Research Article

Andrographolide in atherosclerosis: integrating network pharmacology and in vitro pharmacological evaluation

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Objective: Andrographis paniculata (Burm.f.) Nees is a medicinal plant that has been traditionally used as an anti-inflammatory and antibacterial remedy for several conditions. Andrographolide (AG), the active constituent of A. paniculata (Burm.f.) Nees, has anti-lipidic and anti-inflammatory properties as well as cardiovascular protective effects. The present study aimed to explore the effects of AG on the progression of atherosclerosis and to investigate related mechanisms via network pharmacology.

Materials and methods: Compound-related information was obtained from the PubChem database. Potential target genes were identified using STITCH, SwissTargetPrediction, Bioinformatics Analysis Tool for Molecular mechANism of Traditional Chinese Medicine, and Comparative Toxicogenomics Database. Genes involved in atherosclerosis were obtained from DisGeNet and compared with AG target genes to obtain an overlapping set. Protein–protein interactions were determined by STRING. Gene ontology (GO) analysis was performed at WebGestalt, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was analyzed using Metascape. The final network showing the relationship between compounds, targets, and pathways was constructed using Cytoscape. After that, oxLDL-induced RAW264.7 cells were used to further validate a part of the network pharmacology results.

Result: Eighty-one potential AG target genes were identified. PPI, GO, and KEGG enrichment revealed genes closely related to tumor progression, lipid transport, inflammation, and related pathways. AG improves the reverse cholesterol transport (RCT) through NF-κB/CEBPB/PPARG signaling in oxLDL-induced RAW264.7 cells.

Conclusion: We successfully predict AG’s potential targets and pathways in atherosclerosis and illustrate the mechanism of action. AG may regulate NF-κB/CEBPB/PPARG signaling to alleviate atherosclerosis.

Introduction

Reverse cholesterol transport (RCT) is one of the important mechanisms associated with atherosclerosis (AS) and various cardiovascular diseases. Lipid infiltration is a key risk factor for the increased incidence of atherosclerosis, which leads to stroke, coronary artery disease (CAD), and carotid intima-media thickness [1]. Ischemic stroke and CAD arising from atherosclerosis is the leading cause of death and morbidity worldwide [2]. The annual incidence of sudden cardiac death is 69/100,000 in the United States, and the
number of new stroke cases is over 2 million per year in China [3,4]. The current guidelines for treating AS prescribe statins, such as atorvastatin, simvastatin, and lovastatin, as the primary interventions for atherosclerotic cardiovascular disease (ASCVD). However, musculoskeletal weakness is one of the commonly reported side effects during the treatment [5]. For some ASCVDs induced by autoimmune inflammatory diseases like systemic lupus erythematosus, evidence of the beneficial effects of statin therapy is limited [6]. Moreover, regular use of aspirin for cardiovascular disease primary prevention is associated with the risk of severe bleeding [7]. Therefore, the design and development of new drugs targeting ASCVD remain an active aspect of biomedical research.

One of the successful strategies for novel drug discovery is identifying active compounds from medicinal plants. *Andrographis paniculata* is a medicinal herb from Acanthaceae used in traditional Chinese medicine (TCM) with thousands of years of history. Andrographolide (G), an active ingredient with diterpene lactone structure in *A. paniculata* (Figure 1A), has been demonstrated to have anti-inflammatory properties in *in vitro* and *in vivo* studies [8–11]. AG and its derivatives effectively suppress the development of inflammation-related diseases like stroke, rheumatoid arthritis, and joint pain by inhibiting transcription factor NF-κB signaling pathways [12,13]. Meanwhile, other potential therapeutic applications of andrographolide (AG) and its derivatives, such as antiviral function, antioxidative stress, antitumor, organ protection, and antihyperglycemia, have also been reported [14–17]. Recent evidence indicates that the antiatherosclerosis properties of AG are related to its inhibition of lipogenic gene expression, adipocyte formation, and macrophage foam cell formation [18,19]. Taken together, these findings suggest that the functions of AG are multi-targeted, and network analysis will be necessary for further understanding the mechanisms behind its activities.

The economic and time costs of drug development are large. Network pharmacology is a new method for systematically analyzing and predicting the mechanisms of an active compound or a drug. It was established by updating the research paradigm from the current ‘one target, one drug’ mode to the new ‘network target, multi-components’ mode [20]. The potential candidate targets of an active compound are first selected from different databases, followed by incorporation of genetic information obtained from the disease database. Next, based on the enrichment analysis results of genes, a functional network is generated to facilitate mechanistic studies or new drug development. This method greatly helps decrease the drug attrition rate, and thus, it is vital for rational and cost-effective drug development [21]. It is also suitable for exploring existing mechanisms for developing new drugs. In a similar manner, we have tried to systematically evaluate the mechanism and properties of AG in atherosclerosis by data mining.

![Figure 1. Structure of AG and flow chart of the experiment](image-url)

(A) Structure of AG downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov; PubChem CID: 5318517); (B) flowchart of the process.
In the present study, we first retrieve the target genes corresponding to AG (the structure shown in Figure 1A) through the database. Second, we select genes duplicated in the disease database and conduct related analyses and enrichment. Third, we build the above results into a network. Finally, an oxidized low-density lipoprotein (oxLDL)-induced RAW264.7 model was constructed for further validation. The specific process is shown in Figure 1B.

