

Original Article

A promising drug combination of mangiferin and glycyrrhizic acid ameliorates disease severity of rheumatoid arthritis by reversing the disturbance of thermogenesis and energy metabolism

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ABSTRACT

Background: Activation of immune system in rheumatoid arthritis (RA) consumes amount of energy, and the energy metabolic signals may be a potential target for RA therapy. Baihu-Guizhi decoction (BHGZD) achieves satisfactory therapeutic effects in RA in clinics by recovering the adjacent articular cartilage and bone destruction, and abnormal articular temperature. However, its pharmacological material basis and molecular mechanisms have not been fully elucidated.

Purpose: This study focused on exploring the potential acting mechanism of BHGZD against RA, and identifying its main bioactive compounds (BACs) of the combination of mangiferin and glycyrrhizic acid.

Methods: Key putative targets of BHGZD acting on adjuvant-induced arthritis (AIA)-M rats were screened by the transcriptomic profiling of the whole blood cells and synovium tissues collected from rats in normal control, AIA-M model and AIA-M-BHGZD treatment groups. Then, BACs of BHGZD against RA were identified using Ultra Performance Liquid Chromatography-Mass spectrum/Mass spectrum, molecular docking, surface plasmon resonance and pharmacokinetic analysis. *In vivo* experiments based on AIA-M rats and *in vitro* experiments based on 3T3-L1 preadipocytes were performed to verify the pharmacological effects of BACs against RA and the corresponding mechanisms.

Results: PKA-ADCY5-PPAR γ -PGC 1 α -UCP1-PRDM16 signal axis was demonstrated to be the candidate targets of BHGZD against RA and was involved in maintaining the balance of thermogenesis and energy metabolism, according to the transcriptional regulatory network analysis based on “herbs-putative targets-disease interaction network”. Then, mangiferin from *Rhizoma Anemarrhenae* and glycyrrhizic acid from *Radix Glycyrrhizae* were identified as the main BACs of BHGZD against RA due to their highly accumulation in the blood *in vivo*, strong binding affinities with the two candidate targets of BHGZD against RA-ADCY5 and PPAR γ , as well as the *in vivo* and *in vitro* strong regulation effects on energy metabolism disturbance.

Abbreviations: ADCY5, Adenylate cyclase 5; AIA, Adjuvant induced arthritis; BACs, Bioactive compounds; BHGZD, Baihu-Guizhi decoction; DMARDs, Disease modifying anti-rheumatic drugs; ELISA, Enzyme-linked immunosorbent assay; ETCM, Encyclopedia of Traditional Chinese Medicine; IHC, Immunohistochemical staining; IL-17, Interleukin-17; IL-1 β , Interleukin-1 beta; IL-6, Interleukin-6; LDH, Lactate dehydrogenase; MRT, Mean retention time; MTX, Methotrexate; NCBI, National Center of Biotechnology Information; PGC-1 α , PPAR- γ coactivator 1alpha; PKA, Protein kinase A; PPAR- γ , Peroxisome proliferator activated receptor gamma; PRDM16, PR domain containing 16; RA, Rheumatoid arthritis; SDH, Succinate dehydrogenase; SPR, Surface plasmon resonance; STRING, Search Tool for Known and Predicted Protein-Protein Interactions; T_{1/2 α} , Half-life; T3, Thyroid hormone 3; T4, Thyroid hormone 4; TCM, Traditional Chinese medicine; TCMP, Integrative Pharmacology-based Research Platform of Traditional Chinese Medicine; T_{max}/C_{max}, The maximum plasma concentration; TNF- α , Tumor necrosis factor-alpha; TRH, Thyrotropin releasing hormone; TSH, Thyroid stimulating hormone; UCP1, Uncoupling protein 1; UFLC-Q-TOF-MS/MS, Ultra-fast liquid chromatography-quadrupole time of flight tandem mass spectrometry; UPLC-MS/MS, Ultra-Performance Liquid Chromatography-Mass spectrum/Mass spectrum.

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Conclusions: These findings offer evidence that the combination of mangiferin and glycyrrhizic acid from BHGZD may be a promising candidate drug for RA therapy, and also provide an important reference for the development and modernization of traditional Chinese formulae.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by hyperplasia and chronic inflammation of synovial membrane, leading to articular cartilage and bone erosion (McInnes and Schett, 2017). It affects 0.5 ~ 1.0% of the adult population worldwide, and the disease onset is higher in women (Figus et al., 2021). The etiopathogenesis of RA is partially understood, and the retrospective studies have revealed various genetic, environmental, and hormonal factors as strong inducers of RA (Alpizar-Rodriguez and Finckh, 2017). In chronic inflammatory illnesses, the activation of the immune system consumes energy, and the disturbances in energy metabolism may disrupt the immune system's homeostasis (Yang et al., 2015). Extra-articular manifestations, including abnormal energy metabolism, muscle wasting, anorexia and weight loss, impact more than half of RA patients, leading to lower total energy expenditure, higher resting energy expenditure, and poor therapeutic outcome. Changes of metabolites, which are intermediates or products of metabolism, have been frequently reported primarily in the form of serum and urine as research subjects in RA patients, and have been linked to the overproduction of various proinflammatory cytokines (Choe et al., 2014). These findings suggest that alterations in energy metabolism may be associated with malignant progression of RA and may be a promising target for the disease therapy.

Various treatments, such as symptom-targeting medications and disease-modifying anti-rheumatic drugs (DMARDs), have been extensively utilized to treat RA by preventing joint deterioration, disability, and systemic symptoms (Burmeister and Pope, 2017; Kohler et al., 2019). Although rapid control of RA activity has partially been obtained via assessing the disease status and modifying treatment frequently until the target is achieved and sustained, application of these target-specific prescriptions is difficult to manage due to deleterious side effects, such as vulnerability to severe infection or immune system hyperactivation in individual RA patients, as well as exorbitant prices (Lin et al., 2020). Therefore, it is of great clinical significance to develop safe, effective, and affordable therapeutic prescriptions to treat RA. Emerging evidence reveal that several traditional Chinese medicine (TCM)-originated prescriptions exert promising anti-RA activities, including single Chinese herb and herbal formula, as well as extracts, monomers and active constituents from traditional Chinese herbs (Wang et al., 2021; Lu et al., 2015). Among them, a TCM-originated DMARD prescription, Baihu-Guizhi decoction (BHGZD), recorded in "Synopsis of Golden Chamber", has been used for RA therapy with a satisfying therapeutic efficacy in clinics (Ma, 2016). It consists of five herbs, including *Gypsum Fibrosum* (Shigao), *Rhizoma Anemarrhenae* (Zhimu), *Ramulus Cinnamomi* (Guizhi), *Round-shaped Rice* (Jingmi) and *Radix Glycythizae* (Gancao). Our previous studies identified the chemical constituents containing in BHGZD using ultra-fast liquid chromatography-quadrupole time of flight tandem mass spectrometry (UFLC-Q-TOF-MS/MS) system, and revealed that BHGZD may ameliorate RA partially by restoring the balance of "inflammation-immune" system (Li et al., 2020; Li et al., 2021).

To further investigate the pharmacological material basis and the underlying molecular mechanisms of BHGZD in alleviating disease severity, we herein identified the main bioactive compounds (BACs) of BHGZD against RA and the corresponding candidate targets via a comprehensive strategy integrating transcriptional regulatory network analysis, molecular docking, surface plasmon resonance (SPR), pharmacokinetic analysis, and *in vivo* and *in vitro* experimental validations.

Methods and materials

Animals

Male Lewis rats ($n = 54$, 6 - 8 weeks old, 200 ± 20 g in weight) were purchased from the Experimental Animal Center, Academy of Military Medical Sciences (production license no.: SCXK 2009-0017, Beijing, China), and kept in polycarbonate cages under specific-pathogen free conditions (12h light/12h dark cycle at 25 ± 2 °C, $60 \pm 5\%$ humidity), with free access to food pellets and drinking water. Animals were acclimatized for 1w before the commence of experiments. All procedures had been performed in compliance with the ethical standards of the Center for Laboratory Animal Care, China Academy of Chinese Medical Sciences with the ethical proof No. IBTCMCACMS21-2105-01 (2021.05.04).

Grouping and treatment

A total of 54 rats were randomly divided into two independent sets: a discovery set ($n = 18$) for microarray analysis, and a validation set ($n = 36$) for experimental validations. In the discovery set, rats were randomly allocated into three groups (six per group): (1) Normal control group; (2) adjuvant induced arthritis (AIA) -wind-hot-dampness stimulation modified model group (AIA-M); (3) AIA-M-BHGZD treatment group. In the validation set, 36 rats were randomly allocated into six groups (six per group): (1) Normal control group; (2) AIA model group; (3) AIA-M model group; (4) AIA-M-BHGZD treatment group; (5) AIA-M-Mangiferin/Glycyrrhizic acid (BACs) treatment group; (6) AIA-M-methotrexate (MTX) treatment group.

