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ARTICLE

Imperatorin and β-sitosterol have synergistic activities in alleviating collagen-induced arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic disease with complex molecular network of pathophysiology, single drug is usually not full satisfactory because it is almost impossible to target the whole molecular network of the disease. Drug combinations that act synergistically with each another is an effective strategy in RA therapy. In this study, we aimed to establish a new strategy to search effective synergized compounds from Chinese herbal medicine (CHM) used in RA. Based on multi-information integrative approaches, imperatorin (IMP) and β-sitosterol (STO) were predicted as the most effective pair for RA therapy. Further animal experiments demonstrated that IMP+STO treatment ameliorated arthritis severity of collagen-induced arthritis (CIA) rats in a synergistic manner, whereas IMP or STO administration separately had no such effect. RNA sequencing and IPA analysis revealed that the synergistic mechanism of IMP+STO treatment was related to its regulatory effect on 5 canonical signaling pathways, which were not found when IMP or STO used alone. Moreover, LTA, CD83, and SREBF1 were 3 important targets for synergistic mechanism of IMP+STO treatment. The levels of these 3 genes were significantly up-regulated in IMP+STO group compared to model group, whereas IMP or STO administration separately had no effect on them. In conclusion, this study found that IMP and STO were 2 synergistic compounds from the CHM in RA therapy, whose synergistic mechanism was closely related to regulate the levels of LTA, CD83, and SREBF1.

KEYWORDS
compounds combination, mechanism, prediction, rheumatoid arthritis, synergism

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and destruction of joints and bones. Despite the highly application of antirheumatic drugs including nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), corticosteroids, and biologic agents, this heterogeneous disease is still not well controlled in up to 30% of patients.[1] Therefore, finding new drugs and strategy for RA patients is urgently needed.

The current view is generally accepted that RA has the complex molecular network of pathophysiology, single drug is usually not full satisfactory because it is almost impossible to target the whole molecular network of the disease.[2,3] The discovery of drug combinations that act synergistically with each another is hence of great importance. Recently, several drug combinations have entered clinical trials for the treatment of RA, including adalimumab plus methotrexate and tofacitinib plus methotrexate.[4,5] However, most of the drugs in RA combination therapeutics currently are chemosynthetic molecules or bioengineering products.

Traditional Chinese medicine (TCM), especially herbal medicine, has been used in RA therapy in China for thousands of years. Various TCM-based herbal formulae and extracts have been proved to effectively alleviate the disease progress in RA patients, such as Wu-Tou decoction, Gui-Zhi-Shao-Yao-Zhi-Mu decoction, and so on.[6,7] As well known, Chinese herbal formulae and extracts contain large numbers of compounds and produced multidimensional biological effects through multitarget, multi-pathway, and network regulation. The combination of these compounds follows specific principles and can make the overall effect to be optimal, which is similar to the discovery of drug combinations that act synergistically to some extent. Therefore, it may be a novel strategy to find new synergized compounds from Chinese herbal medicine (CHM).

Based on massive data processing and analysis, some new techniques such as biological computation and network pharmacology facilitate the exploration and reveal of the action mechanism of Chinese herbal formulae and extracts, at the same time, they also provide an effective approach to drug design and drug discovery.[2,8] Therefore, in this study, we want to establish a new strategy based on multi-information integrative approaches to search effective synergized compounds from CHM used in RA therapy.

2 | MATERIALS AND METHODS

2.1 | Text mining

For the treatments of RA, Chinese medicines have been used in paired pattern for synergizing effects. Thus, it is reasonable to explore the 2-dimensional combination patterns with respect to paired Chinese medicines in clinical practices. Clinical reports of RA with Chinese medicines were collected from two major literature databases (CNKI, https://nvsm.cnki.net/KNS/; SinoMed, http://www.sinomed.ac.cn/)

with searching term "rheumatoid arthritis" together with "Chinese medicine." In order to construct the network of paired Chinese medicines, text mining algorithm based on co-occurrence principle was deployed. Specifically, the Chinese medicines in each literature were used to construct the nodes of the network. If two Chinese medicines appeared in the same document, the related edges between the two Chinese medicines were added in the network, otherwise there were no edges added. These nodes and edges formed the co-occurrence network.

2.2 | Chemical components extraction, targets identification, and absorption, distribution, metabolism as well as excretion screening

All compounds of selected herbs and their pharmacokinetic properties were collected from published databases and absorption, distribution, metabolism as well as excretion (ADME) screening strategy were employed for selecting the candidate compounds for further analysis. The detailed information is explained in Supplementary Method 1.

2.3 | Disease-specific signaling network reconstruction

We have developed a data-driven adaptive integrative module for reconstructing signaling networks of diseases based on transcriptome and interactome data. The detailed information for the model implementation is explained in Supplementary Method 2.

2.4 | Functional drug target prediction using network-based recommendation

Drugs often have multiple targets and affect distinct signaling modules, but only parts of them are known for given drugs. The drug communities embed targeting signaling modules of drugs. To uncover the targeting signaling modules of drugs, we proposed a network-based recommendation approach, which could accurately infer the drug-target communities based on the widely used bipartite network projection technique. The detailed information was explained in Supplementary Method 3.

2.5 | Optimization to 2 synergistic compounds

We combined chemical structure, functional prediction, and targeting pathway to develop a biologically plausible model. Our proposed model possessed the ability to identify the dominant combinations in an accurate way, the detailed information for the model implementation is explained in Supplementary Method 4.