Methods

Plant extract
AG was extracted from *A. paniculata* (Burm.f.) Nees as a high purity crystalline powder (purity: 98.4%, LOT. 190402) by the Sichuan Wenlong Pharmaceutical Co., Ltd (Sichuan, China), with inspection Report No. C-01-19013.

Chemical, physical, and toxic properties of AG
The chemical and physical properties related to AG structure and toxicological properties were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov). PubChem is an open chemistry database mainly for small molecules, but larger molecules also are included. The records of PubChem are accumulated from hundreds of data sources, such as chemical vendors, journal publishers, and government agencies [22].

Acute toxicology tests in mice were carried out following the protocol of Liao et al. We divided male Apoe-/- mice into three random groups, each containing eight mice. Two different doses of AG (1000 and 5000 mg/kg) were administered to two different groups of mice. Animals were provided with free access to food and water. Throughout the 14-day experiment, the mice were monitored for any mortality or behavioral changes. The behavioral changes observed included hyperactivity, tremors, ataxia, convulsions, salvation, diarrhoea, lethargy, sleep, and coma. The study was performed in compliance with the Animal Experimental Ethics Committee of Guang’anmen Hospital, China Academy of Chinese Medical Sciences.

Target acquisition and screening
Different types of targets matched to AG were collected from the following databases, which are often used in the literature for monomer research. (1) STITCH (http://stitch.embl.de/cgi/input.pl?UserId=BzUtaFPWtqdq&sessionId=l7vk7FHggCm6) enables users to view the binding affinities of a chemical in an interaction network and the potential effects of the chemical on its interaction targets [23]; (2) Swiss Target Prediction (STP, http://old.swisstargetprediction.ch/) measures the similarity between new molecules and known ligands to predict their targets accurately [24]; (3) Bioinformatics Analysis Tool for Molecular mechANism of Traditional Chinese Medicine (BATMAN-TCM,
http://bionet.ncpsb.org/batman-tcm/) is an online Bioinformatics Analysis Tool for TCM ingredients’ target prediction and functional analysis of targets, including biological pathways and enrichment analyses and visualization [25]; (4) Comparative Toxicogenomics Database (CTD, http://ctdbase.org/) [26] is a premier public resource with manually curated associations between chemicals, targets, diseases, and so on based on literature.

After collection, we input the gene symbols to Uniprot (https://www.uniprot.org/) for gene names and Uniprot IDs. Uniprot ID is an important identification when performing protein alignment between different databases. Genes related to atherosclerosis were obtained from the following databases: (1) DisGeNet (http://www.disgenet.org/search), a versatile platform for various research purposes like investigating the molecular underpinnings of specific human diseases and their comorbidities and analysing the properties of disease genes, etc. [27]; (2) DrugBank database (https://www.drugbank.ca/), a robust and comprehensive bioinformatics database containing data on drugs and disease [28]; (3) Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org/), an authoritative, timely, and comprehensive database about human genes and genetic diseases [29]; (4) Therapeutic Target Database (http://db.idrblab.net/ttd/), which provides data on therapeutic protein and nucleic acid targets for the targeted disease [30]. Homo sapiens was selected as the species. After targets from databases were compared with targets matched to AG, the final 81 targets were successfully identified.

Protein–protein interaction (PPI)
STRING (https://string-db.org/) is a protein-related database that collects, scores, and integrates many publicly available sources of protein–protein interaction information and complements information with computational predictions. Finally, a global network of PPI, including direct (physical) and indirect (functional) interactions, is generated [31].

Homo sapiens was selected as the organism after entering the above target list into the multiple-protein search. To decrease the complexity of the network, 0.95 was set as the minimum required interaction score, and disconnected nodes in the network were hidden. Network parameters were adjusted to get a clearer structure in Cytoscape (v3.6.0; https://cytoscape.org/) [32].

Gene ontology (GO) analysis
The gene ontology (GO) project is a result of efforts to align the functional description of gene products in various databases. GO has developed a standard language (ontologies) with a tertiary structure, which is defined according to the gene product’s relevant molecular functions, biological pathways, and cytological components. WEB-based GEne SeT AnaLysis Toolkit (WebGestalt, http://www.webgestalt.org/option.php) supports 324 gene identifiers from various platforms and computationally analyses 150937 functional categories from public databases [33]. After choosing overrepresentation enrichment analysis (OSA), UniProt IDs of 81 genes were uploaded, and ‘genome’ was selected as the reference set.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment
Metascape (http://metascape.org/gp/index.html#main/step1) is a web-based platform designed to provide a comprehensive gene list annotation and pathway analysis resource for researchers [34]. During the operation, we selected the customized analysis for the enrichment of 81 target genes in KEGG. KEGG, including the GENES, PATHWAY, and LIGAND databases, is a knowledge base for the systematic analysis of gene functions; it links genomic information with different levels of functional information.

Compound-target-pathway network construction
“Compound-Target-Pathway network” was established by Cytoscape 3.6.0 software based on the results in the Metascape platform. The characteristics of multiple targets and multiple pathways of AG were displayed and analysed through the entire complex compound, target, and disease network.