According to our previous investigations, AIA rat model was induced by intradermally injection of 10 mg/ml heat-killed *M. tuberculosis* H37 Ra (Difco, BD company, New Jersey, US) suspended in liquid paraffin. Wind-hot-dampness stimulation were added to stimulate the pathological changes and characteristics of RA in AIA-M rat model, of which male Lewis rats were kept in the model box (production license No: RXZ-380A) for 2 h daily with certain wind velocity (6m/s), temperature (37 °C) and humidity (90%) for a period of 15 days from the day of primary immunization (Zhang et al., 2016). The AIA-M-BHGZD treatment group received 21.4 g/kg BHGZD intragastrically, the dosage of which had been proved to exert the most prominent therapeutic effects against RA among 0.5 (low-dose, 5.4 g/kg), 1 (middle-dose, 10.7 g/kg) and 2 (high-dose, 21.4 g/kg) times the daily RA patient dosage, presenting significantly attenuating the severity, pathological changes, as well as mechanical, cold, and heat hypersensitivities during RA progression based on the AIA rat model in our previous study (Li et al., 2020), therefore chosen for further study. An equal amount of saline was injected at the same region of the normal control group. The AIA-M-BACs treatment group was intragastrical administrated with the combination of mangiferin and glycyrrhizic acid (599.79 µg/kg for mangiferin and 1185.43 µg/kg for glycyrrhizic acid), which was comparable to the corresponding content in 21.4 g/kg BHGZD. The AIA-M-MTX treatment group was treated with 0.2 mg/kg MTX. All treatment were performed once a day, and lasted for a total of 28 days since the day of primary immunization. The preparation of BHGZD was performed as described in **Supplementary File S1-Section 1**. The mangiferin (NATURE STANDARD, CAS NO. 4773-96-0, Purity. 99.72%, Shanghai, China) and glycyrrhizic acid (NATURE STANDARD, CAS NO. 1405-86-3, Purity. 98.00%, Shanghai, China) combination was combined, dissolved by dimethylsulfoxide and then diluted into a concentration of 1 g/ml prior to use. To be noted, a concentration of

dimethylsulfoxide less than 1% of the fluid *in vivo* is appropriate for long-term treatment of rats based on our previous data (Mao et al., 2020). Normal control group, AIA group and AIA-M group both received the same volume of saline. After the rats were sacrificed on the 28 d, synovial tissues were collected, submerged in Trizol (Invitrogen, CA, USA) and immediately transferred into liquid nitrogen for microarray detection. The blood samples were collected by abdominal aortic method after 2% pentobarbital sodium and maintained at room temperature for 1h. After that, the blood was centrifuged for 15min at 3000 rpm, and the serum was kept at -80 °C until the enzyme-linked immunosorbent assay was performed (ELISA). For the western blot examination, the right ankles of rats were excised and maintained at -80 °C prior to use.

Determining energy metabolic changes in RA rats

To detect the energy metabolic changes of rats in normal control group, AIA model group, AIA-M model group and AIA-M drug treatment groups, the temperature of the articular surface, water consumption, energy metabolism indicators, including $\text{Na}^+\text{-K}^+\text{-ATPase}$, $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$, succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), as well as hormone levels, including thyroid hormone 3 (T3), thyroid hormone 4 (T4), thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH) were detected. Specifically, the temperature of the articular surface, awarded to the left hind paw of male Lewis rats, was recorded using an Infrared thermal imager (TESTO-875, Testo AG, Schwarzwald, Germany) once a day from the day when the first signs of inflammatory were observed. Three energy metabolism indicators were measured by colorimetric method, including $\text{Na}^+\text{-K}^+\text{-ATPase}$ (Lot number: A070-2-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ (Lot number: A070-1-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), SDH (Lot number: A022-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and LDH was measured using microplate method (Lot number: A020-2-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) via Unico7200 spectrophotometer (Unocal co. LTD, Shanghai, China). The hormone levels in rat sera were measured via corresponding ELISA kits, according to the manufacturer's instructions using Multiskan MK3 enzyme-labeled instrument (Thermo Fisher Scientific, Waltham, MA USA), including T3 (Lot number: KY0001, kangjia hongyuan biological technology co., LTD, Beijing, China), T4 (Lot number: KY0002, kangjia hongyuan biological technology co., LTD, Beijing, China), TSH (Lot number: KY0005, kangjia hongyuan biological technology co., LTD, Beijing, China) and TRH (Lot number: KY0021, kangjia hongyuan biological technology co., LTD, Beijing, China). Water consumption were daily measured by graduated cylinder in a 12h cycle.

Gene expression profiling and differential data analysis

To obtain RA-related genes (DEGs of AIA-M model group vs. Normal control group) and BHGZD effective genes (DEGs of BHGZD treatment group vs. AIA-M model group), gene expression profiling of whole blood cells and synovium tissues in different groups were respectively detected by RNA-seq and microarray, and significant DEGs were identified by referring to the criteria of P value < 0.05 and $|\log 2 \text{ fold change (FC)}| > 1$ as described in **Supplementary File S1-section 2**. Gene expression profiling are provided in the National Center of Biotechnology Information (NCBI) Gene Expression Omnibus.

Network construction and analysis

Chemical compounds in BHGZD were collected from Encyclopedia of Traditional Chinese Medicine (ETCM, <http://www.tcmip.cn/ETCM/index.php/Home/>) and identified by UFLC-Q-TOF-MS/MS in our previous study (Li et al., 2020; Xu et al., 2019). The corresponding putative targets were predicted based on the structural similarity of

chemical compounds contained in BHGZD to the FDA-approved drugs using the Integrative Pharmacology-based Research Platform of Traditional Chinese Medicine (TCMIP v2.0 <http://www.tcmip.cn/TCMIP/index.php>) (Xu et al., 2017). The prediction results with the similarity score more than 0.8 (moderate-high similarity) were collected for the following analysis. Then, the “disease gene-drug target” interaction network was constructed using the links among RA-related genes, BHGZD putative targets and BHGZD effective genes. The gene-gene interaction data were collected from the public database STRING (Search Tool for Known and Predicted Protein-Protein Interactions, version 10.0, <http://string-db.org/>) and the interaction networks were all visualized by Navigator software (Version 2.2.1) (Szkarczyk et al., 2015). The topological importance of the nodes in the networks were assessed for screening putative targets by calculating node's degree, betweenness and closeness, as described in **Supplementary File S1-section 3**.

Molecular docking stimulation

The direct binding efficiencies of mangiferin and glycyrrhizic acid with the corresponding putative targets were assessed through virtual screening using AutoDock VINA and LeDOCK software. Proteins for AutoDock VINA were prepared as follows. Crystal structures of the target proteins were proceeded using PYMOL (<http://www.pymol.org/>) to remove the water molecules, heteroatoms, ions and original ligands. Ligand binding site was defined in the crystal structure using the reference ligand, the geometry center of which was set as the center of the enclosing box ($60 \text{ \AA} \times 60 \text{ \AA} \times 60 \text{ \AA}$) in PYMOL. Then, polar hydrogen atoms and Gasteiger partial charge were added to the protein in Vina. In terms of LeDock, protein preparation process is more facile, for which the removal of the water molecules, heteroatoms, ions, and additions of hydrogens were conducted automatically by running Lepro. The size of generated box was set at $60 \text{ \AA} \times 60 \text{ \AA} \times 60 \text{ \AA}$. Open Babel was used for transforming various chemical formats of ligands and PYMOL from DeLano Scientific for inspecting docking results visually and presenting images. After that, molecular docking was performed, and docking scores higher than 4 representing strong binding affinity between the ligand and receptor in general were selected (Chen et al., 2017).

SPR detection

SPR detection was performed to confirm the binding affinities of the combination of mangiferin and glycyrrhizic acid with corresponding putative targets obtained by molecular docking using a Biacore 8K instrument (BIAcore 8K, GE, Sweden), and the results were analyzed using the Biacore 8K evaluation software 2.0 (GE Healthcare) for calculating the related K_D values. Detailed information on the protocols of SPR detection is provided in **Supplementary File S1-section 4**.

Pharmacokinetic analysis

To detect the pharmacokinetic parameters of mangiferin and glycyrrhizic acid *in vivo*, quantification analysis was performed by an ultra-high-performance liquid chromatography (Shimadzu Corp., Japan) tandem ion trap quadrupole QTRAP 6500 plus mass spectrometry (AB Sciex, USA), which have been approved by the Guidance for Bio-analytical Method Validation issued by Chinese Pharmacopoeia Commission in 2020. Corresponding pharmacokinetic parameters were examined using Drug and Statistic software (Shanghai, China). The detailed information of the collection of blood samples and UHPLC-QTRAP-MS/MS conditions were provided in **Supplementary File S1-section 5**.