2.6 | Animals

Male Sprague-Dawley (SD) rats (8–10 weeks old) with a mean weight of 180–200 g were purchased from Beijing Vital River Laboratory

Animal Technology Co., Ltd. All rats were fed with food and water ad libitum in an appropriate environment with an air-filtering system, and were allowed to acclimatize themselves for 1 week before the initiation of experiment. The study was approved by the Research Ethics Committee of Institute of Basic Theory of Chinese Medicine, China Academy of Chinese Medical Sciences.

2.7 | Induction of collagen-induced arthritis

The collagen-induced arthritis (CIA) model was established in the SD rats as our previous method.[6] Bovine type II collagen (Chondrex, Redmond, WA, USA) solubilized at 2 mg/ml in 0.05 M acetic acid and equivalent parts of incomplete Freund's adjuvant (Chondrex, Redmond, WA, USA) were mixed into emulsion and then 200 and 100 μl emulsions were intradermally injected into the base of tail on days 0 and 7, respectively. Body weight of rat was measured and arthritis index (AI) was scored twice every week. AI score for each ankle joint was evaluated according to the scoring standard as follows[9]: 0, no swelling or erythema; 1, slight swelling and/or erythema; 2, low-to-moderate edema; 3, pronounced edema with limited joint usage; and 4, excess edema with joint rigidity. The maximum score of each rat was 16.

2.8 | Grouping and administration

After successful induction of CIA model, the rats were randomly divided into 6 groups with 10 rats in each group, which were normal group, model group, imperatorin group (IMP, 1 mg/kg/day), β-sitosterol group (STO, 1 mg/kg/day), imperatorin + β-sitosterol group (IMP, 1 mg/kg/day; STO, 1 mg/kg/day), and methotrexate group (MTX, 0.15 mg/kg/day). All agents were intragastrically administered at a volume of 1 ml/100 g for 4 weeks. The dosage of IMP (purity≥98%, Sigma, St. Louis, MO, USA), STO (purity≥96%, Sigma, St. Louis, MO, USA), and MTX (Sigma, St. Louis, MO, USA) was set as our preliminary experiment and the previous studies.[10-12]

2.9 | Enzyme-linked immunosorbent assay

Levels of IL-1β, IL-6, and TNF-α in sera of rats were detected by commercially available ELISA kits (eBioscience, San Diego, CA, USA) following manufacturer's instructions.

2.10 | Histological examination

Ankle joints were fixed in 4% paraformaldehyde, decalcified with 10% EDTA bone decalcifier, and embedded in paraffin. Tissue sections (4 μm) were prepared and stained with H&E for histological examination. Histopathological characteristics were evaluated blindly according to the following criteria: 0, normal; 1, hyperplasia of the synovial membrane and presence of polymorphonuclear infiltrates; 2, pannus and fibrous tissue formation as well as focal subchondral bone erosion; 3, articular cartilage destruction and bone erosion; 4, extensive articular cartilage destruction and bone erosion.[13]

2.11 | CD4$^+$ T lymphocytes separation and total RNA extraction

PBMCs were separated with a rat PBMC separation kit (Solarbio, Beijing, China) according to manufacturer's instructions. Next, CD4$^+$ T lymphocytes were isolated by magnetic beads separation (Miltenyi Biotec, Germany). The purity of collected CD4$^+$ T lymphocytes was detected by flow cytometry analysis. Finally, the cells were centrifuged and stored at −80°C. Total RNA was isolated by the Trizol extraction method. RNA purity was checked using NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA) according to the manufacturer's instructions.

2.12 | RNA sequence

A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated. We classified the gene as differentially expressed only when the expression difference was >2-fold with adjusted P-value < 0.05.

2.13 | IPA analysis

The dataset from RNA sequence was uploaded into the Ingenuity Pathways Analysis system (IPA, http://www.ingenuity.com) to analyze the canonical pathways of the candidate compounds. We used the IPA analysis system with the 'Core analysis' platform to analyze the identified differentially expressed genes (DEGs) of model group versus normal group (hereafter Model), IMP group versus model group (hereafter IMP), STO group versus model group (hereafter STO), and IMP+STO group versus model group (hereafter IMP+STO). Comparisons of the canonical pathways between these groups were also carried out using the "Comparison" platform in IPA. In this study, the score was −10 logarithms of Fisher's exact test P-values in canonical pathway analysis by IPA.