Cell culture and treatment
RAW264.7 cells were acquired from the Cell Resource Center, Peking Union Medical College (the headquarters of National Infrastructure of Cell Line Resource, NSTI) on March 15, 2018. The cell line was routinely tested for the absence of mycoplasma contamination by PCR and culture. Its species origin was authenticated using PCR. The identity of the cell line was confirmed with STR DNA profiling (FBI, CODIS). All the results are available online at the website (http://cellresource.cn). RAW264.7 cells were maintained in a 25-cm² cell culture flask with a 2 μm vent cap at 37°C in a CO₂ incubator (5% CO₂, 95% air). Cells were cultured in 8 ml DMEM (Cat No. M1805, CELL
Table 1 Antibody information of the anti-NF-κB-p65, anti-CEBPB, anti-PPARG and anti-β actin in RAW264.7 cells

| Cat.No       | Lot.No      | Manufacturer | Specifications | Concentration |
|--------------|-------------|--------------|----------------|---------------|
| NF-κB p65    | ab16502     | Abcam        | 100 μg         | 1 mg/ml       |
| CEBPB        | ab53138     | Abcam        | 100 μg         | 1 mg/ml       |
| PPARG        | ab45036     | Abcam        | 100 μl         | 1 mg/ml       |
| β-Actin      | TDY051F     | TDY bio      | 100 μl         | 1 mg/ml       |

technologies, China) supplemented with 10% Defined Fetal Bovine Serum (FBS, Cat No. SH30070.03, Hyclone laboratories, Inc, U.S.A.), 1.25% L-glutamine, and 2% penicillin/streptomycin. RAW 264.7 macrophages were seeded in 6-well plates (2 × 10^5/well) in 10% FBS DMEM as described [32,33]. The oxLDL was purchased from Shanghai yuanye Bio-Technology Co., Ltd (Cat No. S24879-2mg, China). Cells were classified into three groups: untreated RAW264.7 cells (RAW264.7), oxLDL treated foam cells (RAW264.7+oxLDL), and oxLDL+ 50 μmol/L AG treated foam cells (RAW264.7+oxLDL+AG). After growing to 80% confluency, cells were stimulated with 50 μg/ml oxLDL in a serum-free medium for 24 h. In parallel, 50 μmol/L AG was added together with oxLDL for 24 h. The final concentration of oxLDL was decided by preliminary experiments and previous research [35–37].

**Oil-red staining**

For lipid staining, Oil Red O powder (Cat No. O0625-100G, SIGMA-ALDRICH, U.S.A.) was used according to the BioVision manual. After the indicated treatments, cells were fixed with 4% paraformaldehyde (PFA, Ref No. BL539A, biosharp, Beijing Labgic Technology Co., Ltd, China) in the indicated groups for 30 min and stained with Oil Red O solution for 15 min at 25 ± 2 °C. Finally, The cells were observed with a microscope (Olympus IX70) equipped with a camera at 200× magnification. The percentage of Oil Red O positive area was measured using the ImagePro Plus software.

**Determination of inflammatory cytokines in cell supernatant**

Cell supernatant was collected following each treatment. Meanwhile, the Bio-Plex System was warmed up for at least 30 min. A single vial of standards was reconstituted in 500 μl of a diluent similar to the final sample type or matrix. Standards were incubated on ice for 30 min with vortexing for 5 s. A 4-fold standard dilution of the series and blank were prepared. Samples were vortexed for 5 s between liquid transfers. The 10x single beads were vortexed for 30 s and diluted to 1× in the Bio-Plex Assay Buffer. They were protected from light. The assays were run following the manufacturer’s instructions (Bio-Plex Pro Mouse Cytokine Grp, Catalog NO. #M60009RDPD, Bio-Rad Laboratories, Inc., U.S.A.). The assay measurements were conducted on the Bio-Plex MAGPIX System plate-reader (Bio-Rad Laboratories, Inc.). Finally, the C-C motif chemokine ligand 2 (CCL2), interleukin-6 (IL-6), and tumor necrosis factor α (TNFα) concentration were calculated by the standard curve.

**SDS-PAGE and Western blot**

The methods for protein extraction from RAW264.7 cells, electrophoresis, and subsequent Western blotting are as described. The cells were lysed in RIPA buffer (high) (Cat No. #R0010, Beijing Solarbio Science & Technology Co., Ltd, China) containing haptase and phosphatase inhibitor single-use cocktail for 30 min on ice. The lysates were centrifuged at 12,000 rpm for 30 min at 4 °C. Each sample protein was separated on TGX Stain-Free FastCast Acrylamide Kit, 10% (Cat No. #1610183, Batch No. 64278801, Bio-Rad Laboratories, Inc., www.bio-rad.com, U.S.A.) and immunoblotting with antibodies (Table 1) followed by HRP-conjugated secondary antibody. Visualized bands were analysed on ChemiDocTMMP imaging system (Model No. Universal Hood III, Serial No. 731BR02915, Bio-Rad Laboratories, Inc., www.bio-rad.com, U.S.A.) using ImageLab™ software. Quantification was performed with Image Pro Plus software.

