Integration of network pharmacology and bone marrow mesenchymal stem cells experimental research to reveal the molecular mechanisms for Hai Honghua medicinal liquor against osteoporosis

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Author contributions
DQ contributed article writing and revision. DQ and CX designed this article and performed the online database search. CH and ML contributed to compiling the data as well as creating the network diagram. JG contributed molecular docking related content. HZ participated in verification and inspection. All authors have read and approved the final manuscript.

Competing interests
The authors declare no conflicts of interest.

Acknowledgments
This work was supported by the Project of State Administration of Traditional Chinese Medicine of Sichuan Province of China (No. 2021MS407).

Peer review information
Integrative Medicine Discovery thanks all anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations
OP, osteoporosis; HHML, Hai Honghua medicinal liquor; TCM, traditional Chinese medicine; E, Erythrina varegata L.; SD, Sprague Dawley; PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BMSCs, bone marrow mesenchymal stem cells; ALP, alkaline phosphatase; Q-PCR, quantitative polymerase chain reaction.

Results
Qian D, Xu C, He CX, Li MY, Guo J, Zhang H. Integration of network pharmacology and bone marrow mesenchymal stem cells experimental research to reveal the molecular mechanisms for Hai Honghua medicinal liquor against osteoporosis. Integr Med Discov. 2024;8:e24003. doi: 10.53388/IMD202408003.

Abstract
Background: Hai Honghua medicinal liquor (HHML) formula has been used in clinical practice for a long time, mainly for the treatment of freshly closed fractures, to promote osteogenesis, to increase bone mass, and thus to promote fracture healing. However, the underlying mechanisms of HHML in the treatment of osteoporosis (OP) are still unclear.

Methods: Firstly, Traditional Chinese Medicines Systems Pharmacology Database and Analysis Platform and The Encyclopedia of Traditional Chinese Medicine were used to screen the targets of the active compounds of HHML. At the same time, OP targets were identified through GeneCard, Online Mendelian Inheritance in Man, DisGeNET, Therapeutic Target Database, Comparative Toxicogenomics Database and Human Phenotype Ontology databases. Next, protein-protein interaction and pathway networks were constructed for compound-disease common targets, and core targets and compounds were screened for molecular docking. Furthermore, rat bone marrow mesenchymal stem cells were extracted as model cells, and the osteogenic effects of HHML were verified via in vitro experiments.

Results: Total of 343 common targets of HHML-OP and the top 10 target proteins in the protein-protein interaction network are TP53, AKT1, STAT3, HSP90AA1, ESR1, TNF, IL6, MAPK3, MAPK8. Enrichment analysis yielded that the key molecular pathway was the PI3K/Akt signaling pathway. Molecular docking analysis showed that baicalein, erysodienone and naringenin docked with the target proteins AKT1, STAT3 and TP53, respectively, with low binding energy and high affinity. In addition, in vitro experiments demonstrated that HHML promoted the proliferation of bone marrow mesenchymal stem cells; compared with the control group, HHML-treated cells showed enhanced alkaline phosphatase staining, promoted the expression of OCN, RUNX2, BSP, and COL1 mRNAs as well as the expression of PI3K and AKT phosphorylated proteins. Secondly, the expression of target proteins revealed that HHML promoted the phosphorylation of STAT3 protein and inhibited the expression of P53.

Conclusions: Our study investigated that HHML treatment with OP promotes bone formation possibly through activation of the PI3K/Akt signaling pathway and may involve STAT3 and TP53 target interactions.

Keywords: Hai Honghua medicinal liquor; osteoporosis; network pharmacology; molecular docking; PI3K/AKT signal pathway
Introduction

Osteoporosis (OP) main manifestations are bone loscreased bone mass and degradation of tissue microstructure, ultimately leading to increased bone fragility, which is an endogenous factor that increases the risk of fracture [1, 2]. The prevalence of OP increases with age and is a significant difference in gender, with studies showing that the prevalence is higher in women than in men, and that more bone is lost in postmenopausal women than men because of a decrease in estrogen production [3]. The occurrence of OP affects the satisfaction of millions of patients and significantly increases the healthcare burden on society; therefore, OP is also an urgent challenge that needs to be addressed today.

