

The Therapeutic Potential of Pristimerin in Osteoarthritis: Mechanistic Insights from in vitro and in vivo Studies

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Objective: Osteoarthritis (OA), a degenerative disease marked by cartilage erosion and synovial proliferation, has led to an increased interest in natural plant-based compounds to slow its progression. Pristimerin (Pri), a triterpenoid compound derived from *Tripterygium wilfordii*, has demonstrated anti-inflammatory and antioxidant characteristics. This study explores the protective effects of Pri on OA and its potential mechanisms.

Methods: In this study, we examined the impact of Pri on the expression of inflammatory factors and extracellular matrix (ECM) degradation induced by IL-1 β in chondrocyte experiments. Bioinformatics analysis was then performed to investigate the potential signaling pathways involved in Pri's protective effects. Finally, the efficacy of Pri in reducing cartilage degradation was further evaluated in a destabilization of the medial meniscus (DMM) mouse model.

Results: Utilizing bioinformatics analysis and in vitro studies, it was revealed that Pri inhibits the activation of NF- κ B and MAPK signaling pathways, leading to the reversal of upregulated MMP-13 (matrix metalloproteinases-13), iNOS (inducible nitric oxide synthase), and COX-2 (cyclooxygenase-2) elicited by IL-1 β stimulation, as well as the partial restoration of Collagen-II levels. Furthermore, in a DMM mouse model, the group treated with Pri exhibited reduced cartilage degradation and slowed OA progression compared to the modeling group.

Conclusion: This research highlights Pri as a potential therapeutic agent for delaying OA progression.

Keywords: pristimerin, MAPK/NF- κ B, chondrocyte inflammation, cartilage protection, osteoarthritis, RNA transcriptome sequencing

Introduction

OA is a prevalent condition that affects joints, marked by the progressive deterioration of cartilage within the joint, alterations in the underlying bone structure, and the formation of bony outgrowths.^{1,2} The incidence of OA exhibits a consistent increase with advancing age, significantly influencing the health status of individuals and emerging as a primary reason for disability within the elderly demographic.³

This condition commonly appears due to gradual joint degeneration over time, resulting in stiffness, pain, and decreased mobility.⁴ These symptoms are more common in weight-bearing joints.^{5,6} In advanced stages, OA can also cause significant joint swelling and deformity.⁷ These clinical manifestations highlight the complexity of this degenerative joint disorder and emphasize its multifaceted impact on patients' health.^{8–10} Recent research has indicated that the upregulation of inflammatory factor expression is an integral part of the development of OA.^{11,12} It's worth noting that

excessive expression of IL-1 β significantly accelerates the progression of OA.¹³ Specifically, the elevated secretion initiates the generation and discharge of inflammatory agents and catabolic elements, such as cyclooxygenase-2 (COX-2), matrix metalloproteinases-13 (MMP-13), and inducible nitric oxide synthase (iNOS).¹⁴ The excessive release of inflammatory mediators accelerates the degradation of the extracellular matrix (ECM), critically undermining the structural integrity of cartilage tissue. This degradation results in chondrocytes losing their supportive and protective niche, which consequently disrupts their physiological functions. Essential components of the ECM, including collagen and proteoglycans, play critical roles in sustaining the normal metabolism, proliferation, and differentiation of chondrocytes. Excessive degradation of these ECM components severely impairs chondrocyte function, inducing abnormal cellular stress, elevating apoptosis rates, and ultimately accelerating joint degeneration. Thus, promising prospects for influencing the initiation and progression of OA lie in the effective inhibition of IL-1 β -induced inflammation.

Currently, OA management involves a combination of pharmaceutical and surgical approaches.^{15,16} Pharmacological treatment mainly includes the use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and aspirin to alleviate pain and mitigate inflammation; however, prolonged NSAIDs use may compromise renal and gastrointestinal functions, and these drugs cannot reverse the progression of the disease.¹⁷ Surgical interventions are predominantly directed towards patients in advanced stages, with the primary goal of joint replacement.¹⁸ Nevertheless, these procedures are accompanied by notable drawbacks, including high financial costs and considerable trauma. Emerging research underscores the potential of natural plant compounds in retarding OA progression.^{19–22}