**Real-time quantitative polymerase chain reaction**

NF-κB-p65, CEBPB, and PPARG mRNA levels were quantified by Real Time Quantitative PCR (RT-qPCR). According to the manufacturer’s instructions from Direct-zol™ RNA MiniPrep (Cat No. R2052, www.zymoresearch.com, U.S.A.), total RNA samples were extracted from the frozen mice liver tissues using TRizol Reagent (Ref No. 15596026, Ambion by Life Technologies, U.S.A.). Total RNA was measured by NANODROP 2000 Spectrophotometer (ND2000, Thermo Scientific, Gene Company Limited). cDNA synthesis was carried out with the High Capacity cDNA Reverse Transcription Kit (Ref No. 4368814, applied biosystems by Thermo Fisher Scientific, Thermo Fisher Scientific Baltics © 2022 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC By).
Table 2  Primer of the NF-κB-p65, CEBPB, PPARγ, and β-actin in RAW264.7 cells

| Gene name          | Full name                        | Region  | Sequence                                      |
|--------------------|----------------------------------|---------|-----------------------------------------------|
| NF-κB p65 (mouse)  | Nuclear factor- kappa B p65      | Forward | 5′-ATGGCAGACGATGATCCCTAC-3′                   |
|                    |                                  | Reverse | 5′-CGGAATCGAAATCCCTGTTT-3′                   |
| CEBPB (mouse)      | CCAAT enhancer binding proteins Beta | Forward | 5′-GACAAAGCTGAGCGAGAGTAC-3′                  |
|                    |                                  | Reverse | 5′-TTGCCGACCTTTGQCTCTGC-3′                  |
| PPARγ (mouse)      | Peroxisome proliferators-activated receptors Gamma | Forward | 5′-AAAGAAGCTGACCCATGGTTGC-3′                 |
| β-Actin (mouse)    | Beta cytoskeletal actin          | Forward | 5′-GTGACGTTGACATCCGTAAGA-3′                 |
|                    |                                  | Reverse | 5′-GCCGGACTCATGCTACCC-3′                    |

Table 3  Chemical and physical properties of AG

| Property name                      | Property value        |
|------------------------------------|-----------------------|
| Molecular weight                   | 350.456 g/mol         |
| XLogP3-AA                          | 2.2                   |
| Hydrogen bond donor count          | 3                     |
| Hydrogen bond acceptor count       | 5                     |
| Rotatable bond count               | 3                     |
| Exact mass                         | 350.209 g/mol         |
| Monoisotopic mass                  | 350.209 g/mol         |
| Topological polar surface area     | 87 A2                 |
| Heavy atom count                   | 25                    |
| Formal charge                      | 0                     |
| Complexity                         | 597                   |
| Isotope atom count                 | 0                     |
| Defined atom Stereocenter count    | 6                     |
| Undefined atom Stereocenter count  | 0                     |
| Defined bond Stereocenter count    | 1                     |
| Undefined bond Stereocenter count  | 0                     |
| Covalently bonded unit count       | 1                     |
| Compound is canonicalized          | yes                   |

UAB, Lithuania). Real-time qPCR was performed with the ABI 7900 system (applied biosystems, U.S.A.) using an Power SYBR Green PCR Master Mix (Ref No. 4367659, applied biosystems by Thermo Fisher Scientific, Life Technologies LTD, U.K.). The primers were synthesized by Thermo Fisher and the sequences were listed in Table 2. The thermal conditions of PCR were as follows: 1 cycle of 95°C for 10 min, 45 cycles of 95°C for 15 s, 55–60°C for 20 s, and 72°C for 30 s. The relative expression level of each gene was determined by the 2^{-ΔΔCt} method and normalized to the β-actin mRNA in each sample.

Statistical analysis
Statistical analysis was performed with SPSS software 13.0 (IBM, Almon, NY, U.S.A.). For multiple comparisons, one-way analysis of variance (one-way ANOVA) was performed. All results were expressed as the mean ± SD. P-values <0.05, 0.01, or 0.001 were considered statistically significant. Graphs were plotted using GraphPad Prism Version 8.3 software.

Results
Chemical, physical, and toxic properties of AG
The chemical, physical and toxic properties of AG obtained from the PubChem database are shown in Tables 3 and 4, respectively. Table 3 summarizes some characteristics of AG important for its pharmacological functions, such as molecular weight and number of stereocenters. However, limited toxicological information on AG exists in the PubChem database; only one study of LD50 in mice has been reported (Table 4).
Table 4. Toxic properties of AG

| Organism | Test type | Route        | Dose          | Reference              |
|----------|-----------|--------------|---------------|------------------------|
| mouse    | LD<sub>50</sub> | intraperitoneal | 11,460 mg/kg  | (Handa and Sharma, 1990) |

Figure 3. Results of GO analysis

(A) Bar chart of Biological Process categories. (B) Bar chart of Cellular Component categories. (C) Bar chart of Molecular Function categories.

Behavioural changes were not observed upon oral administration of AG for 14 days, nor was any mortality observed. Therefore, the LD<sub>50</sub> value of AG was concluded to be greater than 5000 mg/kg in mice. As a result, AG showed no apparent acute toxicity in mice.

Putative targets of AG

After searching in different databases, as shown in Figure 1B, 146 genes associated with AG functions were collected from STITCH, STP, CTD, and BATMAN-TCM. Moreover, 2044 atherosclerosis-related genes were found in DisGeNet. Combining these findings, a column graph of highly related genes, according to the gene-disease association (GDA) score in DisGeNet, was generated as shown in Figure 2A. Eighty-one genes regulated by AG were associated with the development of atherosclerosis. The details of the 81 genes are summarized in Table 5.