The metabolic homeostasis of bone is maintained by the coupling of osteoclast-mediated bone resorption and osteoblast-mediated new bone formation, and if this homeostasis is disturbed, OP occurs [4, 5]. Currently, treatment for OP focuses on helping patients reduce symptoms, improve bone volume and strength, and decrease probability of fracture with a view to improving their quality of life. Commonly used treatments include, but are not limited to, estrogen therapy, bisphosphonates, anabolic therapies and other potential therapies [6]. These methods can play a certain curative effect in the treatment of OP patients, but they are not completely ideal treatment methods. Some studies have shown that estrogen prevents postmenopausal bone loss, but the choice of estrogen replacement therapy increases breast cancer risk [7]. Therefore, it is wise to choose relatively safe, non-toxic and inexpensive traditional Chinese medicine to treat OP, which has been used for a long time [8].

Hai Honghua medicinal liquor (HHML), a traditional Chinese medicine (TCM) formula preparation, has been used in clinically to treat diseases for over 30 years, mostly for the treatment of bone fracture and joint pain [9]. The compound formula owes its name to the herbs *Erythrina cortex* and *Carthami flos*, which are the main essentials of the formula. Zhang et al. revealed the role of extracts of the *Erythrina variegata* L. (EV) on ovariectomized rats and showed that the EV was able to prevent the decrease of bone mineral density and ameliorate the biomechanical properties of the skeleton [10]. In addition, it was revealed that EV could inhibit osteoclast differentiation and maturation and exert osteoprotective effects [11]. Furthermore, safflower seed extract has been shown to be effective in promoting the proliferation of osteoblasts and the production of the bone marker osteocalcin, bone-specific alkaline phosphatase, which was able to promote bone formation for the prevention and improvement of OP [12–14]. These prior findings provide strong evidence for the treatment of OP for HHML. Although there were some research bases, the mechanism of HHML clinical formula for OP has not yet been elucidated due to its rich flavor, complex composition, and multiple targets of action.

Bioinformatics techniques, network pharmacology and molecular docking are commonly used to study the underlying mechanism of action between herbal formula and disease [15]. It has the advantage of obtaining results quickly and giving a clear direction to the research that is commonly used. The role of HHML in the treatment of OP was investigated for the first time, so we also analyzed the active ingredients of HHML with the help of this method, as well as the signaling pathways involved in the interaction of the ingredient targets with the disease targets.

In this study, based on the HHML active ingredients screened in previous studies [9], and combined with the OP targets, the important targets and active ingredients were screened for subsequent analysis. According to the pathomechanism of OP, it was investigated whether the effect of HHML in treating OP is to promote bone formation and increase bone mass, thereby achieving relief of OP symptoms through in vitro experiments. In addition, cellular experiments were performed to verify whether HHML promoted the expression of genes associated with osteogenic differentiation and the expression of core proteins and significant signaling pathway proteins involved in the raw letter results, to provide theoretical reference for the application and development of HHML compounding for OP. The graphical abstract is shown in Figure 1.

Materials and methods

Materials

Experimental animal. A total of 4 Sprague Dawley (SD) rats (male, 4-week-old and specific pathogen-free grade), were purchased from Specific Biotechnology Co., Ltd. (Beijing, China). The rats weighed about 90 g. And animal production license is No. SCXK (Beijing) 2019-0010. Due to the stronger survival ability of SD rats, the selected rats were moderate in age, and the bone marrow differentiation of too old rats was more mature and the vitality was weakened. All experimental protocols related animals obeyed the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the Animal Care and Use Committee of Longquan Hospital of Traditional Chinese Medicine, Chengdu (Approval No. 2021.008).

Experimental drugs. HHML formula consists of *Erythrina Cortex* 50 g, *Carthami Flos* 20 g, *Persicae semen* 10 g, *Angelicae Sinensis radix* 10 g, *Chuanxiong rhizoma* processing with Chinese Baijiu 10 g and other traditional Chinese medicine for 19, and these drugs were obtained from Chengdu Longquan District Hospital of Traditional Chinese Medicine.