Pri is derived from *Tripterygium wilfordii*, a widely distributed shrub in China belonging to the Celastraceae family. *Tripterygium wilfordii* is an ancient traditional Chinese medicine with hemostatic, anti-rheumatic, and anti-inflammatory properties. Considerable investigations highlight Pri's pharmacological spectrum, encompassing broad-spectrum anti-tumor, anti-inflammatory, and antioxidant activities.^{23,24} Specifically, Pri mediated anticancer effects and enhancement of susceptibility in human skin cancer cells via adjustment of MAPK signaling pathway.²⁵ Furthermore, Pri alleviates tendon lesions by regulating autophagy to stabilize AIM2-Picard/ASC.²⁶ However, there is currently no research reported on the involvement of Pri in OA. The aim of this research is to clarify the specific therapeutic effects and potential mechanisms of Pri in treating OA through bioinformatics analysis and in vitro and in vivo studies for the first time.

Materials and Methods

Materials and Reagents

In this experimental setup, MedChemExpress (Shanghai, China) supplied the Pristimerin (purity $\geq 98\%$, HY-N1937, CAS number: 1258–84–0) and Adezmapimod (purity $\geq 99\%$, HY-10256, CAS number: 152121–47–6). Primary antibodies against including collagen-II (CatNo. 28459-1-AP), iNOS (CatNo. 18985-1-AP), GAPDH (CatNo. 60004-1-Ig), COX-2 (CatNo. 12375-1-AP), β -actin (CatNo. 66009-1-Ig), LaminB (Cat No. 12987-1-AP), and MMP-13 (Cat No. 18165-1-AP) (with a dilution concentration of 1:3000) were applied by Proteintech (Wuhan, China). Additionally, p65 (Cat No. ab32536), I κ B α (Cat No. ab32518), p-p65 (Cat No. ab31624), and p-I κ B α (Cat No. ab92700) (with a dilution concentration of 1:1000) were acquired from Abcam (Cambridge, UK). P38 (Cat No. 9212S), P-JNK (Cat No. 4668T), P-ERK (Cat No. 4370T), JNK (Cat No. 9252T), ERK (Cat No. 4370T), and P-P38 (Cat No. 4511T) (with a dilution concentration of 1:2000) were sourced from Cell Signaling Technology (America, USA). Zhongshan Golden Bridge Biotechnology provides Goat AntiRabbit IgG (with a dilution concentration of 1:2000). Goat Anti-Mouse IgG was sourced by HuaAn Biotechnology (with a dilution concentration of 1:5000). Penicillin–streptomycin solution (100x), SYBR Green master mix, Collagenase II, 0.25% trypsin and cDNA Synthesis reagent were procured from Yi Sheng Biotechnology (Shanghai) Co., LTD. PageRuler™ Prestained Protein Ladder and Pierce™ BCA were obtained from Thermo Fisher Scientific. Fetal Bovine Serum of Australian Origin was purchased from Hangzhou Ouyuan Biotechnology Co, LTD. The RNA-Quick Purification kit was applied from Shanghai Yi Shan Biological Technology Co, LTD.

Extraction and Cultivation of Primary Murine Chondrocytes

Articular cartilage was harvested from the knees of neonatal wild-type mice (≤ 5 days old) under sterile conditions, with tissue collection performed using a dissecting microscope to ensure precision. Following dissection, cartilage samples

were transferred into sterile tubes containing PBS for temporary storage. Cartilage softening and chondrocyte isolation were subsequently achieved via enzymatic digestion with 2 mg/mL type II collagenase. Samples were incubated at 37°C in a CO₂ incubator for 4–6 hours, with gentle agitation every 30 minutes to promote uniform digestion, then centrifuged at 300 g for 5 minutes to collect chondrocytes. Cells were resuspended in chondrocyte growth medium (DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine) and plated in 6-well plates at a density of 1×10^5 cells per well. Chondrocytes were maintained at 37°C in a 5% CO₂ incubator, with media changes every 2–3 days. Once cells reached 80%–90% confluence, they were detached using 0.25% trypsin-EDTA solution for 2–3 minutes at 37°C. Detached cells were centrifuged, counted, and passaged at a 1:3 ratio. To preserve the phenotypic stability of chondrocytes, only passages F1 to F3 were used in subsequent experiments, as later passages are prone to dedifferentiation. All procedures were performed under aseptic conditions to avoid contamination.