Analysis of protein–protein interaction (PPI)

The PPI network was established to better understand protein–protein interactions (Figure 2B). In this figure, disconnected nodes were not shown, and the confidence score was set to 0.95. The final network embodied 64 nodes and 156 edges; the degree were used to evaluate the importance of proteins in this network.

GO enrichment

To further analyse the functions of the 81 target genes, GO enrichment was performed at WebGestalt (Figure 3). In this platform, the functions are divided into three levels: biological process, cellular component, and molecular functions. The top five genes were attributed to response to stimulus (79/81), biological regulation (76/81), protein binding (75/81), metabolic process (74/81), and cell communication (65/81).

KEGG enrichment

A network figure was mapped using the Metascape platform to predict pathways involving the 81 target genes. As shown in Figure 4, the top five pathways affected by AG were pathways involved in cancer (hsa05200), TNF signaling pathway (hsa04668), fluid shear stress, atherosclerosis (hsa05418), colorectal cancer (hsa05210), and non-alcoholic...
| Num. | UniProt ID | Gene symbol | Gene name | Source |
|------|------------|-------------|-----------|--------|
| 1    | P62736     | ACTA2       | Actin alpha 2, smooth muscle | CTD    |
| 2    | P18089     | ADRA2B      | Adrenoceptor alpha 2B | Stitch |
| 3    | P50052     | AGTR2       | Angiotensin II receptor type 2 | Stitch |
| 4    | P15336     | ATP2        | Activating transcription factor 2 | CTD    |
| 5    | Q07812     | BAX         | BCL2 associated X, apoptosis regulator | CTD    |
| 6    | P10415     | BCL2        | BCL2 apoptosis regulator | CTD    |
| 7    | P42574     | CASP3       | Caspase 3 | CTD    |
| 8    | P56211     | CASP9       | Caspase 9 | CTD    |
| 9    | P04040     | cat         | Catalase  | CTD    |
| 10   | P13500     | CCL2        | C-C motif chemokine ligand 2 | CTD    |
| 11   | P29279     | CCN2        | Cellular communication network factor 2 | CTD    |
| 12   | P24385     | COND1       | Cyclin D1 | CTD    |
| 13   | P61681     | CCR5        | C-C motif chemokine receptor 5 (gene/pseudogene) | Stitch |
| 14   | P61686     | CCR9        | C-C motif chemokine receptor 9 | Stitch |
| 15   | P12830     | CDH1        | Cadherin 1 | CTD    |
| 16   | P24941     | CDK2        | Cyclin dependent kinase 2 | CTD    |
| 17   | P38936     | CDKN1A      | Cyclin dependent kinase inhibitor 1A | CTD    |
| 18   | P49715     | EBP/A       | CCAAT enhancer binding protein alpha | CTD    |
| 19   | P17676     | EBP/B       | CCAAT enhancer binding protein beta | CTD    |
| 20   | Q6UWW8     | CES3        | Carboxylesterase 3 | CTD    |
| 21   | Q9N5E2     | CISH        | Cytokine inducible SH2 containing protein | CTD    |
| 22   | Q99439     | CNN2        | Calponin 2 | CTD    |
| 23   | P08123     | COL1A2      | Collagen type I alpha 2 chain | CTD    |
| 24   | P02461     | COL3A1      | Collagen type III alpha 1 chain | CTD    |
| 25   | Q43927     | CXL13       | C-X-C motif chemokine ligand 13 | CTD    |
| 26   | P10145     | CXL8        | C-X-C motif chemokine ligand 8 | CTD    |
| 27   | P04798     | CYP1A1      | Cytochrome P450 family 1 subfamily A member 1 | CTD    |
| 28   | P05177     | CYP1A2      | Cytochrome P450 family 1 subfamily A member 2 | CTD    |
| 29   | P11712     | CYP2C9      | Cytochrome P450 family 2 subfamily C member 9 | CTD    |
| 30   | P06884     | CYP3A4      | Cytochrome P450 family 3 subfamily A member 4 | CTD    |
| 31   | P05305     | EDN1        | Endothelin 1 | CTD    |
| 32   | Q9H6Z9     | EGLN3       | egfl9 family hypoxia inducible factor 3 | CTD    |
| 33   | Q15910     | EZH2        | Enhancer of zeste 2 polycomb repressive complex 2 subunit | CTD    |
| 34   | P25445     | FAS         | Fas cell surface death receptor | CTD    |
| 35   | P48023     | FASLG       | Fas ligand | CTD    |
| 36   | P17948     | FLT1        | fms-related receptor tyrosine kinase 1 | CTD    |
| 37   | P01100     | FOS         | Fos proto-oncogene, AP-1 transcription factor subunit | CTD    |
| 38   | P48507     | GCLM        | Glutamate-cysteine ligase modifier subunit | CTD    |
| 39   | P49841     | GSK3B       | Glycogen synthase kinase 3 beta | CTD    |
| 40   | P00390     | GSR         | Glutathione-disulffide reductase | CTD    |
| 41   | P28161     | GSTM2       | Glutathione S-transferase mu 2 | CTD    |
| 42   | Q13547     | HDAC1       | Histone deacetylase 1 | CTD    |
| 43   | Q16655     | HIF1A       | Hypoxia inducible factor 1 subunit alpha | CTD    |
| 44   | P04035     | HMGCR       | 3-Hydroxy-3-methylglutaryl-CoA reductase | BATMAN |
| 45   | P09601     | HMOX1       | Heme oxygenase 1 | CTD    |
| 46   | /          | HOTAIR      | HOX transcript antisense RNA | CTD    |
| 47   | P00738     | HP          | Haptoglobin | CTD    |
| 48   | P05231     | IL6         | Interleukin 6 | CTD    |
| 49   | P27011     | ITGAL       | Integrin subunit alpha L | batman |
| 50   | P05107     | ITGB2       | Integrin subunit beta 2 | batman |
| 51   | P05412     | JUN         | jun proto-oncogene, AP-1 transcription factor subunit | CTD    |
| 52   | P05968     | KDR         | Kinase insert domain receptor | CTD    |
| 53   | O14733     | MAP2K7      | Mitogen-activated protein kinase kinase 7 | CTD    |
| 54   | Q99883     | MAP3K5      | Mitogen-activated protein kinase kinase 5 | CTD    |
| 55   | P28482     | MAPK1       | Mitogen-activated protein kinase 1 | CTD    |
| 56   | P27361     | MAPK3       | Mitogen-activated protein kinase 3 | CTD    |