![Figure 1: The graphical abstract of this study. PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BMSCs, bone marrow mesenchymal stem cells; ALP, alkaline phosphatase.](https://www.tmrjournals.com/im)
Network pharmacology analysis

Search target information of active components of HHML. The target information of the active components of TCM was gained from the publicly used database. Commonly used databases include Traditional Chinese Medicines Systems Pharmacology Database and Analysis Platform (http://ibis.hikb.edu.hk/LSP/tcmsp.php), The Encyclopedia of Traditional Chinese Medicine (http://www.tcmip.cn/ETCM/index.php/Home/Index/) and other databases. In the Traditional Chinese Medicines Systems Pharmacology Database and Analysis Platform database, the selecting of chemical compounds in TCM was evaluated by two indicators, oral bioavailability ≥ 30% and drug-likeness ≥ 0.18. Other active ingredients were supplemented from the The Encyclopedia of Traditional Chinese Medicine database and the ingredients retrieved from both databases were combined and duplicates were removed. In addition, components with no corresponding targets were deleted.

Search of disease targets. A total of 6 databases for screening disease targets, Online Mendelian Inheritance in Man (https://omim.org/), GeneCards (https://www.genecards.org/), DisGeNET (https://www.disgenet.org/), Therapeutic Target Database (http://db.idrblab.net/tdt/), Comparative Toxicogenomics Database (https://ctdbase.org/) and Human Phenotype Ontology (http://www.human-phenotype-ontology.org/) databases. Using “osteoporosis” as a keyword, the disease targets were searched from these databases and human genes were combined and duplicates were removed.

Building of protein-protein interaction (PPI) networks. Using the online tool Venny 2.1.0, the active ingredient targets of the HHML formula and the targets of the osteoporosis were entered separately to obtain the intersecting targets of the two. Then, the compound-disease targets were uploaded to the STRING database in txt format, and the parameter of species was set to “Homo sapiens”. In addition, the PPI network was obtained by eliminating the disconnected nodes with a confidence > 0.9 as a filtering condition. Using the Cytoscape 3.9.1 software to visualize the PPI network and adjust the nodes and edges to present it in an appropriate way. Analyzed core gene targets in conjunction with the cytoHubba plugin.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of compound-disease target genes. The DAVID database was able to obtain the results of GO and KEGG enrichment analyses, importing the targets shared by the compound-disease. The information of biological process, cellular component and molecular function were summarized respectively. In addition, KEGG pathway with the top 10 gene counts and the P value > 10^{-5} were selected as a focus for HHML action in osteoporosis.

Construction of compounds-disease-target-pathway network. Based on the target information obtained in the previous stage, it was inputted in the Cytoscape 3.9.1 software in txt format and edited for network visualization. The network presents multiple interrelationships between diseases and pathways, drugs and compounds, and between them and common gene targets, laying the foundation for subsequent analysis.

Molecular docking

The core components structural formulae were drawn by using the ChemBiodraw Ultra 14.0 software, converted to the lowest energy 3D mode and saved. The hub gene targets were selected from Protein Data Bank database (https://www.rcsb.org/) as well as saved in pdb format. Subsequently, the protein molecules were imported into the PyMol software for removing solvent and organic, and other operations. Docking analysis of small molecule ligands to proteins was performed in Autodock software, and lower binding energy was used as the basis for determining whether docking was successful.

Extraction, isolation and culture of bone marrow mesenchymal stem cells (BMSCs)

Whole bone marrow apposition method was used for extraction, four 4-week-old SD rats were taken, anesthetized, lower limbs were shaved, immersed in 75% alcohol for 15 min, separated the femur and tibia of the lower extremity from the whole, aseptically and washed twice in Hanks solution. Complete medium containing 10% fetal bovine serum, 5% dual antibody (penicillin + streptomycin), and α-minimum essential medium was prepared. And it was used to flush the bone marrow 2–3 times until the bone is white. Then, the cells were filtered, 1000 rpm, centrifuged for 5 min, the sediment in the tube was leaved, resuspended and centrifuged, and repeated twice. Finally, the culture medium was added to prepare a single-cell suspension, inoculated in a 100 mm dish and incubated at cell incubator.