3T3-L1 cell culture and differentiation induction

3T3-L1 preadipocytes were purchased from the Cell Bank of Type

Culture Collection of Chinese Academy of Sciences. Cells were cultured and differentiated into adipocytes using classical transformation cocktail named MDI induction, which consisted of insulin (1 mg/ml; Sigma-Aldrich, St. Louis, MO), isobutyl-1-methylxanthine (500mM; Sigma-Aldrich, St. Louis, MO), and dexamethasone (0.25mM; Sigma-Aldrich, St. Louis, MO), and Oil Red O staining were performed according to the routine protocol (Guzman-Ruiz et al., 2020). The detailed information of 3T3-L1 cell culture and differentiation induction was described in **Supplementary File S1-section 6**. BHGZD (2.0 µg/ml), the combination of mangiferin and glycyrrhizic acid (0.056 ng/ml mangiferin and 0.111 ng/ml glycyrrhizic acid, as the same content of that in 2.0 µg/ml BHGZD), and forskolin (20 µM) were added into the differentiation medium on the 2th d of induction. Oil red O staining was performed to observe the lipid droplets intuitively, as described in **Supplementary File S1-section 7**. Total proteins were prepared for western blot analysis. The concentration of dimethylsulfoxide was less than 1% of the solution *in vitro*.

Western blot analysis

To investigate the regulatory effects of BHGZD and the combination of mangiferin and glycyrrhizic acid on the expression levels of the candidate targets that includes adenylate cyclase 5(ADCY5), peroxisome proliferator activated receptor gamma (PPAR-γ), PPAR-γ coactivator 1α (PGC-1α), uncoupling protein 1 (UCP1) and PR domain containing 16 (PRDM16) in both arthritis tissue samples of different AIA-M rats and 3T3-L1 cells. Western blot analysis was performed as described previously (Mao et al., 2020). Detailed information of antibodies is provided in **Supplementary Table S1**.

Immunohistochemical staining (IHC)

To detect the changes of nuclear translocation for nuclear factors PPAR-γ in the ankles of rats in the AIA-M and corresponding treatment groups, an immunohistochemical method was applied using the DAB Kit (Cat No.: ZLI-9018; ZSGB-BIO, Zhongshan Jinqiao Biotechnology, Beijing, China) and rabbit two-step detection kit (Cat No.: PV-9001; ZSGB-BIO, Zhongshan Jinqiao Biotechnology, Beijing, China) according to the routine protocol. The immunoreactive score was obtained by checking three different representative areas of the field of vision in a semi-quantitative manner as described in **Supplementary File S1-section 8**.

Determining the activity of protein kinase A (PKA) in tissues and cellular supernatant

PKA activity in tissues (Lot number: ml037149, Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) and cellular supernatant (Lot number: ml063211, Shanghai Enzyme-linked Biotechnology Co., Ltd.) were detected using ELISA kit. The manufacturer's instructions were strictly followed and the developed color was measured spectrophotometrically at 450nm.

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 6 (GraphPad, San Diego, CA, USA). The parametric data were presented as mean ± SD, and analyzed by one-way ANOVA followed by LSD test. All experiments were performed in triplicate. Differences were considered to be statistically significant when the value of P was less than 0.05.

Results

Key putative targets of BHGZD acting on AIA-M rats

It has been proved in our previous study that BHGZD may exert a

significant therapeutic effect in AIA-M rats by improving the severity of arthritis via reducing the disease incidence, the mean arthritis score, the diameter of the limb and the articular temperature of rats (Li et al., 2020). In the current study, the key putative targets and the underlying mechanisms of BHGZD against RA were further elucidated. The transcriptomic profiling of the whole blood cells and synovium tissues collected from rats in normal control, AIA-M model and AIA-M-BHGZD treatment groups were detected and uploaded via GEO system (GEO accession number: GSE189942; GSE190523). A total of 484 AIA-M related genes (AIA-M vs. normal control, 304 upregulated and 180 downregulated genes) and 1878 BHGZD effective genes (BHGZD treatment group vs. AIA-M model group, 476 upregulated and 1402 downregulated genes) were identified. Then, 635 putative targets hit chemical components contained BHGZD were predicted as described in our previous studies^[12,13]. According to the links among AIA-M related genes, BHGZD effective genes and BHGZD putative targets, the “disease gene-drug target” interaction network was constructed and the nodes' topological features were calculated. After that, 55 major nodes were identified because the Degree, Node betweenness and Closeness values of them in the network were higher than the corresponding median values (**Supplementary Table S2**).

Functionally, the above major nodes were significantly associated with energy metabolism and immune system-related pathways, such as PKA activation in glucagon signaling, PPAR signaling pathway, cAMP signaling pathway, white adipocyte differentiation, regulation of lipolysis in adipocytes, glycolysis, gluconeogenesis, and *etc.* according to the pathway enrichment analysis based on KEGG database (Figs. 1 and 2A). Especially, three key BHGZD effective genes (PKA, ADCY5, PPARγ) with highly topological importance were defined as the BHGZD candidate targets against RA. All of them were upregulated in AIA-M rats compared to normal control rats, while downregulated by BHGZD treatment based on the transcriptomic profiling. Of note, PKA, ADCY5, PPARγ, and their interaction partners (PGC 1α, UCP1 and PRDM16) formed a signal axis which may play an essential role in the malignant progression of RA (Aoh et al., 2013; Liu et al., 2018; Liu et al., 2013; Wong et al., 2020; Yang et al., 2021; Ho et al., 2015; Khalafi et al., 2020; Kim et al., 2021; Li et al. 2017; Shahin et al., 2011).

Mangiferin and glycyrrhizic acid may be the main BACs of BHGZD against RA

To screen the main BACs of BHGZD against RA, a comprehensive analysis of UPLC-MS/MS, molecular docking, SPR detection and pharmacokinetic analysis were performed. In our previous studies, a total of 32 chemical compounds contained in BHGZD were identified in the blood of Lewis rats after drug administration using UPLC-MS/MS (Li et al., 2020). Network analysis based on the “disease gene-drug target” interaction network indicated that those chemical compounds contained in *Radix Glycythizae* (citric acid, formononetin, glabrolide, glycyrrhizic acid, isoliquiritigenin, isoliquiritin, isoschaftoside, liquiritigenin, neoisoliquiritin, schaftoside), and those in *Rhizoma Anemarrhenae* (anemarsaponin E, mangiferin, timosaponin BII, Xilingsaponin B, isovitexin, vitexin) directly targeted multiple putative targets, that were significantly involved in energy metabolism and immune system-related pathways, including ABCB1, ABCG2, ACO2, ADCY2, ADCY5, AKR1B1, AKR1C3, AKT1, ALB, ALDH2, ALOX15, ALOX5, CEBPB, CFTR, CS, CYP11A1, CYP19A1, CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP3A4, GCK, GNAS, HK1, HSD11B1, IDH1, NCOA1, NCOA2, PPARA, PPARG, PPP1CC, PTGS1, PTGS2, SLC2A1, SLC01A2, SLC01B1 and SRC (Figs. 1 and 2A). Then, 13 chemical compounds contained in BHGZD were pre-screened under a comprehensive consideration of their plasma concentration and regulation of energy metabolism and immune system (Li et al., 2022) (**Supplementary Table S3**).

Molecular docking simulation was performed to evaluate the binding affinities of these chemical compounds contained in BHGZD to key