Continued over
Table 5: Information of target genes of AG on atherosclerosis (Continued)

| Num. | UniProt ID | Gene symbol | Gene name                                      | Source   |
|------|------------|-------------|-----------------------------------------------|----------|
| 57   | P09237     | MMP7        | Matrix metalloproteinase 7                    | CTD      |
| 58   | Q16236     | NFE2L2      | Nuclear factor, erythroid 2 like 2            | CTD      |
| 59   | Q9NP32     | NGFB        | Neuroglobin                                   | CTD      |
| 60   | P15559     | NQO1        | NAD(P)H quinone dehydrogenase 1               | CTD      |
| 61   | P16284     | PECAM1      | Platelet and endothelial cell adhesion molecule 1 | CTD      |
| 62   | P57231     | PPARγ       | Peroxisome proliferator activated receptor gamma | CTD      |
| 63   | P17252     | PRKCA       | Protein kinase C alpha                         | STP      |
| 64   | P05771     | PRKCB       | Protein kinase C beta                          | STP      |
| 65   | P23219     | PTGS1       | Prostaglandin-endoperoxide synthase 1         | STP      |
| 66   | P53534     | PTGS2       | Prostaglandin-endoperoxide synthase 2         | STP      |
| 67   | Q04206     | RELA        | RELA proto-oncogene, NF-κB subunit            | STP      |
| 68   | P00441     | SOD1        | Superoxide dismutase 1                        | CTD      |
| 69   | P10451     | SPP1        | Secreted phosphoprotein 1                     | CTD      |
| 70   | Q13501     | SQSTM1      | Sequestosome 1                                | CTD      |
| 71   | P40763     | STAT3       | Signal transducer and activator of transcription 3 | CTD      |
| 72   | Q687X5     | STEAP4      | STEAP4 metalloendopeptidase                    | CTD      |
| 73   | P48775     | TDO2        | Tryptophan 2,3-dioxgenase                     | CTD      |
| 74   | P01137     | TGFβ1       | Transforming growth factor beta 1             | CTD      |
| 75   | P07996     | THBS1       | Thrombospondin 1                              | CTD      |
| 76   | P01575     | TNF         | Tumor necrosis factor                         | CTD      |
| 77   | P04637     | TP53        | Tumor protein p53                              | CTD      |
| 78   | P19320     | VCAM1       | Vascular cell adhesion molecule 1             | CTD      |
| 79   | P15692     | VEGFA       | Vascular endothelial growth factor A          | CTD      |
| 80   | P08670     | vimentin    | Vimentin                                      | CTD      |
| 81   | P98170     | XIAP        | X-linked inhibitor of apoptosis                | CTD      |

Fatty liver disease (NAFLD) (hsa04923). The interactions between the pathways and the heat map, based on rank, are also shown in Figure 4A–C.

Network analysis

The three-layer network was constructed and analysed by Cytoscape 3.6.0, as shown in Figure 5. This network contains 85 nodes and 390 edges and shows the relationship between AG, target genes and pathways.

Effect of Oil Red staining in RAW264.7 cells

Oil Red O staining was performed to identify the foam cell formation under different drug concentrations, as shown in Figure 6A–C. The red area decreased significantly after intervention by AG. This result shown in Figure 6D confirms ROI analysis finding ($P < 0.001$, compared with the RAW264.7+oxLDL group).