2.14 | Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was accomplished by using Graphpad Prism Software 6 (Graph Pad Software Inc.,
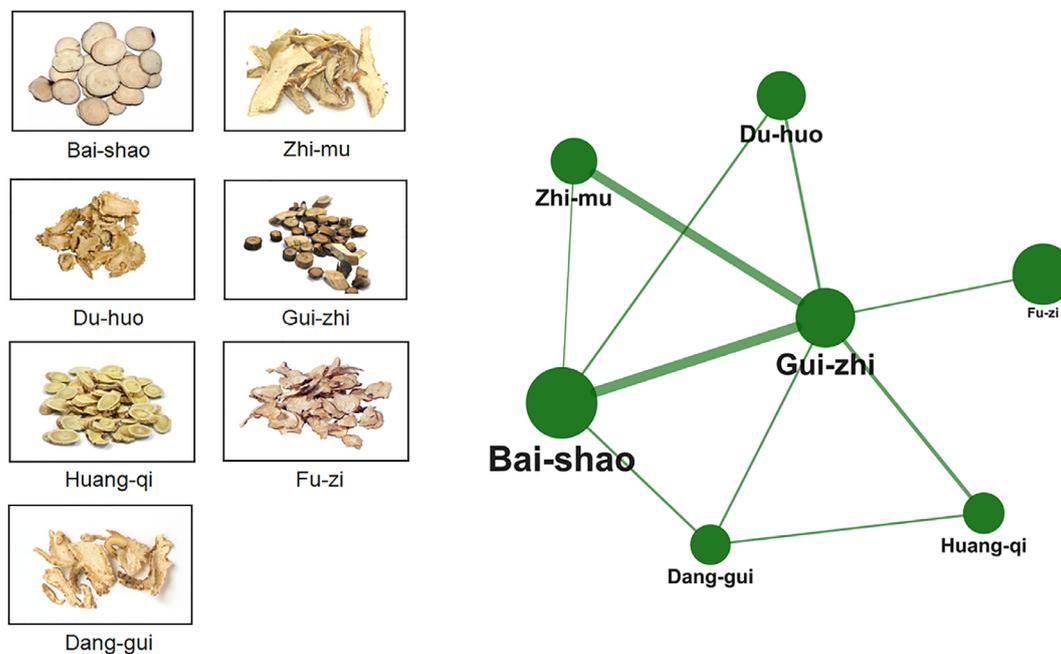
FIGURE 1 Chinese herbal medicine network associated with RA. The network in the right was constructed via mining 2668 pieces of literature associated with RA according to the co-occurrence principle. Font size and node size were calculated by logarithm on values of the frequencies of Chinese herbal medicine extracted from the literature. As to the edges, widths were also calculated by the logarithm of their co-occurrence values in literature

San Diego, CA). Comparisons of numerical data between 2 groups were calculated by Student's t-tests. Differences in mean values of various groups were analyzed by ANOVA. Difference with P-value < 0.05 was considered as statistically significant.

3 | RESULTS

### 3.1 | Frequently pair-used herbs for RA therapy

There were 3179 papers associated with RA and Chinese medicine. From 2668 pieces of literature, text mining returned a network of CHM of 241 distinct CHM and 4037 connections among them. In order to check the consistency of guidelines and clinical reports, the intersection of text mining restricted to 150 CHM in guidelines was filtered out. Consequently, the intersecting network of CHM included 107 distinct CHM and 2388 connections among them. Then, the comparison between them was carried out. Accordingly, the top 100 connections were consistent exactly. To be simplified, top 10 pairs of co-occurred CHM are listed in Supplementary Table 1 and visualized in Fig. 1. From these data, we could find that Bai-shao, Gui-zhi, Zhi-mu, Huang-qi, Du-huo, Dang-gui, and Fu-zi were the most common pairs of CHM used in RA therapy.

### 3.2 | Two synergistic compounds from the herbs by multi-information integrative approaches

These most common pairs of CHM (Bai-shao, Gui-zhi, Zhi-mu, Huang-qi, Du-huo, Dang-gui, and Fu-zi) contain multiple compounds, and only