CCK8 assay

The cytotoxicity of HHML was determined by CCK8 assay. Briefly, BMSCs were inoculated in 96-well plates at 1 × 10^{4}/ml and cultured for 24 h. Then, different concentrations (7.5, 15, 30, 60, 120, 240, 480, 560 μg/ml) of HHML total extract were added to intervene the cell growth, respectively. After the drug was co-cultured with the cells for 24 h, CCK8 reagent was added and co-incubated for 30 min, and its optical density value was detected using an enzyme marker.

Alkaline phosphatase (ALP) staining assay

The osteoblast-induced differentiation solution was prepared in advance, that is, complete medium with 10^{-7} M sodium β-glycerophosphate, and 50 μg/ml ascorbic acid to mix with BMSCs. BMSCs were inoculated in 6-well plates and ALP staining experiments were performed 7 and 14 days after treatment by HHML (30 μg/ml). According to the ALP staining (Sigma-Aldrich, Shanghai, China) instructions, 4% paraformaldehyde was fixed for 20 min and then added to the staining solution. Finally, the staining was observed under a microscope.

Quantitative polymerase chain reaction (Q-PCR) assay

A total RNA of BMSCs was extracted by cellular RNA extraction kit (ForeGENE, Chengdu, China). Briefly, cellular RNA was extracted and purified by adding the appropriate reagents according to the instructions, and detected the size of the concentration it contains. Then, 1 μg RNA was reverse transcribed to cDNA by using 2 × RTOR-Easy™Mix. The total reaction system of 20 μL contains cDNA and Fast SYBR Green qPCR Master Mix UDG amplification reaction reagents. The reaction conditions were all set according to the reagent instructions. The 2^{-ΔΔCt} method was used to analyze the expression levels of related genes. In addition, the sequences information of gene primers used were shown in Table 1.

Table 1 The sequences of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNX2</td>
<td>F: TCACAAATCTCCCTCCCAAGTGGA</td>
</tr>
<tr>
<td></td>
<td>R: GAATGCGCCCTAAATGACTGAA</td>
</tr>
<tr>
<td>OCN</td>
<td>F: GGCCAGTGGACAGAGCCAGG</td>
</tr>
<tr>
<td></td>
<td>R: GGGCTGGGGGCCTCAAGTCCAT</td>
</tr>
<tr>
<td>BSP</td>
<td>F: AGGATCGGCGAGGAGAAAT</td>
</tr>
<tr>
<td></td>
<td>R: TAGGGTGGCCGGTGACTTAAA</td>
</tr>
<tr>
<td>COLI</td>
<td>F: CTGCCCTCCGCSAGGGTTTGG</td>
</tr>
<tr>
<td></td>
<td>R: GCCCTGACATGCTGTGCGCCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: CAGGGTCGCTTCTCTGT</td>
</tr>
<tr>
<td></td>
<td>R: TCCTGTTAGTGACAGCTTC</td>
</tr>
</tbody>
</table>

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Western blot analysis

RIPA lysis solution was used to lyse cells at low temperature for a total of 30 min, scraped off, the collected samples were placed in centrifugation at 12,000 rpm for 10 min and the supernatant was taken. Subsequently, total proteins were separated by using 10% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% bovine serum albumin solution for 1 h, and then primary antibodies PI3K, p-PI3K, Akt, p-Akt, STAT3, TP53 and β-actin (ABclonal, Wuhan, China) were added and incubated at 4 °C overnight. The second antibody HRP-goat anti-rabbit or mouse antibodies (ABclonal, Wuhan, China) were added and incubated at room temperature for 1 h. Finally, washed 3 times with tris buffered saline with Tween 20 solutions, the protein bands on the membrane were imaged with electrochemiluminescence luminescent substrate.

Statistical analysis

The experimental data underwent rigorous statistical analysis employing GraphPad Prism 9 software, and the results were meticulously presented in the format of mean ± standard deviation. A comparative analysis between the two groups was executed through the application of an independent sample t-test, ensuring a robust evaluation of the observed outcomes. P value < 0.05 indicates statistical significance.