Effect of AG on mRNA and protein expression in RAW264.7 cells

To assess the mechanism of AG for promoting the RCT, the protein expression and transcript levels of related genes were measured. In the Western blotting experiment, a clear separation of electrophoretic bands was observed (Figure 6E). Compared with the RAW264.7 group, the expression of PPARγ in the RAW264.7+oxLDL group was significantly down-regulated ($P < 0.01$); however, CEBPB ($P < 0.05$) and NF-κB-p65 ($P < 0.01$) were up-regulated. AG reversed the trend, as shown in Figure 6F ($P < 0.05$). In the RT-qPCR experiment, the mRNA of PPARγ in the RAW264.7+oxLDL group is significantly down-regulated ($P < 0.01$) compared with that in the RAW264.7 group. In contrast, CEBPB ($P < 0.001$) and NF-κB-p65 ($P < 0.01$) are up-regulated. AG reversed the trend, as shown in Figure 6G (PPARG: $P < 0.05$, CEBPB: $P < 0.001$, NF-κB-p65: $P < 0.05$). Besides, the RAW264.7+oxLDL+AG group also showed significantly lower PPARγ mRNA than the RAW264.7 group ($P < 0.05$).

Effect of AG on inflammatory cytokines

The levels of cytokines are shown in Figure 6h. CCL2, IL-6, and TNF-α in the RAW264.7+oxLDL group were significantly higher than those in the RAW264.7 group (CCL2: $P < 0.01$, IL6 and TNF-α: $P < 0.001$). Additionally,
**Figure 4. Results of KEGG pathway enrichment**

(A) Relationship between pathways (color by cluster). (B) Relationship between pathways (color by $P$ value). (C) Heat map of pathways by rank.

AG inhibited the increase in CCL2, IL-6 and TNF-$\alpha$ in RAW264.7+oxLDL group ($CCL2: P<0.05$, $IL6$ and $TNF-\alpha$: $P<0.01$). Besides, the TNF-$\alpha$ levels in the RAW264.7+oxLDL+AG group were also significantly higher than those in the RAW264.7 group ($P<0.05$).

**Discussion**

Atherosclerosis is a crucial underlying pathology of diseases including coronary heart disease, myocardial infarction, stroke, and peripheral artery disease [38]. The aetiology of atherosclerosis is intrinsically linked to cholesterol accumulation at the arterial wall and is caused by an imbalance between deposition and removal [39]. Macrophage accumulation and cholesterol-rich plaques, characteristic of atherosclerosis, can be simulated in oxLDL-induced RAW264.7 cells. Currently, drugs with lipid-lowering and anti-atherosclerotic effects are commonly used in the clinic; however, their use has been accompanied by an increase in liver and kidney damage and other adverse effects [40]. Therefore, there is a great need for developing safe and effective new drugs for patients who cannot tolerate these adverse effects. Previous studies have shown that natural drugs and their derived compounds play an essential role in regulating lipid metabolism and atherosclerosis [41,42]. AG, isolated from *A. paniculata*, could ameliorate...
lipid metabolism disorder and atherosclerosis. It is known to exert multifaceted antiarteriosclerotic effects, such as anti-inflammatory, inhibiting the formation of foam cells, antiplatelet and protecting the endothelium [43–46]. The activation of eNOS-NO / cGMP [45], inhibition of PI3 kinase/Akt-p38 MAPK pathway [46] and NF-κB pathway [43] are reportedly involved. However, the underlying molecular mechanism has not been fully understood. Network pharmacology, a powerful tool to identify alternative targets for herbal medicines, has advantages in facilitating the development of new drugs and assessing their safety [47,48]. In the present study, a total of 146 predicted target genes in AG were obtained. Among these targets, 81 genes were associated with atherosclerosis. In addition, we demonstrate a synergistic treatment strategy for AG featuring multi-target and multi-pathways by applying various methods, including PPI, GO analysis, and Compound-Target-Pathway network. Finally, we performed experiments in RAW264.7 cells to validate part of the mechanism.

First, Lipinski’s rule of five is generally referred to as a guideline for drug optimization. It provides relevant scope to help assess the properties of drugs [49,50]. As shown in Table 1, the pharmacokinetic properties of AG meet these
Figure 6. Results of the experimental verification

(A) Untreated RAW264.7 cells after Oil Red O staining. (B) oxLDL treated RAW264.7 cells after Oil Red O staining. (C) oxLDL+AG treated RAW264.7 cells after Oil Red O staining. (D) The red area of RAW264.7 cells after Oil Red O staining (%), (N = 3 per group). (E) Electrophoretic bands of PPARG, CEBPB, and NF-κB p65. (F) Levels of protein expression (N = 3 per group). (G) Levels of mRNA expression (N = 3 per group). (H) Levels of inflammatory cytokines. The data are expressed as the mean ± SD of the mean and analysed by one-way ANOVA: *P<0.05, **P<0.01, ***P<0.001 versus RAW264.7+oxLDL group.
requirements, suggesting that it has suitable medicinal properties for the development of new commercially available drugs with high efficacies. An optimized AG-loaded nanoemulsion was developed in recent research to improve AG oral bioavailability. The pharmacokinetic results indicate that the AG-loaded nanoemulsion significantly enhanced the absorption of AG in comparison with the AG suspension with a relative bioavailability of 594.3% [51]. Moreover, solid lipid nanoparticles can increase the delivery of AG into the brain, which significantly enhances the rate of AG crossing the blood–brain barrier compared with that of free AG [52], providing strong support for the expansion of AG's efficacy.