a few compounds possess satisfactory pharmacodynamic and pharmacokinetic properties. After ADME screening, 169 active compounds were filtered out of the 892 compounds of these CHM. Then we used HitPick, SEA, STITCH, and Swiss Target Prediction to predict the potential targets of the 169 active compounds and totally got 473 targets. These predicted targets were used to construct the C-T network. Several of these active compounds were related multiple targets, resulting in 1439 compound-target associations between 169 active compounds and 473 targets. The average number of targets per compound was 12.1, and the mean degree of compounds per target was 5.7. The 2D and 3D molecular descriptors of the 169 active compounds were calculated by ChemoPy, and the GO analysis of the 473 targets were calculated by DAVID.[14,15] To determine the relationship between the target and RA ($P < 0.05$), we constructed the RA-target network by application of our published models.[16,17]
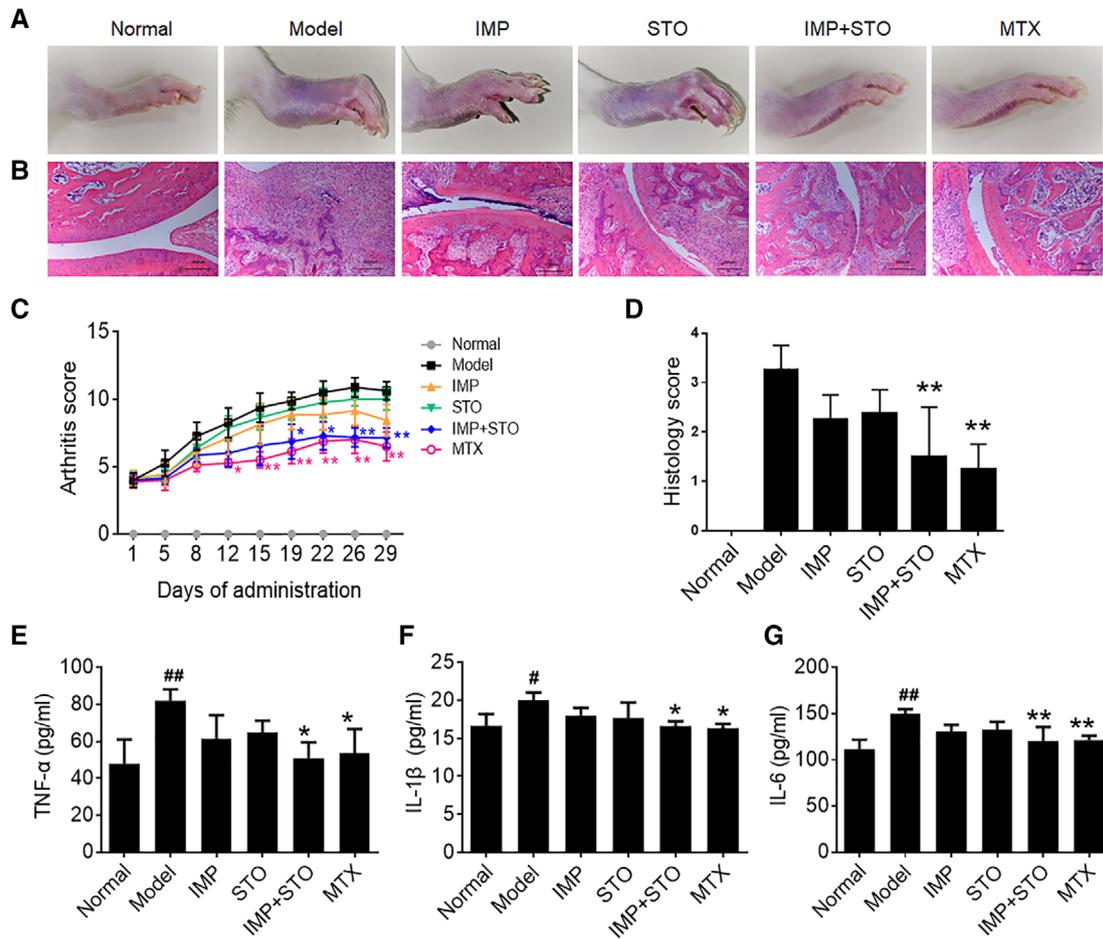
Next, we designed an integrative model that integrated pharmacokinetics similarity, target network, and function analysis to discover the high potential synergistic compound pairs for RA treatment (Fig. 2). The C-T network and RA-target network were used to calculate the target network synergistic score and the 2D and 3D molecular descriptors were used to predict the 2D and 3D similarity synergistic scores, respectively. The GO analysis result of 473 targets were used to calculate the function synergistic score. Thirty-eight pairs of effective synergistic compounds were detected by our scoring model (Fig. 3A). Then we used 3D plot to display the score distribution and found that most of the effective synergistic compounds with high synergistic scores in the 2D and 3D similarities, target network, and function analysis (Fig. 3B). In the 38 pairs of effective synergistic compounds, 7 pairs had high scores (>0.9) in structure similarity,
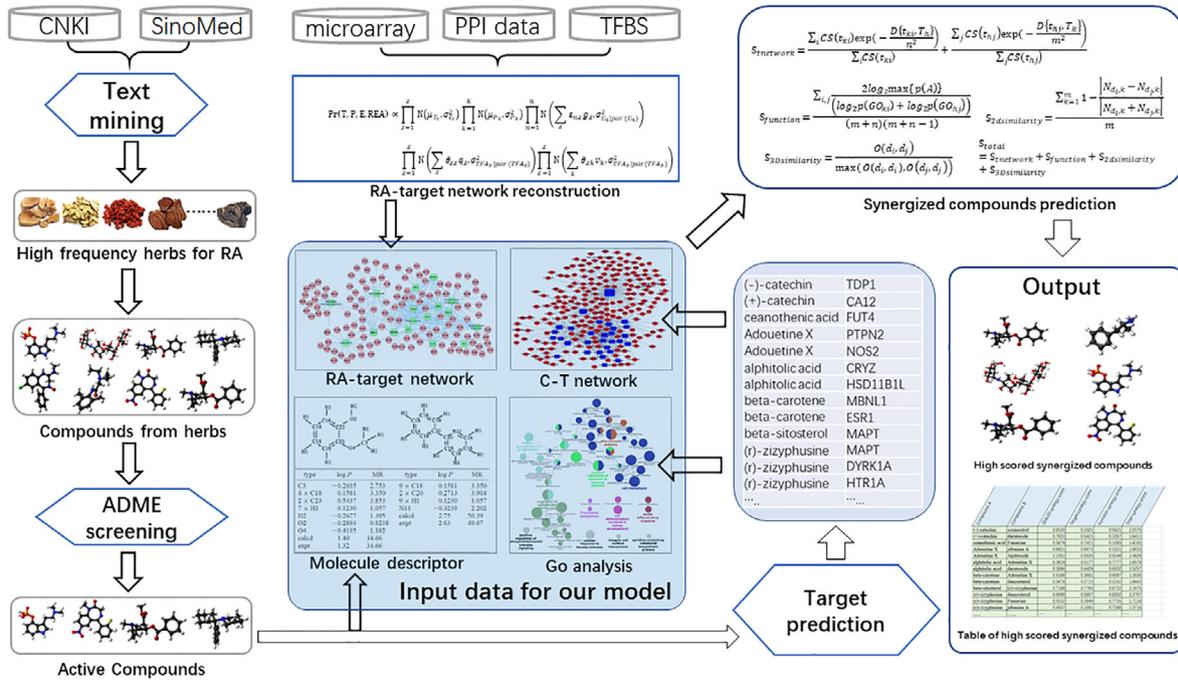
FIGURE 2 Diagram of RA pathogenic network construction and synergistic compounds screening model
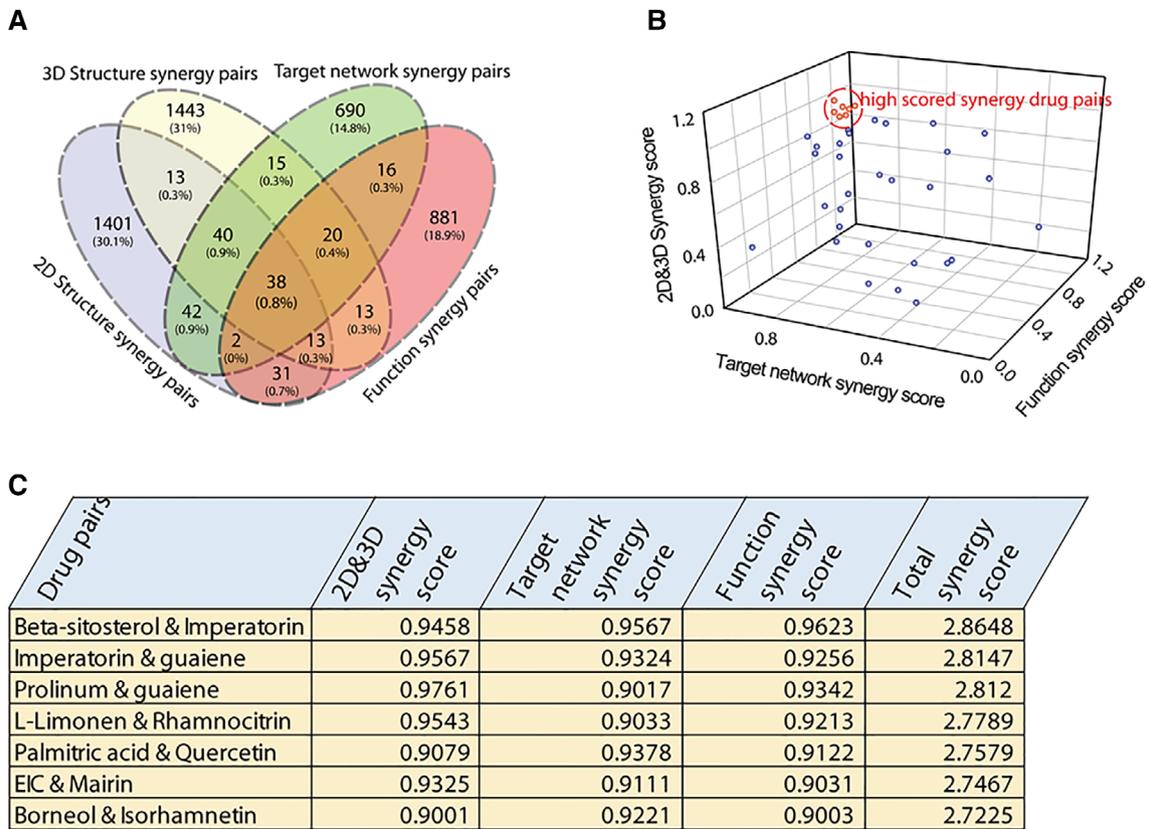
FIGURE 3 Predict the high-scored synergistic compounds for RA. (**A**) Venn diagram of high scored combination compounds in 4 score-models. (**B**) 3D plot of high-scored combination compounds in structure, target network and function synergy score-models. (**C**) The structure, target network, function and total synergy score of high-scored combination compounds