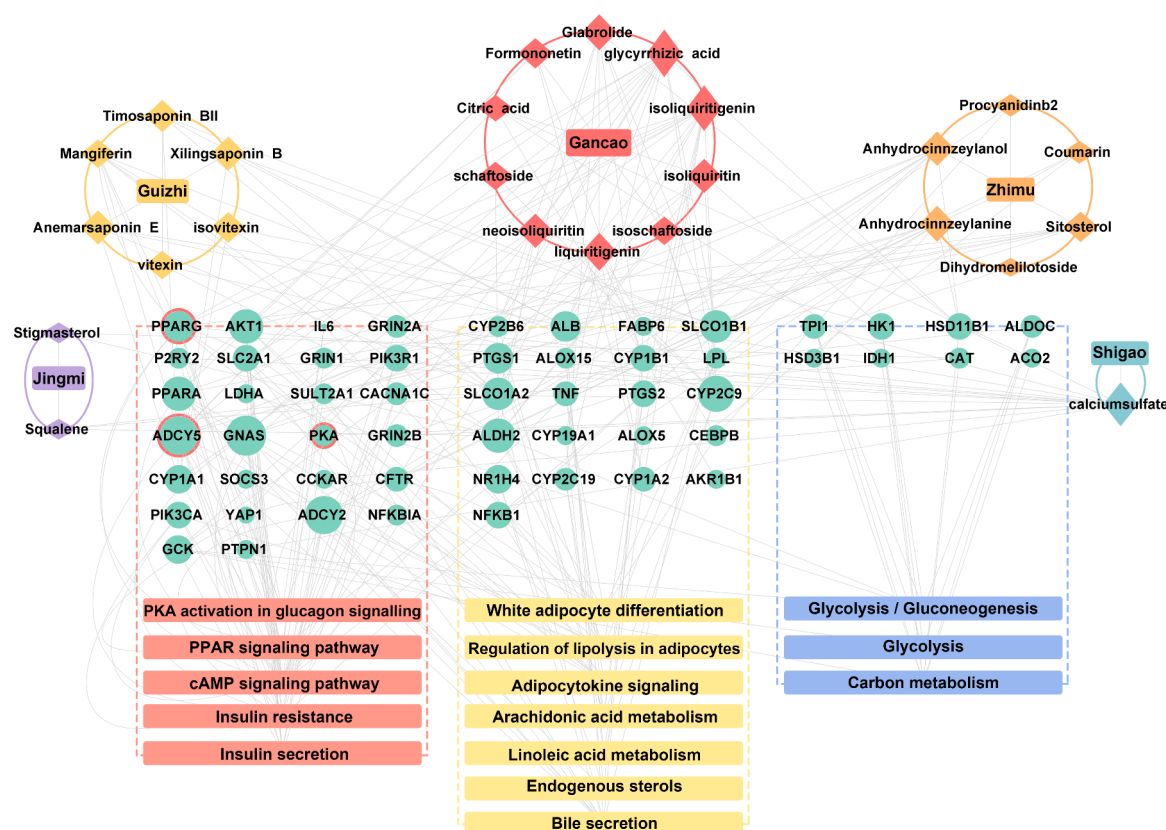


Fig. 1. Network based on the direct interactions among chemical compounds of five herbs contained in BHGZD (*Gypsum Fibrosum*, *Rhizoma Anemarrhenae*, *Ramulus Cinnamomi*, *Round-shaped Rice* and *Radix Glycythizae*), BHGZD effective genes, RA-related genes and the corresponding pathways (herb-BHGZD effective gene-RA-related gene-pathway network). Rhombus nodes refer to the chemical compounds of five herbs contained in BHGZD; Circular nodes refer to the BHGZD effective genes; Circular nodes cycled with red refer to the key candidate genes of BHGZD against RA. The size of the nodes were ordered according to their degree in the network.

proteins ADCY5 and PPAR- γ (7AWC) in PKA-ADCY5-PPAR γ -PGC 1 α -UCP1-PRDM16 signal axis using AUTODOCK VINA and LEDOCK, further validated by SPR detection. The concentration of mangiferin from *Rhizoma Anemarrhenae* and glycyrrhizic acid from *Radix Glycythizae* were 8535.02 ng/ml and 2253.26 ng/ml at 12 h after drug administration, respectively, that is the highest among those chemical compounds. Mangiferin were found to bind with PPAR γ with docking score -5.56, and SPR detection verified the strong binding affinity of mangiferin with both ADCY5 and PPAR γ proteins with a K_D value around 10^{-5} μ M. Similarly, glycyrrhizic acid also binds with ADCY5 protein efficiently (Fig. 2B, D, F, G, H, I). However, the docking score of mangiferin-ADCY5 pair and glycyrrhizic acid-ADCY5 pair were not obtained due to the lack of crystal structure of active ADCY5 protein from human-derived X-ray diffraction. Quantification analysis of the content of mangiferin and glycyrrhizic acid were 0.337 μ g and 0.666 μ g in 1mg of BHGZD, respectively, with the conversion ratio of 8.3%, calculated by the weight of BHGZD lyophilized powder divided by the weight of crude herbs in BHGZD. Therefore, a two-BAC-combination consisting of 599.79 μ g/kg mangiferin and 1185.43 μ g/kg glycyrrhizic acid were formed, which were equivalent to the dosage administration of 21.4 g/kg BHGZD.

UHPLC-QTRAP-MS/MS system was applied to explore the pharmacokinetic properties of mangiferin and glycyrrhizic acid in the serum of rats after BHGZD administration. A non-compartment model analysis was used for analyzing several pharmacokinetic parameters, which were presented in (Li et al., 2022) Supplementary Table S4 for glycyrrhizic acid, and in our previous studies for mangiferin. Moreover, the mean plasma concentration-time profiles of mangiferin and glycyrrhizic acid from time 0 to 24 h after 21.4 g/kg BHGZD treatment was shown in

Fig. 2C, E. After the treatment of BHGZD, the maximum plasma concentration (C_{max}) of mangiferin and glycyrrhizic acid were 132.01 μ g/l and 46.46 μ g/l, respectively, and the time to reach the maximum plasma concentration (T_{max}) for those were 4.00 h and 4.46h, respectively. The plasma concentration-time curve AUC (0 - 24h) of mangiferin and glycyrrhizic acid were 578.42 μ g/l•h and 255.15 μ g/l•h, respectively, and the half-life ($T_{1/2}$) and mean retention time (MRT) of mangiferin (1.26h and 4.23h, respectively) and glycyrrhizic acid (2.16 h and 5.67 h, respectively) were also measured, all of which indicated the good pharmacokinetic properties of mangiferin and glycyrrhizic acid contained in BHGZD.

Mangiferin and glycyrrhizic acid combination alleviates RA disease severity and energy metabolic disorders by regulating the PKA-ADCY5-PPAR γ -PGC1 α -UCP1-PRDM16 signal axis

To further verify the pharmacological effects of the two-BAC-combination of mangiferin and glycyrrhizic acid against AIA-M, the *in vivo* and *in vitro* experiments were performed. Our previous studies revealed that 21.4 g/kg BHGZD significantly alleviated disease severity of arthritis in AIA-M rats by reducing the arthritis score and the diameter of limbs, the levels of inflammatory cytokines that includes interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and interleukin-17 (IL-17), the thymus and spleen indexes, and simultaneously elevated the body weight and the pain thresholds (mechanical, thermal and acetone-induced hyperalgesia) (Li et al., 2020). It also apparently improved histopathological alterations of ankle joints in AIA-M rats by promoting the proliferation and maturation vascular granulation tissues, preventing bone destruction, cartilage degeneration