Second, pathogenic mechanisms and interactions of potential targets were also elucidated in our study. As shown in Figure 2A, targets with a high GDA score are closely related to chronic inflammation in atherosclerosis. Figure 2B analyses the interaction according to the degree. The potential antiatherosclerotic properties of AG are probably mediated by two different types of proteins. One type is closely related to tumor proliferation, possibly through JUN, STAT3, TP53, FOS, MAPK3, MAPK1, PPARG, and CCND1. The other types of proteins, like IL6, TNF, RELA, CXCL8, and CEBPB, are associated with the regulation of inflammation. A subset of proteins performs the two distinct functions simultaneously. The regulation of the above targets by AG occurs in numerous diseases, especially cancer. TP53 genes that correlate significantly with STAT3 may be especially critical nodes in human cancers [53,54]. In human cervical and colorectal cancer cells, TP53 and STAT3 are simultaneously regulated by miR-214 [55]. JUN and FOS are the subunit proteins of the AP-1 transcription factor. AG suppresses angiogenesis in the tumour microenvironment in HCT116 colorectal cancer cells partly because of the inhibition of AP-1 and MAPKs expression [56]. AG also inhibits cell-cycle progression in human colorectal carcinoma lovo cells with a marked decrease in the protein expression of CCND1. Although cancer increases the risk of many other diseases, the mechanism of tumour progression is too complex to interpret as atherosclerosis. Notably, PPARG is a ligand-activated nuclear transcription factor that has a central role in controlling lipid metabolism [57]. PPARG signaling has also been implicated in the control of atherosclerosis [57]. Research about the effects of AG on PPARG activation focusses chiefly on lipid metabolism and the inflammatory response, which may be more closely related to atherosclerosis.

Third, the GO project and KEGG pathway enrichment analysis revealed the correlation of 81 target genes with their response to stimulus, biological regulation, protein binding, metabolic process, and cell communication. They regulate lipid transport [58], ameliorate inflammation [59], attenuate oxidative stress, and promote anti-tumour effect [60], which are intersecting, interacting, and synergetic as described in the literature.

The compound-target-pathway network shown in Figure 5 further indicated that AG plays different roles in multiple targets or pathways.

Fourth, AG-treated foam cell formation seemed to be significantly decreased, as demonstrated by Oil Red O staining. Thus, AG may be involved in macrophage cholesterol efflux, which is the first step of RCT. AG inhibits inflammation by interacting with NF-κB, the factor which regulates the transcription of pro-inflammatory cytokine genes, such as those coding for TNF-α, IL-6, and CCL2 [61]. Similar observations have been described for CEBPB interaction with NF-κB. Activation of CEBPB is induced by up-regulation of NF-κB [62]. Notably, the down-regulation of CEBPB has been reported to induce PPARG in vascular protection [63]. CEBPB and PPARG modulate macrophage function, including M2 macrophage polarization [64]. They are also similarly involved in lipid metabolism [65]. Activation of both could contribute, at least in part, to the amelioration of lipid homeostasis and atherosclerosis [66]. The variation in the expression of the above three genes has established a role for AG in the regulation of lipid metabolism and inflammation in atherosclerosis.

In conclusion, we successfully predict the potential targets of AG for application in atherosclerosis and help illustrate the mechanism of action on a systematic level. This study not only provides new insights into the chemical basis and pharmacology of AG but also demonstrates a feasible method for evaluating potential molecular drugs from herbal medicine. Our results show that AG may regulate NF-κB/CEBPB/PPARG signalling to play a therapeutic role in atherosclerosis.

Data Availability
Some or all data, models or used during the study are available from the corresponding author by request. Direct requests for these materials may be made to the provider as indicated.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.
Funding

This work was supported by the Scientific and technological innovation project of China Academy of Chinese Medical Sciences [grant number CI2021A03801]; National Natural Science Foundation of China [grant number 81373833]; Fundamental Research Funds for the Central public welfare research institutes [grant number ZZ15-YQ-054]; and Fundamental Research Funds for the Central public welfare research institutes [grant number Z0745].

CRediT Author Contribution

Shuai Shi: Conceptualization, Resources, Data curation, Software, Writing—original draft, Project administration, Writing—review & editing. Xinyu Ji: Writing—original draft. Jingjing Shi: Software, Formal analysis. Shuqing Shi: Visualization, Methodology. Qiuyan Zhang: Software, Formal analysis. Yu Dong: Conceptualization, Resources, Project administration. Hanming Cui: Conceptualization, Resources, Writing—review & editing. Yuanhui Hu: Conceptualization, Resources, Project administration, Writing—review & editing.

Abbreviations

AG, andrographolide; AS, atherosclerosis; ASCVD, atherosclerotic cardiovascular disease; BATMAN-TCM, A bioinformatics analysis tool for molecular mechanAism of traditional Chinese medicine; CAD, coronary artery disease; CTD, Comparative Toxicogenomics Database; GDA, gene-disease association; GO, Gene ontology; IL-6, interleukin-6; KEGG, Kyoto encyclopedia of genes and genomes; NAFLD, nonalcoholic fatty liver disease; OMIM, Online Mendelian Inheritance in Man; one-way ANOVA, one-way analysis of variance; OSA, overrepresentation enrichment analysis; oxLDL, oxidized low-density lipoprotein; PPI, protein–protein interaction; RCT, reverse cholesterol transport; RT-qPCR, real-time quantitative PCR; STP, Swiss target predition; TCM, traditional Chinese medicine; TNF, tumor necrosis factor; WebGestalt, WEB-based GEne SeT AnaLysis toolkit.

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