Drug pairs	2D&3D synergy score	Target network synergy score	Function synergy score	Total synergy score
Beta-sitosterol & Imperatorin	0.9458	0.9567	0.9623	2.8648
Imperatorin & guaiene	0.9567	0.9324	0.9256	2.8147
Prolinum & guaiene	0.9761	0.9017	0.9342	2.812
L-Limonen & Rhamnocitrin	0.9543	0.9033	0.9213	2.7789
Palmitric acid & Quercetin	0.9079	0.9378	0.9122	2.7579
EIC & Mairin	0.9325	0.9111	0.9031	2.7467
Borneol & Isorhamnetin	0.9001	0.9221	0.9003	2.7225

FIGURE 4 Effects of IMP and STO synergism on arthritis severity of CIA rats. (**A**) Representative photograph of ankle joint from each group. (**B**) Representative histological finding of ankle joint from each group. Tissue sections from ankle joints were stained with H&E. Original magnification 200×. (**C**) Arthritis score of each group. (**D**) Histology score of each group. (**E–G**) TNF-α, IL-1β, and IL-6 concentration of each group. Concentrations of TNF-α, IL-1β, and IL-6 in serum of rats were determined by ELISA. [#]$P < 0.05$, [##]$P < 0.01$ vs. normal group. [*]$P < 0.05$, [**]$P < 0.01$ vs. model group

target network, and function analysis. The pair β-sitosterol (STO) and imperatorin (IMP) was predicted as the most effective one for RA treatment with highest score (Fig. 3C), Thus, we chose this pair for further experiment validation.

3.3 | IMP and STO synergism alleviated the arthritis severity of CIA rats

As shown in Fig. 4A and C, no visible morphological features of arthritis could be seen in the normal rats over the entire period. By contrast, erythema and swelling could be markedly observed in the ankle joints of CIA rats, which was reflected by the significantly increased AI score. The elevation of AI scores was significantly inhibited by the combination treatment of IMP and STO. However, there were no apparent therapeutic effects when IMP and STO were administered to CIA rats separately. To further investigate the effect of IMP, STO, and their combination in CIA rats, the pathological changes in the ankle joints of rats were observed through H&E staining. As shown in Fig. 4B, massive influx of inflammatory cells, synovial hyperplasia, pannus formation, and severe erosion of cartilage and bone in ankle joints of CIA rats were observed and these pathological changes were mitigated by

the combination treatment of IMP and STO. The histological score of IMP+STO group was remarkably lower than that in model group after treatment, whereas there was no significant difference between IMP group and model group, as well as STO group and model group (Fig. 4D). In addition, the levels of several important pro-inflammatory cytokines in sera of CIA rats were detected. As shown in Fig. 4E–G, compared to normal group, the levels of IL-1β, IL-6, and TNF-α were significantly higher in model group, whereas the levels of these pro-inflammatory cytokines were significantly lower in IMP+STO group when compared to the model group. However, IMP or STO administration separately did not observe this effect. All these results showed that IMP and STO combination showed good synergistic effect and effectively alleviated the arthritis severity of CIA rats.