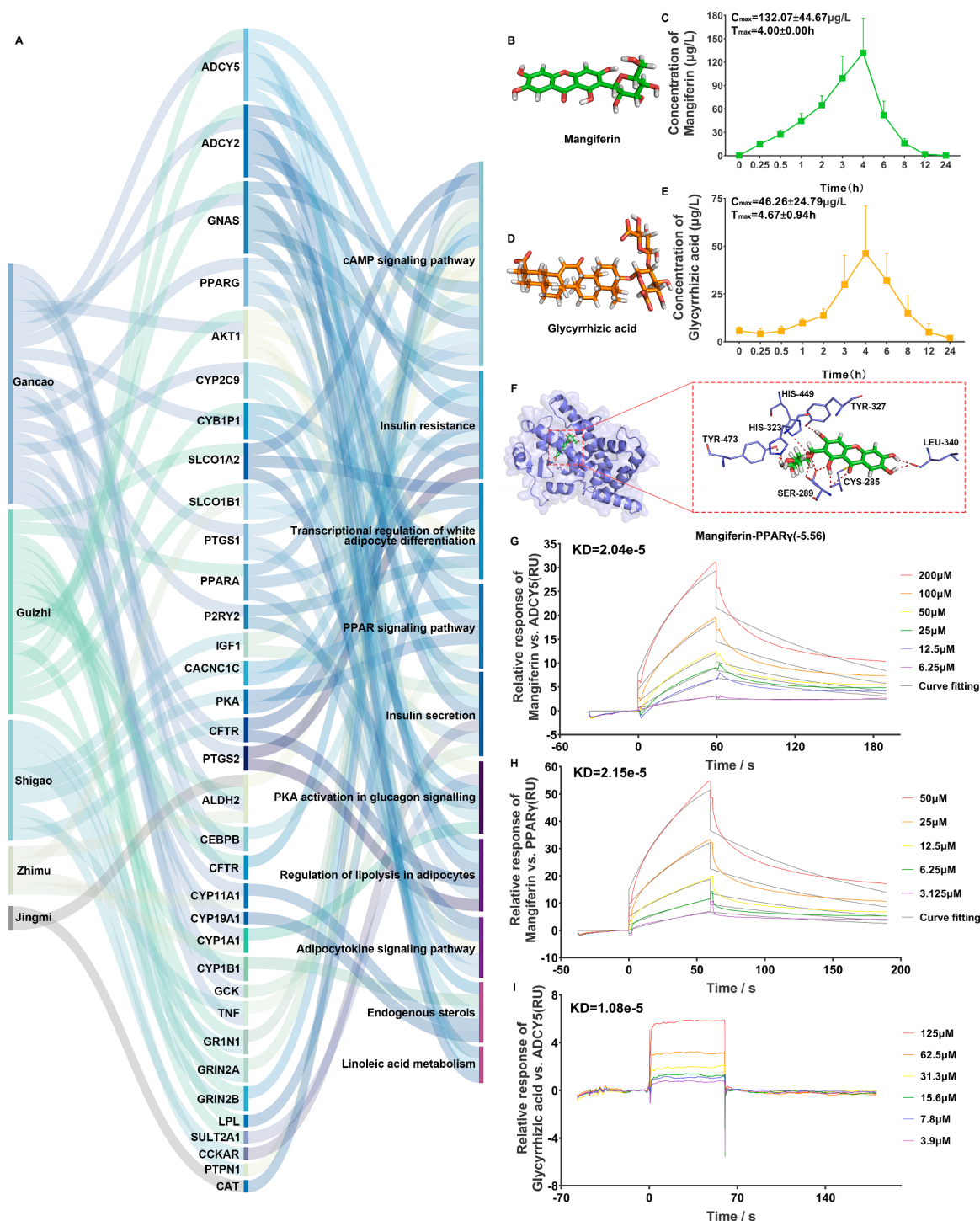


Fig. 2. Sankey diagram of five herbs contained in BHGZD (*Gypsum Fibrosum*, *Rhizoma Anemarrhenae*, *Ramulus Cinnamomi*, *Round-shaped Rice* and *Radix Glycythizae*), BHGZD effective genes, RA-related genes and the corresponding pathways, the chemical structures of mangiferin and glycyrrhizic acid, their molecular docking simulation and SPR analysis with PPAR γ and ADCY5 proteins, as well as the mean plasma concentration-time profiles from time 0 to 24 h after 21.4 g/kg BHGZD treatment. (A) Sankey diagram of five herbs contained in BHGZD (*Gypsum Fibrosum*, *Rhizoma Anemarrhenae*, *Ramulus Cinnamomi*, *Round-shaped Rice* and *Radix Glycythizae*), BHGZD effective genes, RA-related genes and the corresponding pathways. Longer lines represent more importance of genes and pathways in BHGZD alleviating RA. (B) The chemical structures of mangiferin downloaded from Pubchem website (<https://pubchem.ncbi.nlm.nih.gov/>). (C) The mean plasma concentration-time profiles of mangiferin from time 0 to 24 h after 21.4 g/kg BHGZD treatment. (D) The chemical structures of glycyrrhizic acid downloaded from Pubchem website (<https://pubchem.ncbi.nlm.nih.gov/>). (E) The mean plasma concentration-time profiles of glycyrrhizic acid from time 0 to 24 h after 21.4 g/kg BHGZD treatment. (F) The molecular docking simulation of the binding pattern of mangiferin with PPAR γ . PPAR γ are presented with cartoon and bioactive compound are presented with green sticks. Red dotted lines refer to hydrogen bond interactions. White sticks refer to amino acid residues. (G) The surface plasmon resonance (SPR) curves of the interaction of mangiferin with PPAR γ . (H) The SPR curves of the interaction of mangiferin with ADCY5. (I) The SPR curves of the interaction of glycyrrhizic acid with ADCY5. All binding affinity values are determined according to three parallel experiments.

and decreasing inflammatory cell infiltration (Li et al., 2020). Herein, we found serious redness and swelling of joints in AIA and AIA-M groups, of which the latter is more serious, and both were markedly improved by the administration of the two-BAC-combination of mangiferin and glycyrrhizic acid (equivalent to BHGZD at the dosage of 21.4 g/kg) and similar to the pharmacological effects of BHGZD and positive drug MTX (Fig. 3A~B). In terms of key indicators reflecting the energy metabolic changes, compared with normal control group, rats in AIA and AIA-M group showed higher energy metabolism levels, and more obvious in AIA-M group. The administration of the two-BAC-combination, BHGZD and MTX significantly recovered the abnormal upregulated articular temperature and decreasing water consumption, as well as regulated the abnormal alterations of energy metabolism parameters ($\text{Na}^+\text{-K}^+\text{-ATPase}$, $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$, SDH, LDH) and hormones (T3, T4, TRH, TSH) of AIA-M rats (Fig. 4A~K).

Furthermore, the regulatory effects of the two-BAC-combination and BHGZD on the PKA-ADCY5-PPAR γ -PGC 1 α -UCP1-PRDM16 signal axis were detected using ELISA, western blot analysis and IHC based on the inflamed joints of AIA-M rats. The expression levels of PKA, ADCY5, PPAR γ , PGC 1 α , UCP1 and PRDM16 were significantly increased in AIA-M group compared to the normal control group, and both the two-BAC-combination and BHGZD significantly reversed the upregulation of these proteins, the effects of which were similar to that of MTX (Fig. 5A~H).

In addition, both of the two-BAC-combination and BHGZD effectively inhibited the biological process of adipocyte differentiation by increasing the account of lipid droplets in 3T3-L1 cells. ELISA and western blot analyses revealed that abnormal changes of corresponding proteins in PKA-ADCY5-PPAR γ -PGC 1 α -UCP1-PRDM16 signal axis in 3T3-L1 cells can be obviously reversed by the two-BAC-combination

(0.056 ng/ml mangiferin and 0.111 ng/ml glycyrrhizic acid, equivalent to the corresponding content of 2.0 $\mu\text{g/ml}$ BHGZD) and 2.0 $\mu\text{g/ml}$ BHGZD treatment, similar to that of MTX, which was in consistent with our *in vivo* results. However, the regulatory effects of single mangiferin or glycyrrhizic acid on morphological changes and protein expression levels were not as obvious as that of two-BAC-combination and BHGZD. To further detect the role of upstream gene PKA, 20 μM forskolin, a PKA agonist was added to BHGZD treatment group. It has been revealed that inhibitory effects of BHGZD on lipid droplets accumulation was weakened after forskolin intervention, and the same decreasing trend was observed on its regulation of PKA-ADCY5-PPAR γ -PGC 1 α -UCP1-PRDM16 signal axis, suggesting that both the two-BAC-combination and BHGZD inhibited adipogenesis and lipogenesis by regulating the corresponding target proteins in PKA-ADCY5-PPAR γ -PGC 1 α -UCP1-PRDM16 signal axis (Fig. 6A~H).

Discussion

RA patients exhibit the enhanced resting expenditure and wasting of fat-free mass, with elevated anaerobic catabolism and reduced aerobic oxidation (Roubenoff et al., 1994). Changes in energy metabolism of RA patients can be attributed partially to chronic inflammation driven by excessive secretion of several proinflammatory cytokines. In this regard, the activation of immune system consumes more than 2,000 kJ energy per day in rheumatic and other chronic inflammatory diseases, and serve as the background machinery, that is, multilevel interactions exist between immunology and metabolism, and play an essential role in the pathophysiology of RA (Buttgereit et al., 2000; Spies et al., 2012). The application of TCM-originated prescriptions has been prevalent for thousands of years in RA treatment, with the benefits of whole-body

