.[42,43] It also provides new effective formulas design of Chinese medicine and effectively promotes the modernization and development of Chinese medicine.[44] At present, network pharmacology is mainly used to study the effective components and action mechanism of a single Chinese medicine or Chinese medicine formulas in the treatment of complex diseases.[45–49] Taken together, network pharmacology is practical in the study of single herbs, drug pairs, and traditional Chinese medicine formulas. Therefore, the development of network pharmacology techniques that can predict multiple drug–target interactions may hold the key to future drug discoveries in complex diseases such as RA and Osteoporosis.

Network pharmacology approach has been used to determine the active components and potential targets of *Curculigo orchioides* in the treatment of Osteoporosis.[18] On the basis of this article, we further applied network pharmacology to predict the potential targets of CA to exert anti-RA and anti-osteoporosis effects, the interaction of targets with CA, the signaling pathways and networks involved. GO analysis showed that the predicted targets of CA are mainly enriched in GO: 0004089 ~ carbonate

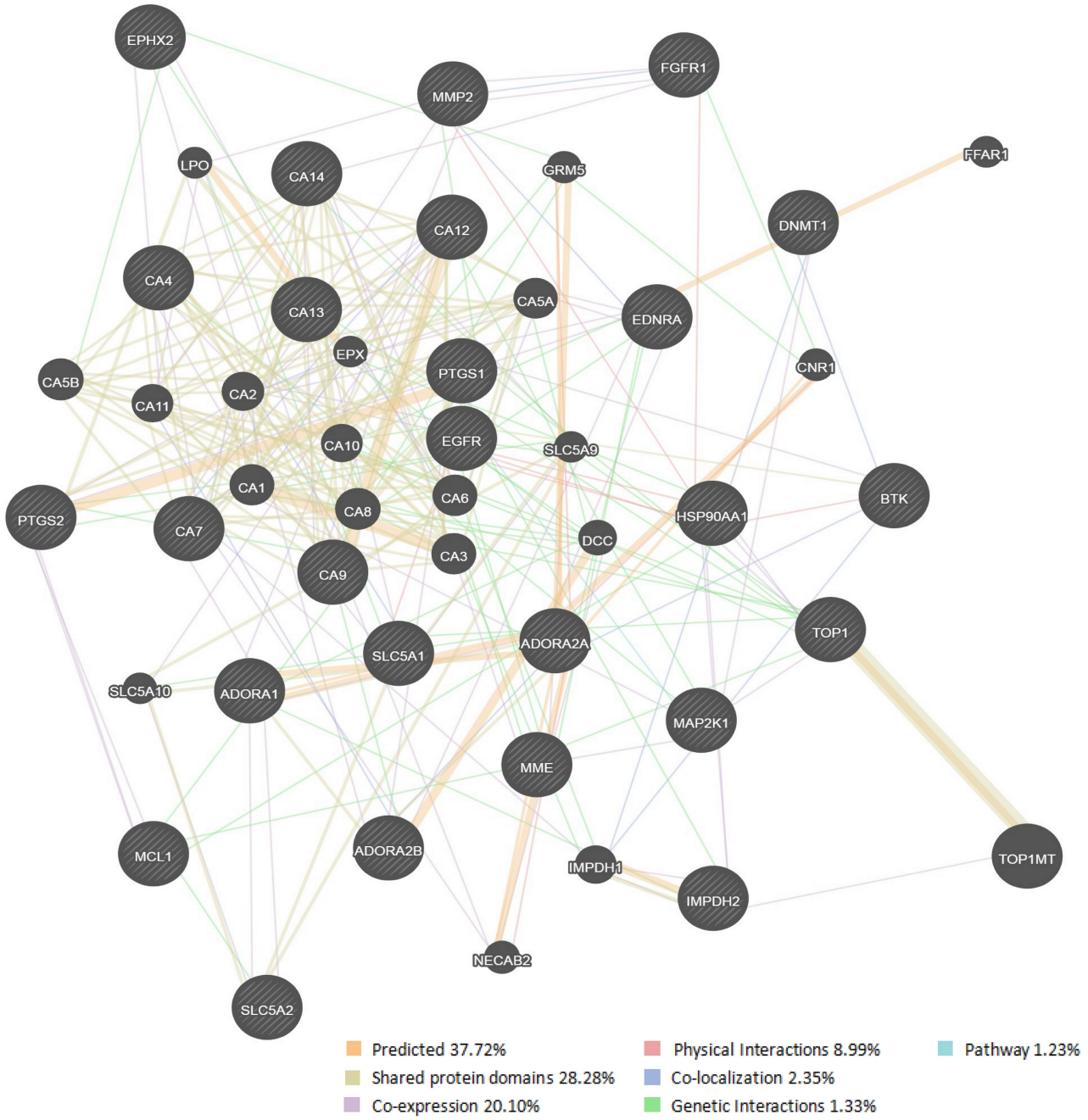

Figure 3 The network of predicted CA targets. Each node represents a predicted target gene or a gene associated with these target genes. The node size represents the strength of interactions. The inter-node connection lines represent the types of gene-gene interactions, and the line color represents the types of interactions. Firstly, genes corresponding to the aforementioned protein targets were submitted for further query, then the functional correlation between targets was analyzed by GeneMANIA.

dehydratase activity, GO: 0015701 ~ bicarbonate transport, GO: 0006730 ~ one-carbon metabolic process, GO: 0005886 ~ plasma membrane, and GO: 0001609 ~ G-protein-coupled adenosine receptor activity. Several studies in vitro and in vivo suggest that inhibition of bicarbonate transport leads to changes in pH, which in turn affects the activity of pH-dependent cells and exerts anti-inflammatory and anti-RA effects.[50] Inhibition of key enzymes in intracellular folate metabolism and further limitation of the availability of methyl groups derived from one-carbon metabolism can treat RA.[51,52] The combination of adenosine and G protein-coupled adenosine receptors can exert the effects of inhibiting osteoporosis and RA, by promoting osteoblast differentiation and bone repair, regulating osteoclast function, and pathological bone remodeling.[53,54] Consistent with our expectations,