3.4 | RNA sequencing revealed the synergistic mechanism involved in IMP and STO combination

To further illustrate the synergistic mechanism of IMP+STO treatment in CIA rats, we detected differentially expressed genes (DEGs) of CD4^{+} T cells from peripheral blood using RNA sequencing. The results
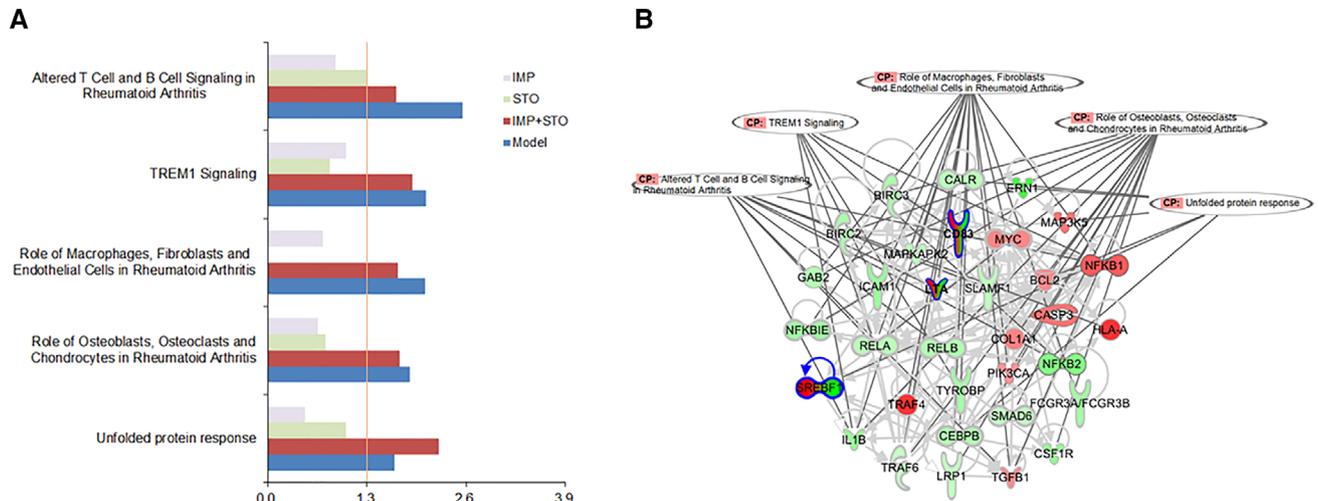
FIGURE 5 Canonical signaling pathway and network analysis between IMP+STO group and model group. (A) Shared canonical signaling pathways of IMP+STO group and model group. Threshold > 1.3. (B) The molecular network of shared canonical signaling pathways of IMP+STO group and model group. Notes: red: up-regulated genes. Green: down-regulated genes. Circle with blue: shared DEGs in IMP+STO group and model group

showed that compared with normal group, there were 347 DEGs in model group. Compared with model group, there were 183 DEGs in IMP group, 243 DEGs in STO group, and 202 DEGs in IMP+STO group (Supplementary Tables 2–5).

Canonical pathway analysis indicated that the synergistic mechanism of IMP+STO treatment in CIA rats was related to its regulatory effect on altered T cell and B cell signaling in RA, TREM1 signaling, role of Mϕs, fibroblasts, and endothelial cells in RA, role of osteoblasts, osteoclasts, and chondrocytes in RA, and unfolded protein response, whereas IMP or STO administration separately did not lead to these effects (Fig. 5A). Among these 5 signaling pathways, the 3 most important target genes of IMP+STO treatment were LTA, CD83, and SREBF1. The expression levels of these genes were remarkably down-regulated in model group but up-regulated in IMP+STO treatment group, whereas there was no significantly change in expression levels of these genes in IMP group and STO group (Fig. 5B).

Further, we analyzed the target genes of each treatment in these 5 signaling pathways, which were shown in Supplementary Fig. 1. In "altered T cell and B cell signaling in rheumatoid arthritis," the expression levels of NF-κB, RELB, NF-κB2, and LTA were down-regulated in model group. IMP+STO treatment could up-regulate the expression level of LTA and TGF-β, at the same time, down-regulate IL-1. IMP or STO administration separately could down-regulate the levels of NF-κB2 and NF-κB. In "TREM1 signaling," the levels of NF-κB, CD53, and CD83 were down-regulated in model group. IMP+STO treatment up-regulated CD83 and down-regulated DAP12. IMP and STO administration separately down-regulated NF-κB. In role of Mϕs, fibroblasts, and endothelial cells in rheumatoid arthritis, the expression levels of LTA, TRAF, PI3K, RELA, IκB, TRAF6, MAPK/APK2, ICAM1, and c-MYC were down-regulated in model group. IMP+STO treatment up-regulated the expression level of LTA, whereas IMP or STO administration separately down-regulated the levels of PI3K and TRAF. In role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid

arthritis, the expression levels of TRAF6, PI3K, RELA, IκB, CIAP, and SMAD6 were down-regulated in model group. IMP+STO treatment down-regulated the levels of IL-1, LRP1/5/6, M-CSFR, and ASK1. IMP or STO administration separately down-regulated the level of PI3K. In unfolded protein response, the expression levels of SREBF1, IRE1, and CALR were down-regulated in model group. IMP+STO treatment could up-regulate SREBF1, at the same time, down-regulate ASK1 and c/EBP. These results suggested that compound used alone and in combination acted to different targets.