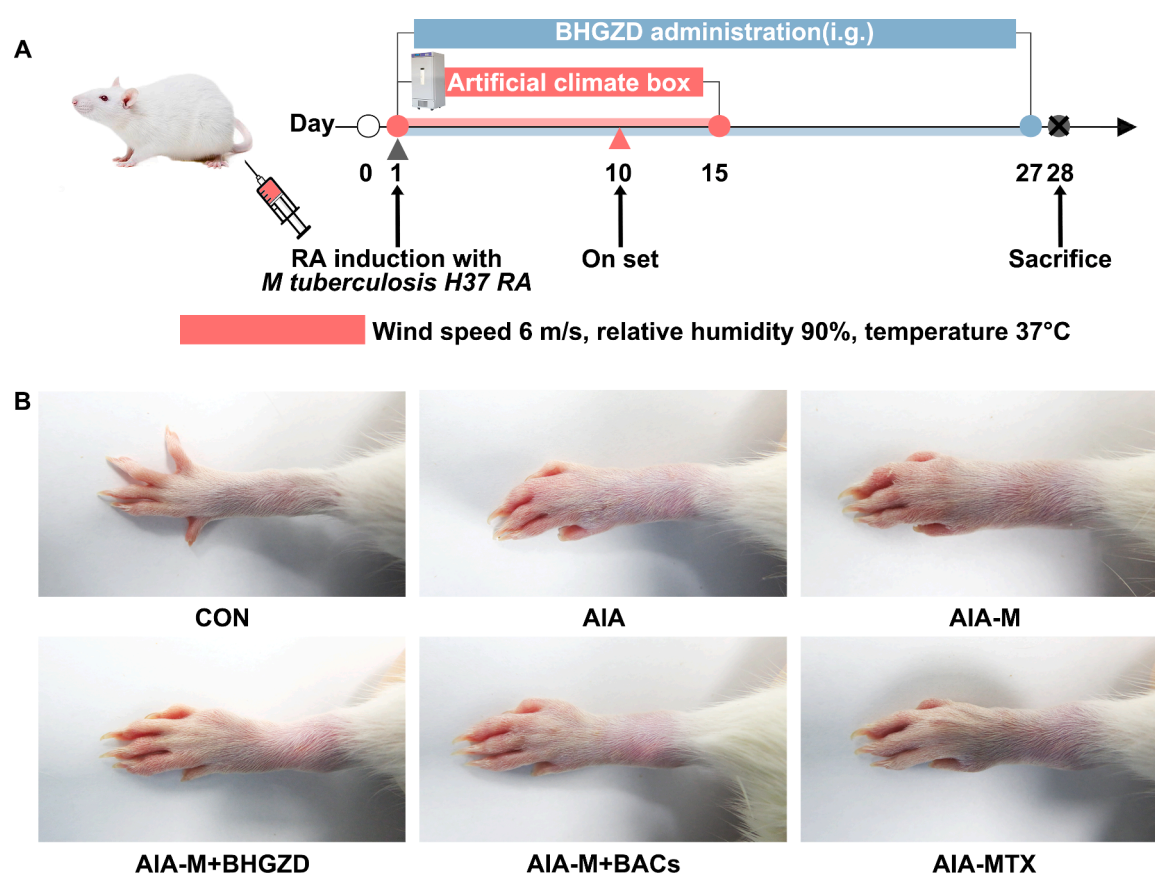


Fig. 3. Alleviating effects of BHGZD, two-BAC-combination and MTX on the severity of arthritis in AIA and AIA-M rats. (A) Induction protocol of AIA and AIA-M in Lewis rats and drug treatment during experiments. (B) Macroscopic evidence of arthritis in different groups. [normal control group ($n = 6$); AIA model group ($n = 6$); AIA-M model group ($n = 6$); AIA-M-BHGZD treatment group ($n = 6$); AIA-M-BACs treatment group ($n = 6$); AIA-M-MTX treatment group ($n = 6$)].

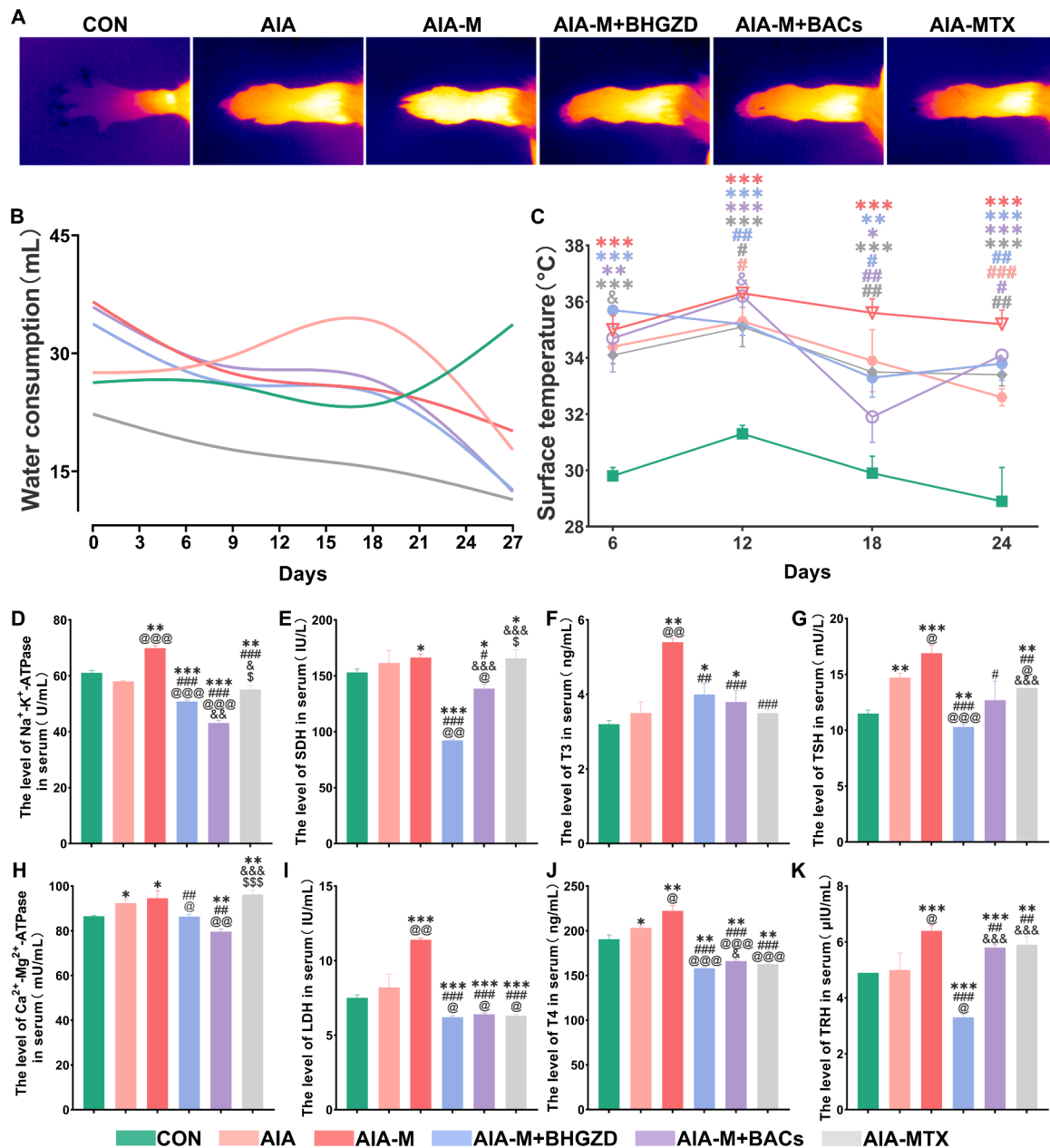


Fig. 4. Alleviating effects of BHGZD, two-BAC-combination and MTX on the indicators for thermogenesis and energy metabolism in AIA-M rats in terms of articular temperature, the levels of $\text{Na}^+\text{-K}^+\text{-ATPase}$, $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$, SDH, LDH, T3, T4, TRH, TSH and daily water consumption. (A) Infrared thermography. (B) Daily water consumption. (C) Articular temperature. (D) $\text{Na}^+\text{-K}^+\text{-ATPase}$. (E) SDH. (F) T3. (G) TSH. (H) $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$. (I) LDH. (J) T4. (K) TRH. [normal control group ($n = 6$); AIA-M model group ($n = 6$); AIA-M-BHGZD treatment group ($n = 6$); AIA-M-BACs treatment group ($n = 6$); AIA-M-MTX treatment group ($n = 6$)]. Data are expressed as the mean $\pm$ S.D. ‘*’, ‘***’, and ‘***’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the normal control group. ‘@’, ‘@@’, and ‘@@@’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the AIA-M model group. ‘#’, ‘##’, and ‘###’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the AIA-M-BHGZD treatment group. ‘\$’, ‘\$\$’, and ‘\$\$\$’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the AIA-M-BACs treatment group.

adjustment, safety and lower expenditure. Among them, BHGZD has been proved to achieve satisfactory therapeutic effects in clinics in the treatment of active RA by recovering the imbalance in immune system and energy metabolism (Li et al., 2020).

In the current study, following the transcriptomic profiling and network calculation, a list of major nodes of “disease gene-drug target” interaction network were identified and demonstrated to be significantly enriched in energy metabolism and immune system related pathways, including PKA activation in glucagon signaling, PPAR signaling pathway, cAMP signaling pathway, white adipocyte differentiation, regulation of lipolysis in adipocytes, glycolysis, gluconeogenesis,

and etc, which was in line with the *in vivo* studies showing that 21.4 g/kg BHGZD obviously recovered the abnormal changes of indicators in AIA-M model rats that reflect thermogenesis and energy metabolism disturbance, including articular temperature, daily water consumption, as well as energy metabolism parameters ($\text{Na}^+\text{-K}^+\text{-ATPase}$, $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$, SDH, LDH) and hormones (T3, T4, TRH, TSH).

Moreover, the PKA-ADCY5-PPAR γ -PGC1 α -UCP1-PRDM16 signal axis was identified to be one of the therapeutic targets of BHGZD against RA. In this axis, cyclic adenosine monophosphate PKA, the primary effector of the glucose-induced developmental program, has been indicated to participate in the transient stoppage of endosomal traffic during