Results

Analysis of bioinformatics results

Prediction of bioactive ingredients and disease targets. After several databases as well as setting the corresponding parameters, 80 active ingredients of traditional Chinese medicine and 1532 compound targets in HHML were screened. In addition, osteoporosis targets were obtained from 6 databases, and the target information retrieved from each database was as follows, 6 in Online Mendelian Inheritance in Man database, 5630 in GeneCards, 1118 in DisGeNET, 28 in Therapeutic Target Database, 516 in Comparative Toxicogenomics Database and 317 in Human Phenotype Ontology database (Figure 2A). The HHML formula compounds were taken to intersect with disease targets to obtain 343 common targets (Figure 2B).

Analysis of PPI network and core target. To identify the core targets, we build a PPI network diagram as shown in Figure 3. The results obtained by analyzing with the STRING database showed that there are 342 nodes with 1014 edges, and the average degree is 5.93. The main data were inputted in the Cytoscape 3.9.1 software to visualize the PPI network and calculate the target-related properties include betweenness, closeness and degree value. According to the degree value of protein targets, the top 10 targets were extracted in descending order: TP53, AKT1, STAT3, HSP90AA1, ESRI, TNF, IL6, MAPK1, MAPK3 and MAPK8. The cytoHubba plugin analyzed this core protein targets as shown in Figure 4. In addition, the other parameters of core targets are presented in Table 2.

Enrichment analysis. The biological properties of these genes and proteins in the cell were obtained by GO analysis, with 903 GO terms in biological process, 100 in cellular component and 176 in molecular function. As shown in Figure 5A, the top 10 were selected for analysis based on the –log10P value. In order to investigate the mechanism and function exerted by HHML in the treatment of osteoporosis, we obtained 207 pathways, organized in a table, and the results showed that it was closely associated with the PI3K-Akt signaling pathway and MAPK signaling pathway (Figure 5B).

Compounds-target-pathway-drug-disease network analysis. To explore the mechanisms associated with OP symptom relief in HHML, we constructed a “compound-target-pathway-drug-disease” network. Among them, the large circle indicates the common target of the compound-diseases, and the color from deep to shallow is based on the degree value of the target from large to small; the green in the small circle denotes HHML, which connects blue denotes the 80 active ingredients, and the red in the circle denotes OP, which connects the top 10 signaling pathways screened (Figure 6). Based on the attributes such as strong correlation, degree value in the network diagram, 3 active ingredients were screened and the more detail was shown in Table 3.

Molecular docking analysis. Combined with the results of PPI network analysis above, we chosen AKT1, STAT3 and TP53 as the large molecule protein receptors, and the active ingredients in Table 3 were selected as the docked small molecule ligands. The molecular docking results of AKT1 (PDB ID: 6s9w), STAT3 (PDB ID: 6njs) and TP53 (PDB ID: 8f2h) with the 3 active ingredients, including baicalein, erysodienone and naringenin, respectively, were shown in Table 4, which showed good interactions with a range of binding energies from −4.04 to −7.114. As shown in Figure 7A, the AKT1 docking with 3 active ingredients, baicalein could from H bonds with ASP-274 (2.0 Å and 2.0 Å), TYP-272 (2.0 Å and 3.2 Å), erysodienone could develop H bonds with ASP-292 (3.3 Å), GLN-79 (2.8 Å), ASN-54 (2.5 Å), and naringenin could form H bonds with ASP-292 (1.9 Å), GLY-294 (1.7 Å), TLR-272 (2.8 Å), VAL-271 (3.3 Å), ASN-54 (1.9 Å). As shown in Figure 7B, the STAT3 docking with 3 active ingredients, baicalein could from H bonds with GLU-324 (2.7 Å), ILE-258 (2.2 Å), ASN-257 (1.9 Å), PRO-256 (1.8 Å and 2.4 Å), erysodienone could develop H bonds with ILE-258 (2.1 Å), GLN-326 (1.6 Å), and naringenin could form H bonds with ILE-258 (1.8 Å), GLN-247 (3.0 Å), ALA-250 (2.7 Å), GLN-326 (1.9 Å). As shown in Figure 7C, TP53 docking with 3 active ingredients, baicalein could from H bonds with GLY-279 (1.8 Å), ARG-283 (2.1 Å and 2.2 Å), LYS-120 (1.7 Å), erysodienone could develop H bonds with GLN-136 (2.0 Å), ALA-276 (2.2 Å), LYS-120 (2.1 Å), and naringenin could form H bonds with GLY-279 (2.0 Å), LYS-120 (1.8 Å), PRO-301 (1.8 Å). In a word, the docking results indicated that the target protein AKT1 had strong affinity with erysodienone than other proteins. The binding energy values of the other two compounds are not much different, and both are negative, showing a relatively stable state. From another perspective elucidates that HHML has multiple compounds and multiple targets anti-OP effects.