4 | DISCUSSION

The complexity of RA limits the efficacy of commonly used single drug therapeutics. Emerging evidence showed that combination therapeutics would become an effective strategy to address this issue.[18] Computational tools in this area has recently been made in computing synergistic effects for combinatorial therapy. While the current in silico models are mainly based on the limited synergy information, such as chemical structure, targeting pathway, gene expression profile on drug dealing. This is not enough for decoding the effective drug combination. To address this challenge, we combined chemical structure, functional prediction, and targeting pathway to develop a biologically plausible model. Our proposed model possessed the ability to identify the dominant combinations in an accurate way. In our model, the effective combination was defined as a function of structure and pathway occupancies and kinetic parameters. Unlike existing integrative analyses that treated structure and targeting pathway as 2 separate processes, our approach put these 2 types of data into 1 single model, which was more biologically meaningful. One obstacle was that the combination kinetic function was essentially nonlinear, which made it difficult to develop computational methodologies. Here, we adopted Taylor expansion to convert the nonlinear kinetic function to a

polynomial function. The usage of the polynomial function provided a general mathematic form to simultaneously involve different combinations. By assuming each combination had a probability of being involved in a potential function, we were able to construct the model equation. Solving the model equations could lead to a determination of the key combinations. In this study, we found 2 new synergized compounds, IMP and STO, from CHM in RA therapy by using our multi-information integrative approaches. AI scoring, histological analysis, and inflammatory cytokines examination showed the efficacy and synergy of IMP and STO combination treatment in CIA rats, which further proved the accuracy of our screening strategy.

Based on the RNA sequencing technique, we found that the expression levels of some genes could be regulated by the administration of IMP or STO alone or by the combination of IMP and STO. Interestingly, IMP and STO combination caused several new effects when compared with IMP or STO administration separately, including regulatory effect on altered T cell and B cell signaling in rheumatoid arthritis, TREM1 signaling, role of Mϕs, fibroblasts, and endothelial cells in rheumatoid arthritis, role of osteoblasts, osteoclasts, and chondrocytes in RA, and unfolded protein response. As well known, T cells, B cells, Mϕs, fibroblasts, endothelial cells, osteoblasts, osteoclasts, and chondrocytes are all important cell types in RA progression.[3] They participated in mediating the joint inflammation and destruction through multifarious ways, including producing inflammatory factors, cell-cell interaction, and so on.[3,19,20] Our results again showed good synergistic effect of IMP and STO combination. Moreover, we found that most of the genes regulated by IMP and STO combination treatment were located in the upstream of these 5 signaling pathways, which might also explain the reason why combination treatment was superior to single compound treatment.

We found that lymphotoxin alpha (LTA), CD83, and sterol regulatory element-binding protein 1 (SREBP1) were 3 most important target genes in IMP and STO combination treatment. LTA, also known as TNF-β, mediates a large variety of inflammatory and immunostimulatory responses.[21] In vitro studies showed that LTA induced proliferation and inflammatory cascade signaling in RA synovial-fibroblasts (RASFs) and chondrocytes.[22,23] CD83, dendritic cell marker, belongs to the immunoglobulin superfamily and has previously been associated with autoimmune diseases. Previous studies have reported that the levels of soluble CD83 (sCD83) were elevated in RA plasma and synovial fluid, whereas anti-TNF-α treatment had no effect on the sCD83 plasma level.[24,25] SREBP1 is a transcription factors that functions as master regulators of genes that control cellular lipid homoeostasis.[26] It plays a crucial role in numerous pathogenic processes such as inflammation and apoptosis, and contributes to many chronic diseases.[27] In this study, we found that the levels of LTA, CD83, and SREBP1 was obviously down-regulated in CD4+ T cells from peripheral blood of model group, whereas IMP and STO combination could significantly up-regulated the expression levels of these 3 genes. Interestingly, IMP or STO administration separately had no effect on these 3 genes, which also showed the synergized effect of 2 compounds combination treatment. As these 3 genes were not the targets of conventional chemical drugs and biological agents in RA, IMP and STO combination

might become a candidate treatment used in those RA patients who were not response well with conventional treatment.

In this study, we only used 1 dose in combination treatment group. We did not examine the optimum proportion of IMP and STO to achieve the best synergistic effect. In addition, we only selected the compound combination ranked first for further experimental study. Whether other candidate compound combinations also have synergistic therapeutic effects is still not clear. We will continue to do these studies in the future.

In conclusion, we found 2 synergistic compounds, IMP and STO, from the CHM in RA therapy. In vivo experiment indicated that IMP and STO combination could effectively alleviate the arthritis severity of CIA rats in a synergistic manner, and the synergistic mechanism was closely related to targeting LTA, CD83, and SREBF1. We think IMP and STO combination will become a new candidate treatment in RA in the future.

AUTHORSHIP

Q.G., L.L., and K.Z. performed the major research in equal contribution. G.Z. performed text mining. H.S. and Y.S. collected samples. C.L. and J.S. provided the technical support. D.G. performed the biological computation, bioinformatics analysis, and revised the manuscript. A.L. and X.H. designed the study and revised the manuscript. Q.G., L.L., and K.Z. contributed equally to this work.

DISCLOSURE

The authors declare no conflict of interest.