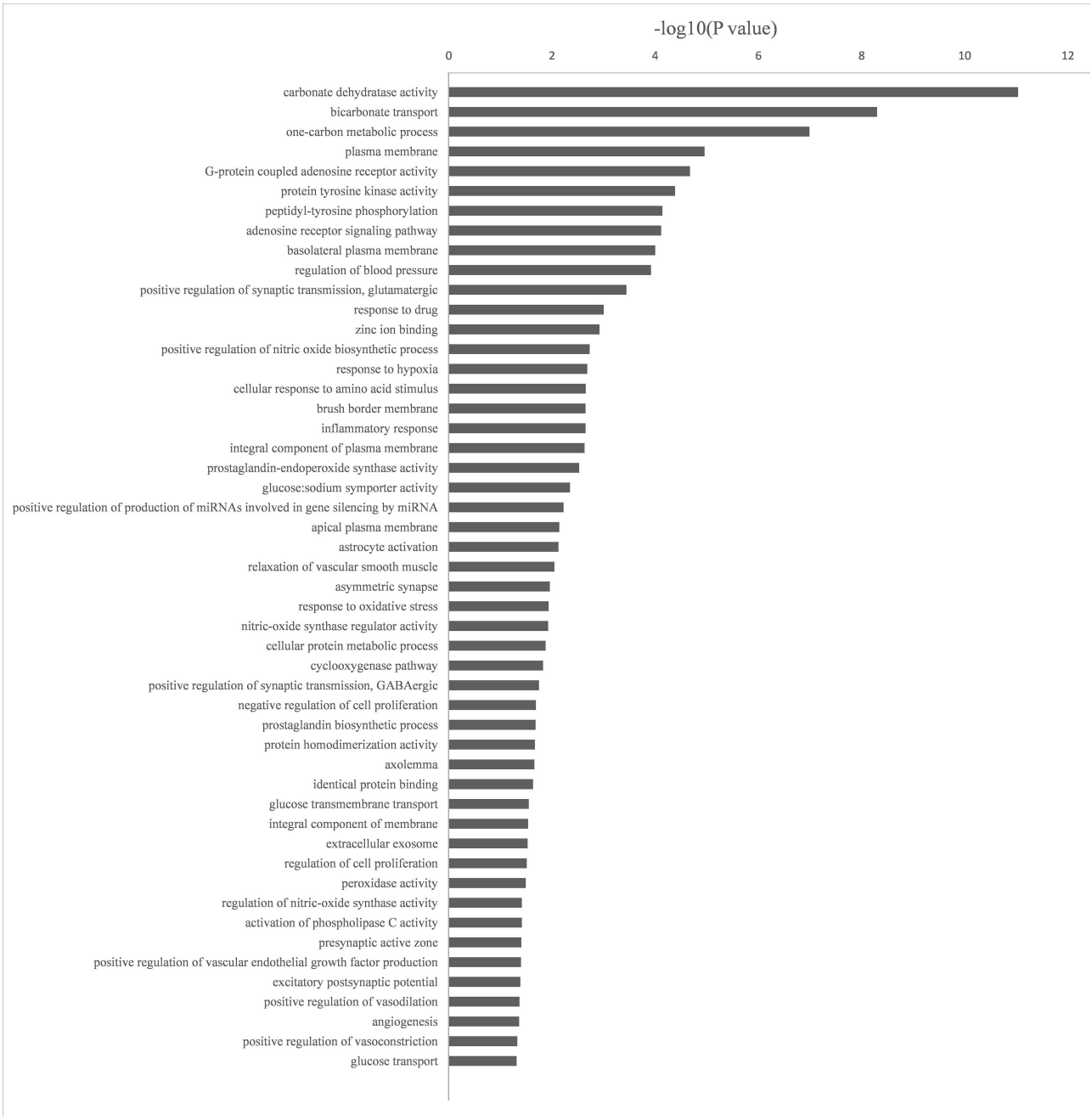

Figure 4 GO analysis of putative targets by DAVID6.8. The Y-axis shows significantly enriched Biological Process categories that the targets participate in, and the X-axis shows the enrichment scores (-log10 (P value)) of these terms (P<0.05).

these GO corresponding to the predicted targets of CA is mainly related to RA and osteoporosis. Pathway analysis suggested that CA mainly regulates nitrogen metabolism, estrogen signaling pathway, Rap1 signaling pathway, and PI3K/Akt signaling pathway in RA and osteoporosis. These results were consistent with several studies in vitro and in vivo. Reactive nitrogen (RNS) indirectly induces the formation of autoantibodies, further aggravating RA[55] and it is used as a potential biomarker of disease activity and antioxidant effects.[56] In RA, estrogen withdrawal during menopause indirectly affects osteoclast bone resorption, accelerated bone loss, osteoporosis by increasing production of proinflammatory cytokines.[1,57] Rap1 plays a vital role in promoting angiogenesis and maintaining vascular stability, and it is closely related to further RA.