Cell Viability Assay

The viability of chondrocytes was assessed through the Cell Counting Kit-8 (CCK-8) assay. Chondrocytes were initially seeded into 96-well microplates at a density of 8×10^3 cells per well and then treated with varying concentrations of Pristimerin (25 nm, 50 nm, 100 nm, 200 nm, 400 nm, 800 nm, 1000 nm, 2000 nm, and 4000 nm). After incubation periods of 24 and 48 hours, 10% CCK-8 reagent was added to the culture medium, followed by an additional 1–2 hours of incubation. Absorbance was subsequently measured using a spectrophotometer to evaluate cell viability.

RNA Extraction and Real-Time Polymerase Chain Reaction

Full RNA was extracted from chondrocytes according to the manufacturer's protocol. Firstly, the RNA was reverse-transcribed into cDNA. Then, Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the Roche LightCycler 480 detection system. Normalization of target mRNA levels to GAPDH levels was conducted, and the relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method. The sequence of the target gene is as follows: MMP-13: forward: TGTTCGAGAGCACTACTTGAA, reverse: CAGTCACCTCTAAGCCAAAGAAA (length: 150bp); iNOS: forward: GGAGTGACGGCAAACATGACT, reverse: TCGATGCACAACCTGGGTGAAC (length: 200bp); Collagen-II: forward: CAGGATGCCCCGAAAATTAGGG, reverse: ACCACGATCACCTCTGGGT (length: 180bp); GAPDH: forward: TGACCTCAACTACATGGTCTACA, reverse: CTTCCCATCTCTCGGCCTTG (length: 140bp); COX-2: forward: TTCCAATCCATGTCAAACCGT, reverse: AGTCCGGGTACAGTCACACTT (length: 170bp).

Protein Extraction and Western-Blot Assay

After washing cartilaginous cells three times with pre-chilled PBS, protein extraction was performed by adding lysis buffer. The lysate was prepared by combining PMSF and RIPA buffer (1:100) and underwent a 40-minute ice-cold lysis. Following that, the lysate underwent high-speed centrifugation at 4 °C (14,400 rpm, 15 minutes). Subsequently, the obtained supernatant was collected, and the protein content in the samples was assessed utilizing the BCA protein assay kit. Total proteins were electrophoretically separated on an 8% SDS-polyacrylamide gel at 80 V for 1 hour and 120 V for an additional hour, and subsequently transferred onto a PVDF membrane. After 2 hours of room temperature incubation with 5% skim milk for membrane blocking, the membrane underwent three washes with TBST, each lasting 10 minutes. The membrane was incubated overnight at 4 °C with the primary antibody dilution buffer. Following an overnight incubation period, the membrane was rinsed three times with 1% TBST for 10 minutes each. Subsequently, the membrane was incubated with a specific secondary antibody at room temperature for 2 hours. After three additional washes with TBST, protein expression was detected using the ChemiDoc™ MP imaging system.

Immunofluorescence

After cultivating chondrocytes in confocal dishes, the chondrocytes were treated with IL-1 β or IL-1 β +Pri (200nmol) for 24 hours. They were fixed with 4% paraformaldehyde for 30 minutes, followed by an additional three washes with PBS. Subsequently, membrane permeabilization was achieved using 0.3% Triton X-100 for 25 minutes, after being blocked with 5% BSA for 50 minutes, the cells were incubated with the primary antibody for overnight. After the overnight incubation,

cells underwent a triple wash with PBS, followed by 2 hours of incubation with the secondary antibody. Subsequently, DAPI staining was performed for 5 minutes. Finally, fluorescence intensity was assessed using laser confocal microscopy.

RNA Transcriptome Sequencing

Chondrocytes from the F1 generation were treated with IL-1 β or for 24 hours. Subsequently, RNA was extracted from the control group, IL-1 β group, and IL-1 β +Pri (200nmol) group for transcriptome sequencing. Specifically, the raw data of transcriptome sequencing were filtered to obtain high-quality data. The DESeq v1.40.2 software was employed to study differential gene expression among the groups, with conditions set as $|\log_2FC| > 1$ for fold change and $P < 0.05$ for significance, to filter differentially expressed genes. To explore the functional roles of significantly differentially expressed mRNAs and their associated signaling pathways, we conducted enrichment analyses on KEGG and GO pathways. The relevant information was then visualized and analyzed using the R language.