Figure 2 The venn diagram of the interaction between HHML and OP targets. (A) Venn diagram of the Osteoporosis Target Database and (B) intersection of HHML-OP targets. OP, osteoporosis; HHML, Hai Honghua medicinal liquor; OMIM, Online Mendelian Inheritance in Man; TTD, Therapeutic Target Database; CTD, Comparative Toxicogenomics Database; HPO, Human Phenotype Ontology.
Figure 3 The PPI network of HHML-OP protein targets. PPI, protein-protein interaction; HHML, Hai Honghua medicinal liquor; OP, osteoporosis.

Figure 4 The core protein targets network of HHML-OP in cytoHubba. HHML, Hai Honghua medicinal liquor; OP, osteoporosis.

Table 2 The core targets of HHML for OP and the topological parameters

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Symbol name</th>
<th>Degree</th>
<th>Betweenness</th>
<th>Closeness</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>Cellular tumor antigen p53</td>
<td>122</td>
<td>0.249328551</td>
<td>0.419306184</td>
</tr>
<tr>
<td>AKT1</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
<td>80</td>
<td>0.075578977</td>
<td>0.380821918</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
<td>76</td>
<td>0.059830037</td>
<td>0.378231293</td>
</tr>
<tr>
<td>HSP90AA1</td>
<td>Heat shock protein HSP 90-alpha</td>
<td>68</td>
<td>0.080198222</td>
<td>0.377717391</td>
</tr>
<tr>
<td>ESR1</td>
<td>Estrogen receptor</td>
<td>66</td>
<td>0.079467248</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
<td>58</td>
<td>0.042763531</td>
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<td>IL6</td>
<td>Recombinant Interleukin 6</td>
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<td>0.033125901</td>
<td>0.343634116</td>
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<td>MAPK1</td>
<td>Mitogen-activated protein kinase 1</td>
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<td>Mitogen-activated protein kinase 8</td>
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<td>0.05598477</td>
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</tbody>
</table>

HHML, Hai Honghua medicinal liquor; OP, osteoporosis.
Figure 5 The results of GO and KEGG enrichment analysis. (A) The BP, CC and MF of GO enrichment analysis of core genes. (B) Counting the top 30 pathways in KEGG of core genes. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

Figure 6 Compounds-target-pathway-drug-disease network of HHML-OP. HHML, Hai Honghua medicinal liquor; OP, osteoporosis.

Table 3 The effective compounds and their properties and structures

<table>
<thead>
<tr>
<th>Mol ID</th>
<th>Compound name</th>
<th>OB%</th>
<th>DL</th>
<th>Degree</th>
<th>Structure</th>
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<tbody>
<tr>
<td>MOL004328</td>
<td>naringenin</td>
<td>59.29</td>
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<tr>
<td>MOL002714</td>
<td>baicalein</td>
<td>33.52</td>
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<tr>
<td>MOL000451</td>
<td>erysodienone</td>
<td>37.29</td>
<td>0.44</td>
<td>24</td>
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</tr>
</tbody>
</table>

OB, oral bioavailability; DL, drug-likeness.

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Table 4 The binding energy of AKT1, STAT3 and TP53 docked with 3 active compounds

<table>
<thead>
<tr>
<th>Target</th>
<th>PDB ID</th>
<th>Compounds</th>
<th>Auto-dock energy</th>
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<tbody>
<tr>
<td>AKT1</td>
<td>6s9w</td>
<td>baicalein</td>
<td>-6.837</td>
</tr>
<tr>
<td></td>
<td></td>
<td>erysodienone</td>
<td>-7.114</td>
</tr>
<tr>
<td></td>
<td></td>
<td>naringenin</td>
<td>-6.763</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>naringenin</td>
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</table>

Figure 7 Molecular docking results of active ingredients of HHML. The baicalein, erysodienone and naringenin in the figure docked with (A) AKT1, (B) STAT3 and (C) TP53 proteins respectively, b, baicalein; e, erysodienone; n, naringenin; HHML, Hai Honghua medicinal liquor.