[58] Besides, deficiency in Rap1 compromises in vivo bone resorption, resulting in an osteoporotic phenotype.[59] Activation of the PI3K/Akt signaling pathway affects the

Table 4 KEGG PATHWAY Analysis of Potential Targets by DAVID6.8 (P<0.05)

Term	Count	P value
hsa00910:Nitrogen metabolism	6	1.58E-09
hsa05200:Pathways in cancer	7	0.00147002
hsa05215:Prostate cancer	4	0.002977326
hsa04915:Estrogen signaling pathway	4	0.004154887
hsa04015:Rap1 signaling pathway	5	0.00473551
hsa04270:Vascular smooth muscle contraction	4	0.00662614
hsa05219:Bladder cancer	3	0.00810137
hsa05206:MicroRNAs in cancer	5	0.0138639
hsa04923:Regulation of lipolysis in adipocytes	3	0.014759858
hsa00590:Arachidonic acid metabolism	3	0.017363013
hsa05230:Central carbon metabolism in cancer	3	0.01901226
hsa04020:Calcium signaling pathway	4	0.020926395
hsa05218:Melanoma	3	0.0231066
hsa04151:PI3K-Akt signaling pathway	5	0.025838382
hsa04024:cAMP signaling pathway	4	0.02722417
hsa05205:Proteoglycans in cancer	4	0.027940509
hsa04912:GnRH signaling pathway	3	0.036574443