Molecular Modeling

AlphaFold was employed to generate predictive models for the target protein's structures. When setting the protonation state of the small molecule to pH = 7.4, the compound was transformed into its three-dimensional structure. AutoDock Tools 3 was utilized to prepare the receptor protein and ligand, including the generation of docking boxes through AutoGrid. Subsequently, we performed molecular docking using AutoDock Vina. Ultimately, the protein-ligand interaction diagram was created using PyMOL.

Animal Model

Eight-week-old male wild-type mice were purchased from Jiangsu Jicui Yaokang Biotechnology Co., Ltd with an average weight ranging from 25 to 30 g. Eighteen mice were randomly divided into three groups: sham surgery group, model group, and Pri treatment group. The OA model was induced according to established protocols, four weeks post DMM surgery. The drug treatment group received an intra-articular injection of Pri solution at a volume of 5 μ L with a concentration of 200 nmol,²⁷ administered weekly for four consecutive weeks. The modeling group and sham surgery group were administered a matching quantity of normal saline weekly for four consecutive weeks. In the end, euthanasia was performed on the 18 mice two months post-DMM surgical operation, followed by the collection of joint tissue specimens for further assessment.

Histological Analysis

Knee joints were procured, and each sample was fixed in 4% paraformaldehyde for 48 hours. Subsequently, the specimen was decalcified in a 10% EDTA solution for one month. The decalcified specimen embedded in paraffin was sectioned into 5 μ m-thick slices. To assess the extent of cartilage erosion, Safranin-O/Fast Green and Hematoxylin-Eosin staining were employed for evaluation. The evaluation of histological changes in root sections was performed using an optical microscope and the OARSI scoring system. The sections were subjected to an overnight incubation with MMP-13 and Collagen-II antibodies, subsequently, the corresponding enzyme-linked secondary antibodies were incubated at ambient temperature for two hours. Alterations in expression were observed and documented. Stained sections were carefully photographed under an optical microscope.

Statistical Analysis

All experiments were independently conducted three times. GraphPad Prism 9 was utilized for graphical representation. One-way analysis of variance (ANOVA) was applied for comparing multiple groups, followed by Tukey's test for pairwise comparisons. A significance level of $P < 0.05$ was deemed statistically significant.

Results

Effect of Pri on the Viability of Chondrocytes

Figure 1A shows the molecular structure of Pri. Chondrocytes underwent treatment with various concentrations of Pri (25 nm, 50 nm, 100 nm, 200 nm, 400 nm, 800 nm, 1000 nm, 2000 nm, 4000 nm) for 24 and 48 hours. The effects of Pri on

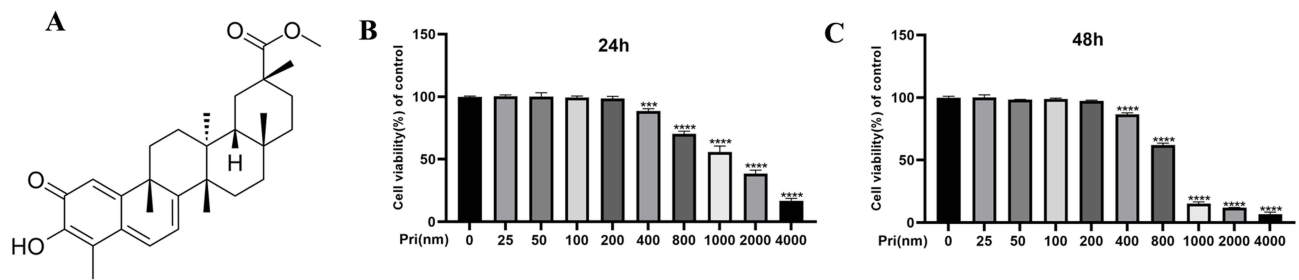


Figure 1 The molecular structure of Pri and its effect on the viability of chondrocytes. Pri's impact on the viability of chondrocytes. **(A)** Pri's chemical make-up and structure. **(B and C)** Cells were treated by several concentrations (25nm, 50nm, 100nm, 200nm, 400nm, 800nm, 1000nm, 2000nm, 4000nm) for 24 or 48 h. The viability of chondrocytes was assessed by CCK-8 assay. The results from three independent experiments are presented as mean $\pm$ standard deviation. (** $p < 0.001$, *** $p < 0.0001$.) indicates statistical significance, $n=3$.

chondrocyte viability were assessed utilizing the CCK-8 (a widely utilized assay for assessing cell proliferation and viability) test. Pri concentrations below 400 nmol showed no apparent effect on chondrocyte viability. In contrast, concentrations at or above 400 nmol led to a gradual decrease in cell viability (Figure 1B and C). Consequently, Pri concentrations of (50 nm, 100 nm and 200 nm) were selected for ensuing trials.