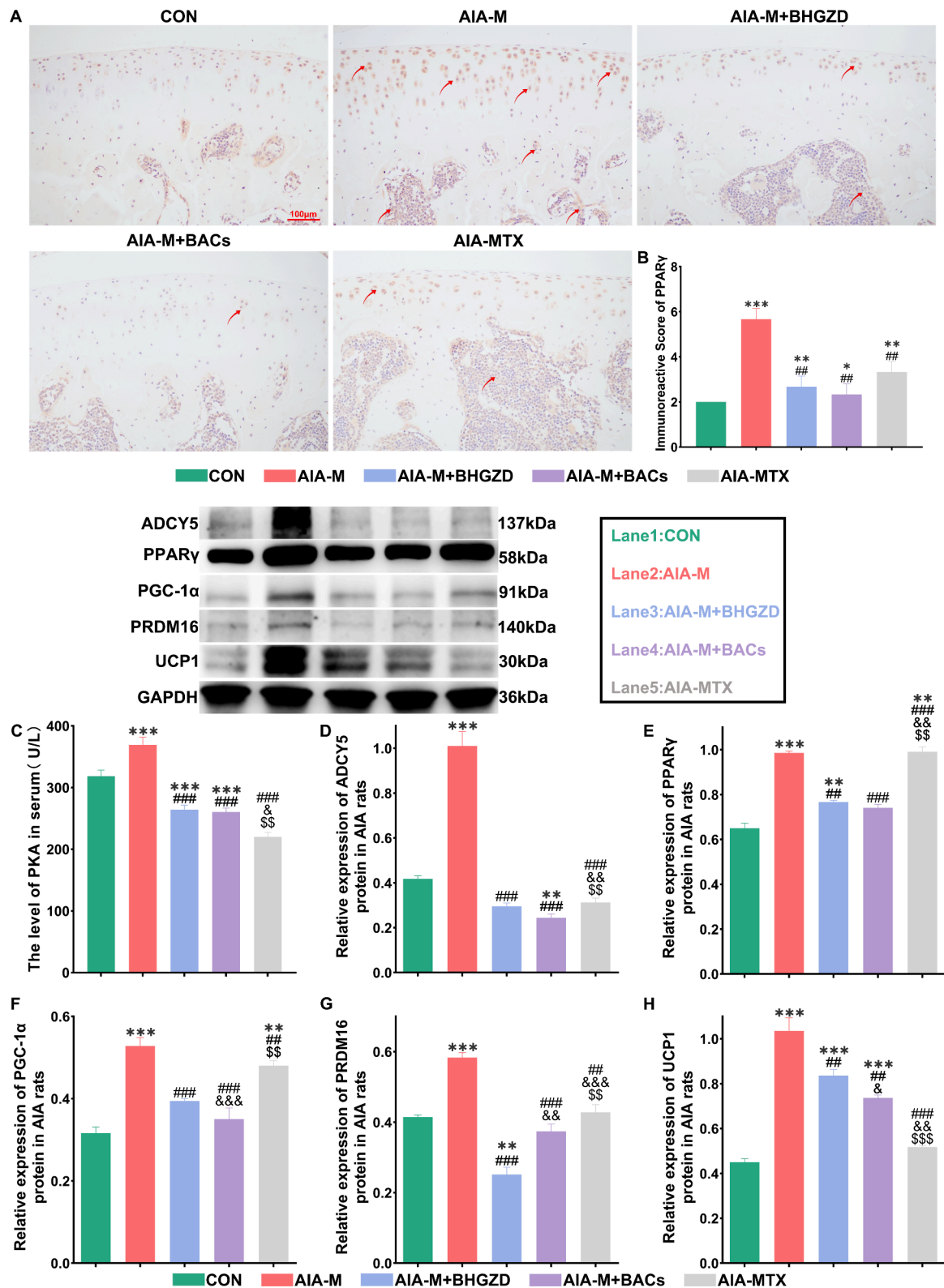
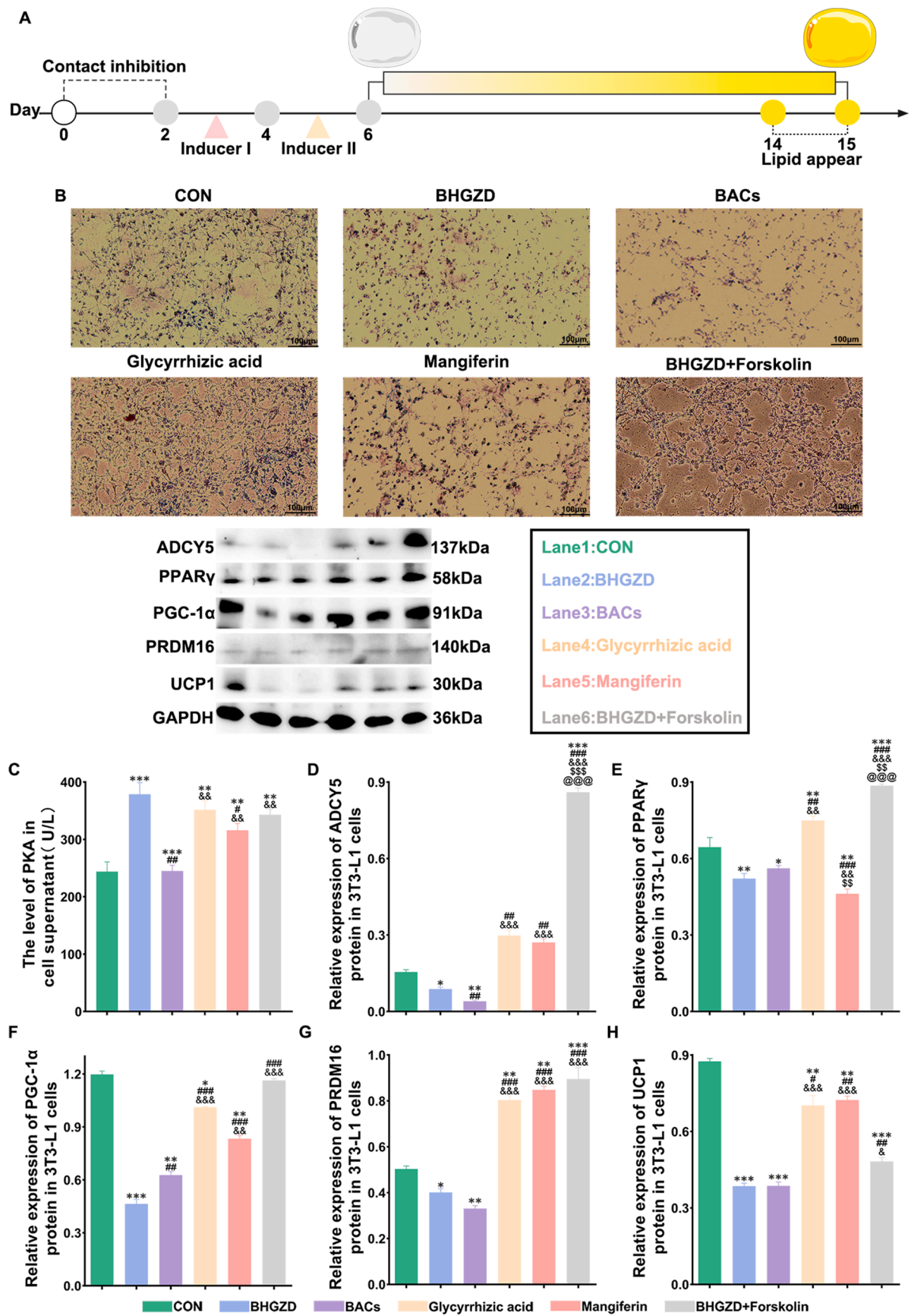


Fig. 5. Regulatory effects of BHGZD, two-BAC-combination and MTX on the expression of corresponding proteins in PKA-ADCY5-PPAR γ -PGC1 α -UCP1-PRDM16 signal axis in the joints of AIA-M rats. (A) The expression and cellular distributions of PPAR- γ in the joints of rats in different groups detected by immunohistochemical assay ($\times 20$), and red arrows indicated the expression intensity of PPAR- γ in chondrocytes of rat ankles. (B) The value of immunoreactive score of PPAR- γ in the joints of rats in different groups. (C) The level of PKA in serum of rats in different groups detected by ELISA. (D~H). The expression levels of ADCY5, PPAR γ , PGC1 α , UCP1 and PRDM16 in affected joints of rats in different groups detected by western blot. Data are expressed as the mean $\pm$ S.D. ‘*’, ‘**’, and ‘***’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the normal control group. ‘@’, ‘@@’, and ‘@@@’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the AIA-model group. ‘#’, ‘##’, and ‘###’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the AIA-M model group. ‘&’, ‘&&’, and ‘&&&’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the AIA-M-BHGZD treatment group. ‘\$’, ‘\$\$’, and ‘\$\$\$’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the AIA-M-BACs treatment group.