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REFERENCES

1. Koenders MI, van den Berg WB. Novel therapeutic targets in rheumatoid arthritis. *Trends Pharmacol Sci*. 2015;36:189-195.
2. Hopkins AL. Network pharmacology: the next paradigm in drug discovery. *Nat Chem Biol*. 2008;4:682-690.
3. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med*. 2011;365:2205-2219.
4. Smolen JS, Emery P, Fleischmann R, et al. Adjustment of therapy in rheumatoid arthritis on the basis of achievement of stable low disease activity with adalimumab plus methotrexate or methotrexate alone: the randomised controlled OPTIMA trial. *Lancet*. 2014;383:321-332.
5. Burmester GR, Blanco R, Charles-Schoeman C, et al. Tofacitinib (CP-690,550) in combination with methotrexate in patients with active rheumatoid arthritis with an inadequate response to tumour necro-

sis factor inhibitors: a randomised phase 3 trial. *Lancet*. 2013;381:451-460.

6. Guo Q, Zheng K, Fan D, et al. Wu-Tou decoction in rheumatoid arthritis: integrating network pharmacology and in vivo pharmacological evaluation. *Front Pharmacol*. 2017;8:230.

7. Huang L, Lv Q, Xie D, Shi T, Wen C. Deciphering the potential pharmaceutical mechanism of Chinese traditional medicine (Gui-Zhi-Shao-Yao-Zhi-Mu) on rheumatoid arthritis. *Sci Rep*. 2016;6:22602.

8. Li S, Fan TP, Jia W, Lu A, Zhang W. Network pharmacology in traditional Chinese medicine. *Evid Based Complementary Altern Med*. 2014;2014:138460.

9. Shahrara S, Amin Ma Fau-Woods JM, Woods Jm Fau - Haines GK, Haines Gk Fau - Koch AE, Koch AE. Chemokine receptor expression and in vivo signaling pathways in the joints of rats with adjuvant-induced arthritis. *Arthritis Rheum*. 2003;48(12):3568-3583.

10. Cuzzocrea S, Mazzon R, Fau di Paola E, et al. Synergistic interaction between methotrexate and a superoxide dismutase mimetic: pharmacologic and potential clinical significance. *Arthritis Rheum*. 2005;52(12):3755-3760.

11. Oh HA, Kim HM, Jeong HJ. Distinct effects of imperatorin on allergic rhinitis: imperatorin inhibits caspase-1 activity in vivo and in vitro. *J Pharmacol Exp Ther*. 2011;339:72-81.

12. Wong HS, Chen JH, Leong PK, Leung HY, Chan WM, Ko KM. beta-sitosterol protects against carbon tetrachloride hepatotoxicity but not gentamicin nephrotoxicity in rats via the induction of mitochondrial glutathione redox cycling. *Molecules*. 2014;19:17649-17662.

13. Thwin MM, Douni E, Aidinis V, et al. Effect of phospholipase A2 inhibitory peptide on inflammatory arthritis in a TNF transgenic mouse model: a time-course ultrastructural study. *Arthritis Res Ther*. 2004;6:R282-294.

14. Cao DS, Xu QS, Hu QN, Liang YZ. ChemoPy: freely available python package for computational biology and chemoinformatics. *Bioinformatics*. 2013;29:1092-1094.

15. Huang DW, Sherman BT, Tan Q, et al. The DAVID gene functional classification tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biol*. 2007;8:R183.

16. Guan D, Shao J, Zhao Z, et al. PTHGRN: unraveling post-translational hierarchical gene regulatory networks using PPI, ChIP-seq and gene expression data. *Nucleic Acids Res*. 2014;42:W130-136.

17. Guan D, Shao J, Deng Y, et al. CMGRN: a web server for constructing multilevel gene regulatory networks using ChIP-seq and gene expression data. *Bioinformatics*. 2014;30:1190-1192.

18. He B, Lu C, Zheng G, et al. Combination therapeutics in complex diseases. *J Cell Mol Med*. 2016;20:2231-2240.

19. McInnes IB, Leung BP, Liew FY. Cell-cell interactions in synovitis. Interactions between T lymphocytes and synovial cells. *Arthritis Res*. 2000;2:374-378.

20. Politti U. Lymphocytic choriomeningitis and alpha-chemokines. *Clin Ter*. 2015;166:e197-202.

21. Hirose T, Fukuma Y, Takeshita A, Nishida K. The role of lymphotoxin-alpha in rheumatoid arthritis. *Inflamm Res*. 2018;67:495-501.

22. Calmon-Hamaty F, Combe B, Hahne M, Morel J. Lymphotoxin alpha revisited: general features and implications in rheumatoid arthritis. *Arthritis Res Ther*. 2011;13:232.

23. Buhrmann C, Shayan P, Aggarwal BB, Shakibaei M. Evidence that TNF-beta (lymphotoxin alpha) can activate the inflammatory environment in human chondrocytes. *Arthritis Res Ther*. 2013;15:R202.

24. Kristensen AM, Stengaard-Pedersen K, Hetland ML, et al. Expression of soluble CD83 in plasma from early-stage rheumatoid arthritis patients is not modified by anti-TNF-alpha therapy. *Cytokine*. 2017;96:1-7.

25. Hock BD, O'Donnell JL, Taylor K, et al. Levels of the soluble forms of CD80, CD86, and CD83 are elevated in the synovial fluid of rheumatoid arthritis patients. *Tissue Antigens*. 2006;67:57-60.

26. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*. 2002;109:1125-1131.

27. Shimano H, Sato R. SREBP-regulated lipid metabolism: convergent physiology - divergent pathophysiology. *Nat Rev Endocrinol*. 2017;13:710-730.

SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.