Results verified by cell experiments in vitro

BMSGs passing and culture. Primary cells were extracted from SD rat tibial bone marrow for culture (Figure 8A). The primary cells were expressed as P0, and the cells were recorded on day 3, day 6 and day 8, respectively (Figure 8B). As a result, it was observed that the number of cells gradually increased, the growth was extended, and the cell morphology was in the form of long spindle or fibroblasts, with an evident nucleus in the center of the cell and radially growth. In addition, P1, P2 and P3 of the passaged culture of BMSGs were recorded on day 6, and it was observed that the cells were in good growth condition and could be continued for subsequent experimental studies (Figure 8C).

Effect of HHML on cell viability. To validate the proliferative effect of HHML on BMSGs as postulated from above analysis, CCK8 assay was performed. When cells were treated with different concentrations (7.5-560 µg/mL) of HHML, it was found that HHML promoted cell growth rather than inhibited it. The promotion of cell proliferation after 24 h of HHML (50 µg/mL) treatment was significant compared to the control group ($P < 0.05$) (Figure 9).

Effect of HHML on osteogenic differentiation of BMSC cells. Generally, well-grown cells of the 2nd or 3rd generation were selected to induce differentiation into osteoblasts. ALP is a marker of early osteoblasts and can directly reflect the activity and functional status of osteoblasts. As shown in Figure 10, ALP staining was performed after 7 and 14 days of HHML treatment of BMSC cells, and it was found that the cells were stained blue, and the color was deepened at 14 days, indicating that HHML exerts positive effects on promoting the differentiation of BMSGs into osteoblasts.

Effect of HHML on the expression of osteogenesis-related genes. In order to investigate the expression of osteogenesis-related genes after HHML treatment of cells, we detected the expression of RUNX2, OCN, BSP and COL1 genes by q-PCR experiments, and the analysis of mRNA expression levels are illustrated in Figure 11. The relative expression of RUNX2, OCN, BSP and COL1 genes was remarkably increased ($P < 0.05$) after treatment in the HHML group compared with the control group, suggesting that HHML promotes the expression of genes associated with osteogenic differentiation.

Effect of HHML on the expression of PI3K/Akt pathway proteins and target proteins. To probe into the mechanism of HHML in alleviating OP, we analyzed key PI3K/Akt signaling pathway proteins and core target proteins for molecular docking results. As exhibited in the Figure 12, the phosphorylation of PI3K and Akt proteins were remarkably higher after HHML treatment compared to the control group ($P < 0.05$). Carefully analyzing the results of molecular docking, the main core target proteins were verified by Western blot experiments, which revealed that the phosphorylation level of STAT3 protein was significantly elevated in the HHML group vs. control group ($P < 0.05$), while the expression of P53 protein was inhibited ($P < 0.05$). It has demonstrated that the improvement of OP by HHML may be associated with the activation of PI3K/Akt signaling and the promotion of STAT3 protein phosphorylation as well as the inhibition of P53 protein expression.
Figure 8 The cultivation of BMSCs. (A) The brief flowchart of BMSC cell extraction; (B) growth of primary cells for 3, 6 and 8 days; (C) cells were cultured for the 1st, 2nd and 3rd passage. BMSCs, bone marrow mesenchymal stem cells; SD, Sprague Dawley.

Figure 9 The effect of HHML treatment on the viability of BMSCs. HHML, Hai Honghua medicinal liquor; BMSCs, bone marrow mesenchymal stem cells; OD, optical density.

Figure 10 The results of ALP staining in BMSCs. ALP, alkaline phosphatase; BMSCs, bone marrow mesenchymal stem cells.
Figure 11 The levels of relative expression of RUNX2, OCN, BSP and COL1 genes. The data were repeated three times. *P < 0.05, **P < 0.01 vs. the control group. HHML, Hai Honghua medicinal liquor.