(caption on next page)

Fig. 6. Effects of BHGZD, two-BAC-combination, mangiferin, glycyrrhizic acid and BHGZD/forskolin on the promotion of adipogenesis in 3T3-L1 adipocyte and thermogenesis-related proteins. (A) The induction protocol of adipogenesis in 3T3-L1 adipocyte by MDI approach and corresponding drug intervene during experiments. (B) Oil Red O staining of adipocyte differentiation in 3T3-L1 adipocytes treated by BHGZD, two-BAC-combination, mangiferin, glycyrrhizic acid and BHGZD/forskolin ($\times 4$). (C) Regulatory effects of BHGZD, two-BAC-combination, mangiferin, glycyrrhizic acid and BHGZD/forskolin on PKA level in 3T3-L1 adipocyte detected by ELISA. (D–H) Regulatory effects of BHGZD, two-BAC-combination, mangiferin, glycyrrhizic acid and BHGZD/forskolin on the expression level of ADCY5, PPAR γ , PGC1 α , UCP1 and PRDM16 proteins in 3T3-L1 adipocyte detected by western blot. Data are expressed as the mean $\pm$ S.D. ‘*’, ‘**’, and ‘***’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the normal control group. ‘#’, ‘##’, and ‘###’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the BHGZD treatment group. ‘&’, ‘&&’, and ‘&&&’, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, comparison with the BACs treatment group.

glucose starvation, subsequently to regulate both peripheral and central energy homeostasis (Aoh et al., 2013). It is mediated by the transcription factor cAMP responsive element binding protein, which is highly expressed in white adipose tissues and affects brown fat function by upregulating PGC 1 α , UCP1 and PRDM16 expression (Liu et al., 2018). The activation of PKA may promote osteoblast differentiation and angiogenesis via increasing protein stability in RA, accompanied with facilitating Th17 differentiation and suppressing Treg differentiation (Liu et al., 2013; Wong et al., 2020). It has been reported that PKA-ADCY5 pair can mediate lipolysis and fueling support for osteoblasts, bridging between fat and bone (Yang et al., 2021). ADCY5 belongs to the adenylyl cyclase families, and functions in catalyzing the conversion of ATP into the second messenger cAMP. Knockdown of ADCY5 improves glucose tolerance and insulin sensitivity, manifested with body weight loss in rats fed with standard and high-fat diet (Ho et al., 2015). PPAR γ , a well-known master transcriptional regulator in brown adipogenesis, is up-regulated in brown adipocytes maturation. PRDM16 also acts as a master regulator that induces a complete program of differentiating brown fat through the activation of thermogenic related genes PGC 1 α and UCP1. In particular, UCP1 regulates diet-induced thermogenesis, the deletion of which induced increasing weight gain in animals. Similarly, PGC 1 α mainly regulate mitochondrial biogenesis and serve as a primary activator of PPAR γ , which is regulated by calorie restriction and exercise training. Its overexpression enhances numerous mitochondrial genes and thermogenic genes in white fat cells (Khalafi et al., 2020; Kim et al., 2021). Interestingly, the latest achievement obviously reported that PPAR γ may contribute to the persistent expression of pro-inflammatory cytokines in RA, and play an essential role in fibroblast-like synoviocytes proliferation and migration (Li et al. 2017). Its agonist pioglitazone appears to be a promising therapeutic strategy for RA patients (Shahin et al., 2011). These findings suggest that the PKA-ADCY5-PPAR γ -PGC1 α -UCP1-PRDM16 signal axis might be a promising therapeutic target of RA disease.

Accumulating studies indicated that only a few BACs contained in herbs or herbal formulae play pivotal roles in disease treatment (Xu et al., 2016). Thus, the identification of BACs from TCM herbs or herbal formulae may benefit new drug discovery. Herein, mangiferin from *Rhizoma Anemarrhenae* and glycyrrhizic acid from *Radix Glycythizae* were identified to be the main BACs of BHGZD against RA for the following reasons: (1) Both mangiferin and glycyrrhizic acid were demonstrated to be high accumulated in the blood of Lewis rats according to UPLC-MS/MS *in vivo*. (2) Mangiferin were found to have a strong binding affinity with ADCY5 and PPAR γ (key proteins in PKA-ADCY5-PPAR γ -PGC1 α -UCP1-PRDM16 signal axis), and glycyrrhizic acid binds with ADCY5 protein efficiently determined by molecular docking and SPR analysis. (3) Mangiferin and glycyrrhizic acid displayed good pharmacokinetic properties in Lewis rats after BHGZD administration. (4) The administration of the two-BAC-combination of mangiferin and glycyrrhizic acid, and BHGZD significantly alleviated RA disease severity and recovered energy metabolic disorders in Lewis rats by decreasing the expression levels of proteins in PKA-ADCY5-PPAR γ -PGC1 α -UCP1-PRDM16 signal axis, the therapeutic effects of which were similar to that of MTX. (5) *In vitro* experiments demonstrated that both the two-BAC-combination and BHGZD obviously inhibited the adipogenesis of adipocytes by down-regulating thermogenesis-related proteins in PKA-ADCY5-PPAR γ -PGC1 α -UCP1-PRDM16

signal axis. We also successfully used agonist of PKA named forskolin to observe its regulation effects, and further supporting the medicine-signal axis modulatory relations. To the best of our knowledge, mangiferin is a naturally occurring polyphenol that possess prominent anti-inflammatory and antioxidative properties, and has been reported to effectively attenuate the destructive behavior in RA by inhibiting proliferation, migration, inflammatory mediators' secretion and promoting apoptosis (Wang et al., 2021). Its control of inflammatory response and mechanical hyperalgesia associated with PKA regulation, and that of lipid metabolic disorder and mitochondrial dysfunction related with PPAR γ regulation (El-Sayyad et al., 2017; Li et al., 2019). Regarding to glycyrrhizic acid, a natural triterpene glycoside isolated from licorice roots, has been found to exert obvious anti-inflammatory and anti-arthritis effects (Ansari et al., 2021). Glycyrrhizic acid also affects lipid metabolism gene expressions and modification of fatty acids via regulating PPAR γ expression (Yaw et al., 2015).

Conclusions

Our results indicate for the first time that mangiferin from *Rhizoma Anemarrhenae* and glycyrrhizic acid from *Radix Glycythizae* may be the main BACs of BHGZD against RA, focusing on recovering energy metabolism and immune system imbalance, the mechanism of which may be associated with the regulation on the PKA-ADCY5-PPAR γ -PGC1 α -UCP1-PRDM16 signal axis (Fig. 7). These findings unravel the potentiality of the mangiferin and glycyrrhizic acid combination as a novel strategy for the treatment of RA.

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CRedit authorship contribution statement

Xia Mao: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Yudong Liu:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Weijie Li:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Kexin Wang:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Congchong Li:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Qian Wang:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Wenjia Chen:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Zhaochen Ma:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Xiaoyue Wang:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Zihe Ding:** Data curation, Formal analysis,

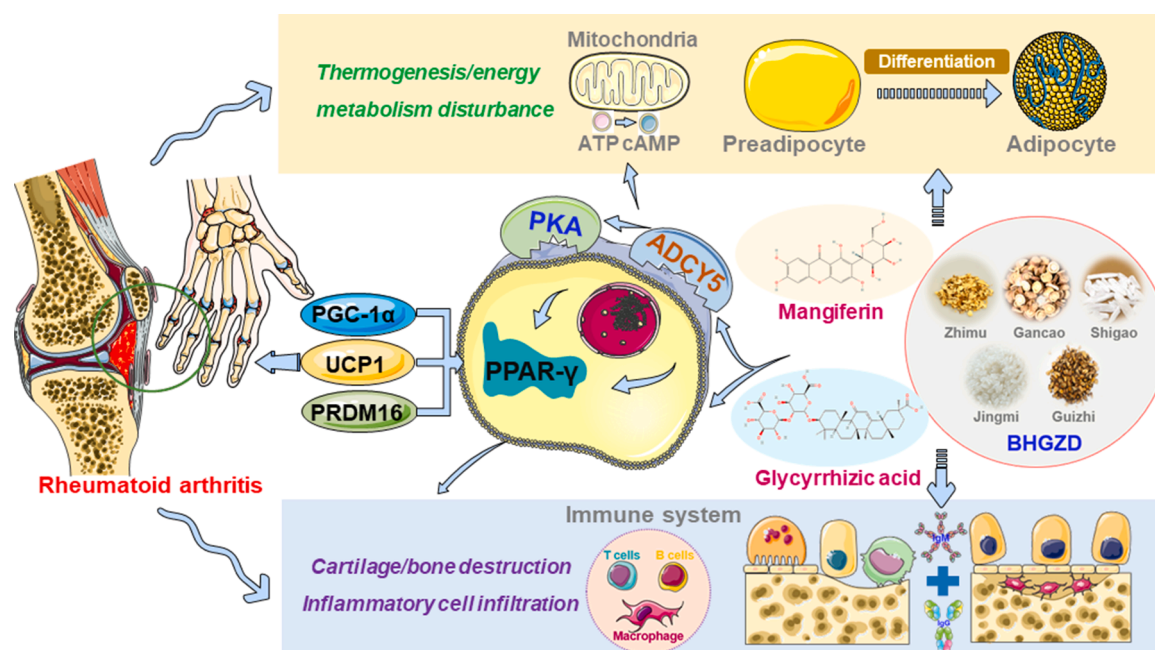


Fig. 7. Schematic diagram of the underlying mechanisms of BHGZD and BACs against RA via recovering the thermogenesis and energy metabolism disturbance by targeting PKA-ADCY5-PPAR γ -PGC 1 α -UCP1-PRDM16 signal axis.

Investigation, Validation, Visualization, Writing – original draft. **Yan-qiong Zhang**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Na Lin**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

Xia Mao, Yudong Liu, Weijie Li, Kexin Wang, Congchong Li, Qian Wang, Wenjia Chen, Zhaochen Ma, Xiaoyue Wang, Zihong Ding, Yanqiong Zhang and Na Lin declare that they have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2022.154216.

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