anti-apoptotic properties of synovial fibroblasts to affect synovial proliferation in RA.[60–62] Activation of the PI3K/Akt signaling pathway can regulate the production of RA-related inflammatory mediators.[63] PI3K/Akt signaling pathway is also involved in regulating osteoporosis.[64] Thus, these KEGG pathways corresponding to the predicted targets of CA were also mainly related to RA and osteoporosis. In addition, Gene interaction analysis by GeneMANIA indicated that the 26 genes may comprehensively interact with each other. Besides, it also further implied that the 26 genes may function through alliance mechanisms in anti-RA and anti-osteoporosis process. It was consistent with the results of GO and pathway analysis. GO and pathway analyses were completed by DAVID 6.8, and the network was constructed using Cytoscape 3.0. As shown in Figure 5, the network indicated that CA might act on multiple targets, further causing complex and diverse pharmacological effects to exert its anti-osteoporosis and anti-RA effects. Although network pharmacology is an efficient method for predicting multiple drug targets in complex diseases, it is still necessary to verify the predicted targets using in vitro experiments.

RA is a chronic autoimmune inflammatory arthritis associated with persistent inflammation, and osteoporosis commonly occurs in the setting of inflammatory arthritis. Thus, suppression of inflammation has been the goal of their treatment.[65,66] Macrophages are essential for the pathophysiology of RA and they are a major source of pro-inflammatory cytokines and chemokines (such as TNF, IL-1β, and IL-6).[67] The large number of macrophages is a major feature of inflammatory lesions, and removal of these macrophages from inflammatory tissue has profound therapeutic benefits.[68] Therefore, when studying the pathogenesis of RA, we often use inflammation models induced with RAW264.7 cells and THP1 cells in vitro.[69–72] According to previous experience, some drugs that work in mouse are not effective in humans. In order to avoid this mistake, we carry out qPCR verification in vitro inflammation models induced with them both, to get a more accurate and widely applicable result in this study. By detecting the expression levels of predicted gene targets, we further verified how CA exerted its anti-osteoporosis and anti-RA pharmacological effects. Experimental verification suggested that CA stimulated monocytes/macrophages in a concentration-dependent manner and effectively down-regulate the expression of the predicted five major gene targets (*EGFR*, *MAP2K1*, *MMP2*, *FGFR1*, and *MCL1*) enriched on these KEGG pathways associated with osteoporosis and RA, in vitro inflammation models induced with RAW264.7 cells or THP1 cells. As mentioned above, the relationship between these five gene targets and the pathogenesis, pathological process and related treatment of RA and osteoporosis had also been reported by researchers. *EGFR* is newly identified as a direct inhibition target of miR-573. It regulates IL-6 production and human umbilical vein endothelial cells (HUVECs) angiogenesis by miR-573, which alleviates RA.[27] Consistent with the conclusion of this article, our experimental data suggested that CA may play anti-RA and anti-osteoporosis role by inhibiting *EGFR* expression. It confirms that our prediction may be precise that *EGFR* is indeed a therapeutic target of CA in RA and osteoporosis. It has been demonstrated that Cocoa Polyphenols directly inhibited the activity of *MAP2K1* to reduce the TNF-α-induced up-regulation of *VEGF* which exasperates RA.[28] Besides, *MAP2K1* may be also involved in the mechanism of tomatidine in improving osteoporosis.[29] Consistently, our result suggested that CA may exert anti-RA and anti-osteoporosis effects by inhibiting *MAP2K1* expression. The expression of *MMP-2* can be inhibited by artesunate, leading to remarkable suppression of the migration and invasion of RA-fibroblast-like synoviocytes (FLS).[30] *MMP-2* also plays a negative regulatory role in