Figure 12 Expression of critical pathway and core target proteins. (A) Expression of p-PI3K, PI3K, p-Akt and Akt protein was tested and the gray values of p-PI3K/PI3K and p-Akt/Akt were counted. (B) Expression of P53, p-STAT3 and STAT3 protein was detected and the gray values of P53 and p-STAT3/STAT3 were counted. The data were repeated three times. *P < 0.05, **P < 0.01 vs. the control group. HHML, Hai Honghua medicinal liquor.
Discussion

Our current study demonstrated that HHML exhibited promotion of differentiation of BMSCs into osteoblasts and expression of osteogenic hallmark genes in in vitro experimental studies. Network pharmacological analyses showed that HHML action on OP was closely related to the PI3K/Akt signaling pathway, and the role of this pathway with osteogenic differentiation was also verified. HHML can promote the generation of phosphorylated states of PI3K and Akt proteins, which may accelerate bone formation by activating this pathway. In addition, the molecular docking demonstrated that the three active ingredients screened docked with high affinity and stable binding to the three core target proteins. Among them, erysodienone was the active ingredient of E, echoing the existing studies [10, 11]. Furthermore, we verified the effects of HHML on the core target proteins STAT3 and TP53 by western blot experiments, promoting the phosphorylation of STAT3 protein and instead inhibiting the expression of TP53 protein. What we can gain from these findings is that the mechanism of functional compounds of HHML for OP is intricate and may involve multiple targets and pathways.

It is well known that OP is mainly caused by bone loss and destruction of bone structure, as well as defective bone formation failing to replace lost bone in a timely manner, and therefore research from promoting its bone formation is of interest [16, 17]. BMSCs are extensively used in bone tissue engineering because of their pluripotent differentiation potential, and are capable of differentiating into osteoblasts, chondrocytes, and adipocytes, among other cell lines, when stimulated under specific conditions [18]. BMSCs are often used as in vitro experimental model cells, either endogenous or exogenous, in studies to treat bone-related diseases such as OP and bone repair [19]. In the present study, we extracted BMSC cells from rat tibia, femur bone marrow to investigate whether HHML promotes the proliferation and differentiation of BMSC into osteoblasts for bone formation.

Furthermore, the PI3K/Akt is a classical molecular pathway involved in complex cellular biological processes and regulates multicular functions such as cell proliferation, apoptosis and metabolism [20, 21]. PI3K/Akt was found to be taken part in the regulation of OP, and inhibition of the PI3K pathway in osteoblasts revealed a reduction in mRNA expression of OCN, RUNX2, and likewise ALP activity [22]. In this study, we found that the effect of HHML on the differentiation of BMSC cells into osteoblasts also involves the PI3K/Akt pathway and promotes the expression of osteogenesis-related genes, a result that is consistent with other studies. In addition, the regulation of the PI3K/Akt pathway has been involved in studies of traditional Chinese medicinecompounding to treat OP and improve bone loss and bone microarchitecture, and activation of the pathway, regulates other relevant cytokines [23, 24]. And then, a few points of rationale need to be pointed out in response to the study of the core target. Target proteins including STAT3 and TP53, obtained based on molecular docking results, have been shown to play vital roles in bone homeostasis. There has been much evidence that STAT3 can regulate skeletal development and bone homeostasis by mediating osteoblast and osteoclast generation and differentiation, and if STAT3 is of loss, it is detrimental to bone development [25–27]. TP5, an oncogene, is involved in bone metabolism-related pathways. Studies have shown that P53 inhibits osteoblast proliferation and reduces bone formation, leading to an imbalance in bone homeostasis, which in turn accelerates the development of OP [28, 29]. We showed that HHML could elevate the phosphorylation of STAT3 protein and inhibit P53 protein, thus suggesting that STAT3 or P53 may be a target to focus on in subsequent studies of OP.

Conclusion

In conclusion, combining the above results we draw the following conclusions, the formula HHML containing 19 Chinese herbs has a certain improvement effect on OP and advanced the proliferation and differentiation of BMSC cells into osteoblasts; the occurrence of this effect may be associated with the activation of the PI3K/Akt molecular pathway, and HHML is able to promote the phosphorylation of PI3K and Akt proteins; because of the complexity of its composition, the treatment of the disease may involve a number of targets, among which STAT3 and TP53 may be the potential targets, and HHML is able to promote the phosphorylation of STAT3 proteins or inhibit P53, which promotes the formation of bone.

References


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