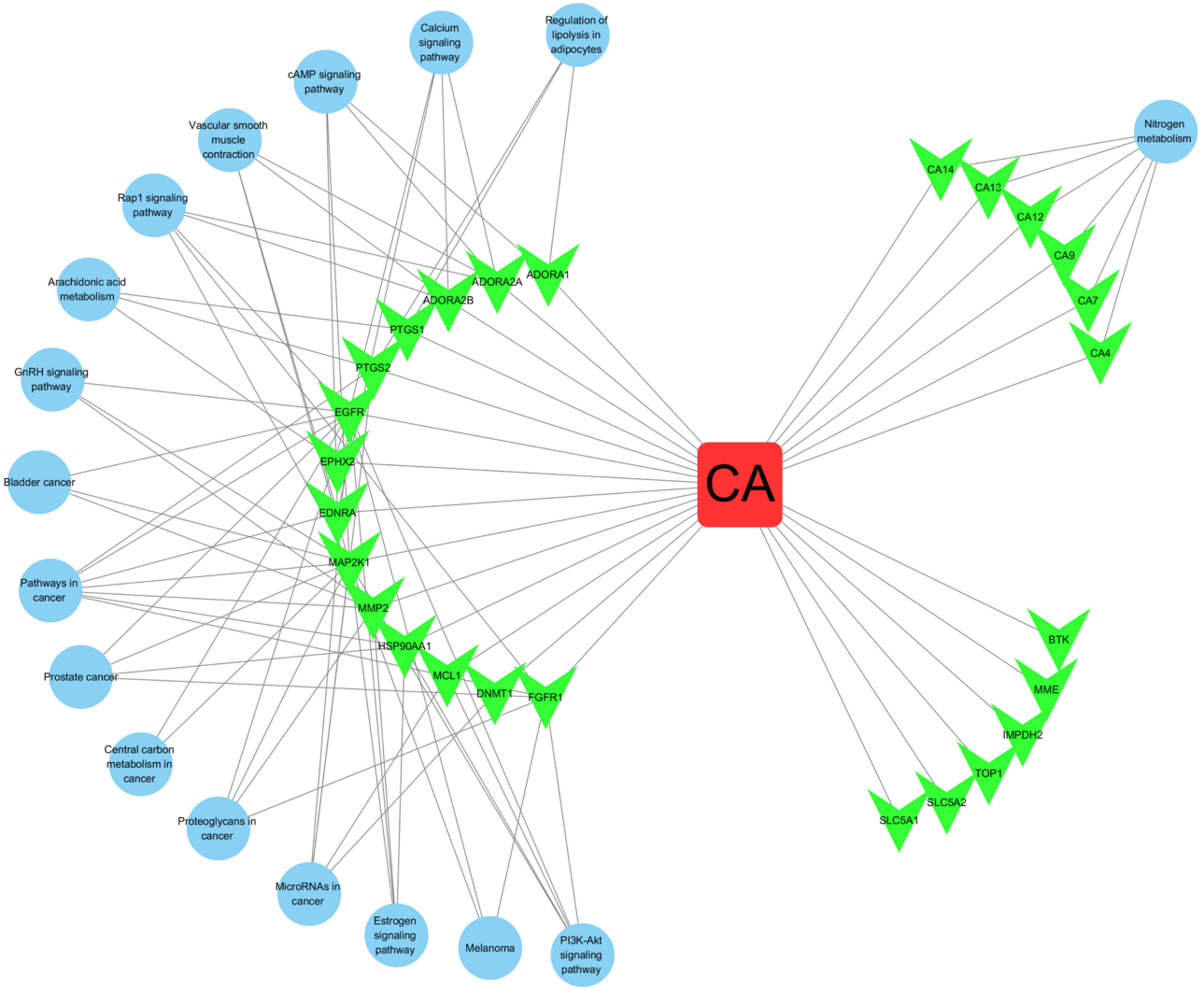

Figure 5 CA-target-pathway network. The red oblong, green inverted triangles, and blue circles correspond to CA, target proteins, and related pathways, respectively.

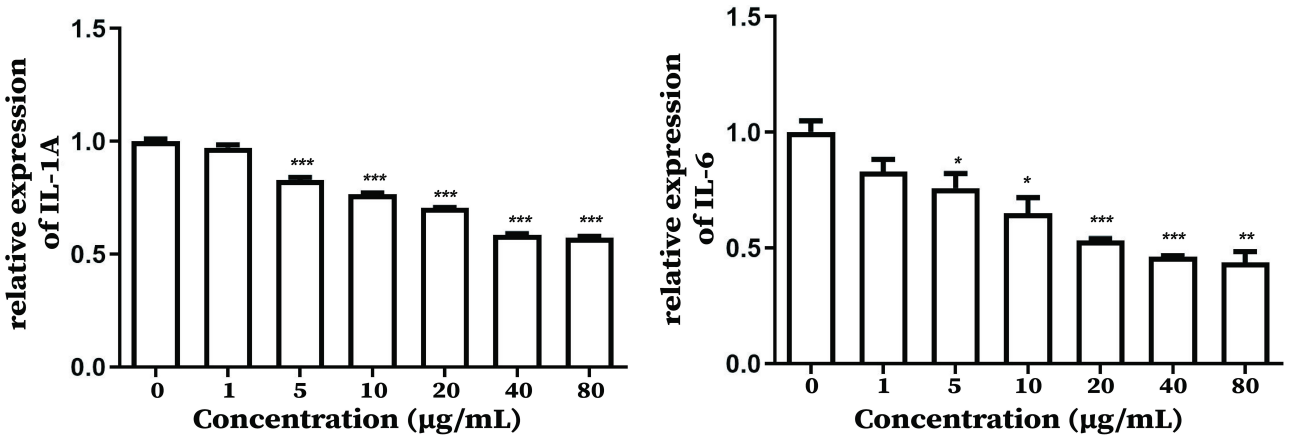

Figure 6 Real-time PCR analysis for pro-inflammatory factors (IL-1β and IL-6) genes expression in activated RAW264.7 cells not pretreated or pretreated with CA at different concentrations. *P<0.05 was considered statistically significant. ** represented P<0.01, *** represented P<0.001.

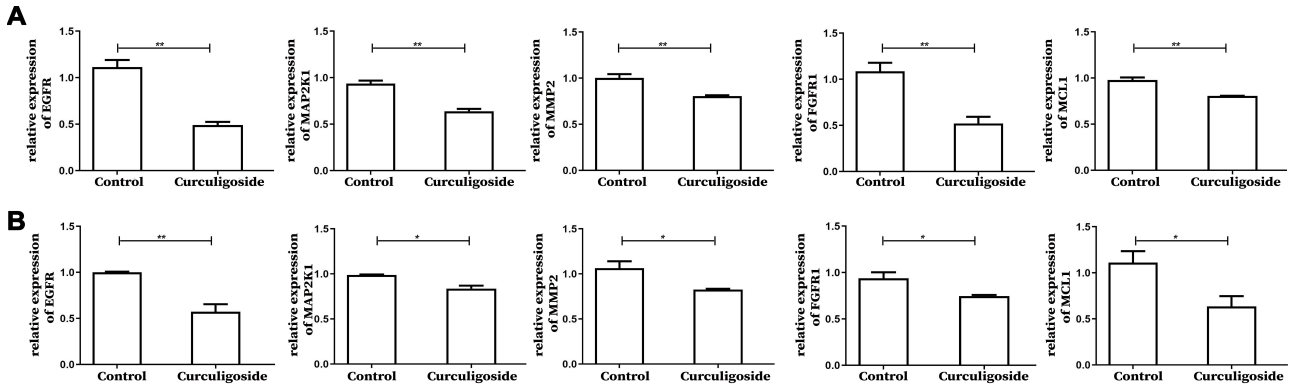

Figure 7 Real-time PCR analysis for five predicted genes expression in two kinds of activated cells (RAW264.7 cells and THP1 cells) pretreated or not pretreated with CA with a concentration of 40μg/mL. (**A**) in RAW264.7 cells. (**B**) in THP1 cells. *EGFR*, Epidermal growth factor receptor erbB1; *MAP2K1*, Dual specificity mitogen-activated protein kinase kinase 1; *MMP2*, Matrix metalloproteinase 2; *FGFR1*, Fibroblast growth factor receptor 1; *MCL1*, Induced myeloid leukemia cell differentiation protein Mcl-1. *P<0.05 was considered statistically significant. ** represented P<0.01.

osteoporosis by MMP-2/B7-H3.[31] The expression of *FGFR1* is regulated by the SHH-Gli signaling pathway, which subsequently leads to the abnormal proliferation of RA-synovial fibroblast (RA-SF). Blocking the SHH-Gli pathway can inhibit RA-SF cells proliferation and increase apoptosis to treat RA.[32] Besides, inhibition of *FGFR1* and ERK1/2 signaling rescues the anti-osteogenic effects.[33] The up-regulation of *MCL-1* promotes the anti-apoptotic ability of FLS through the activation of the PI3K/Akt and STAT3 pathways to aggravate RA.[34] Therefore, they can represent therapeutic targets for RA and osteoporosis. Overall, experimental results were consistent with these studies in vitro and in vivo suggesting that *EGFR*, *MAP2K1*, *MMP2*, *FGFR1*, and *MCL1* all can directly or indirectly participate in the pathogenesis, pathological process, and related treatment of RA and osteoporosis. So *EGFR*, *MAP2K1*, *MMP2*, *FGFR1*, and *MCL1* are potential targets of CA for RA and osteoporosis treatment. CA may exert anti-RA and anti-osteoporosis effects by inhibiting their expressions. However, the validated targets used in experimental verification are based on the network pharmacology prediction and may be deviated from the actual targets. In addition, our results can only prove that CA down-regulated the expression of these five targets, and further research is needed on how CA regulates downstream pathways to exert anti-osteoporosis and anti-RA effects. Thus, our study just suggested there was a possibility that CA might act on osteoporosis and RA associated KEGG pathways (enriched with these five genes) such as nitrogen metabolism, estrogen signaling pathway, Rap1 signaling pathway, and PI3K/Akt signaling pathway to exert anti-osteoporosis and anti-RA effect. In summary, experimental verification preliminarily proved that our prediction based on network pharmacology on how CA exerted its anti-osteoporosis and anti-RA effects may be correct and credible.

Our results were derived from network pharmacology-based prediction. In order to improve the accuracy of prediction, SwissTarget and TargetNet were used to predict and overlap the targets of CA for RA and osteoporosis. The prediction of SwissTarget was based on a combination of 2D and 3D similarity with a library of 370′000 known actives on more than 3000 proteins from three different species. Its success rate that at least one of the experimentally known targets can be found among the predicted top-15 was 72%.[23] Besides, the TargetNet can predict the activity of the user's molecule across 623 human proteins by establishing the high-quality QSAR model for each human protein. Its AUC scores were 75%-100%.[22] Although using two methods to predict and overlap the targets can increase the credibility of the results, some prediction errors and missing will occur due to the incomplete coverage of the library/QSAR model and the accuracy of prediction methods. So there may exist some deviations due to that important real targets are not predicted or are not among the 26 overlapping genes when target prediction and overlapping are performed at the beginning. Therefore, targets other than our predicted results should not be ignored in future studies. In this study, our intention was to initially construct a drug-target-pathway network of CA in osteoporosis and RA and to briefly verify the accuracy of this network by qPCR experiments. However, we did not further explore the pivotal targets that play a key role in this network. In

future research, we will conduct the network analysis for screening the pivotal targets and explore precise mechanism on how CA exerts anti-osteoporosis and anti-RA effects. Besides, for further verification, more specific and accurate experimental verification still needs us to carry out in future studies in addition to qPCR. Nevertheless, our results still provide a relatively credible prediction about the pharmacological mechanism of how CA treats osteoporosis and RA, which is useful for further developing new CA-based drugs in RA and applications of network pharmacology in drug discovery.

Conclusion

Collectively, the present study revealed a systematic and visual overview of the probable molecular mechanisms and signaling pathways of how CA exerted its anti-RA and anti-osteoporosis effects. CA can be considered as a good drug candidate for osteoporosis and RA. Consistent with the prediction, experimental results also proved that the prediction based on network pharmacology methods may be accurate and credible.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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