Effect of Pri on IL-1 β -Induced Inflammatory Factors in Chondrocytes

Elevated IL-1 β levels serve as a pivotal driver in the progression of OA, markedly upregulating the expression of additional inflammatory mediators, thereby intensifying the inflammatory cascade. This cascade of heightened inflammatory mediators contributes to enhanced cellular apoptosis and inhibits the synthesis of the cartilage matrix. After stimulation with IL-1 β or Pri+IL-1 β , the impact of Pri on the mRNA and protein of iNOS and COX-2 was assessed. The findings reveal an increase in the expression of iNOS and COX-2 stimulated by IL-1 β , the weakening is diminished in a concentration-dependent fashion after treatment with Pri (Figure 2A-E). Specifically, higher concentrations of Pri result

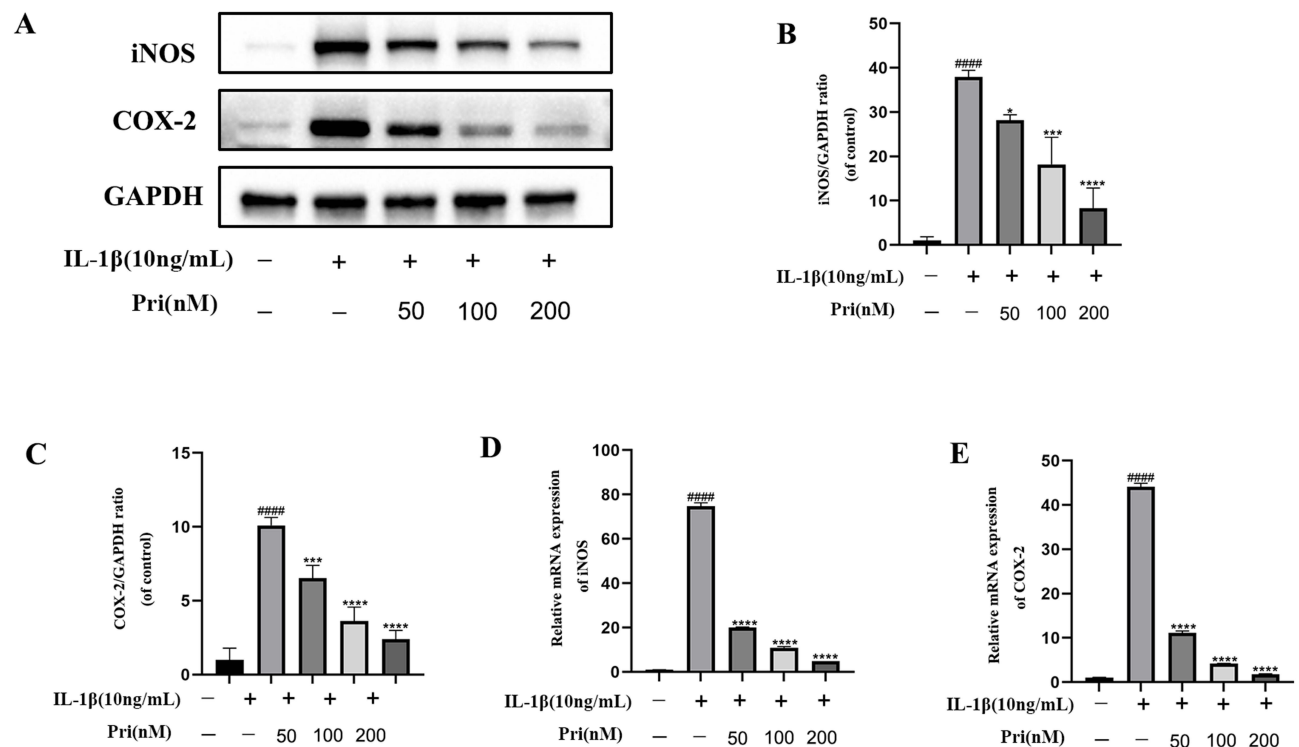


Figure 2 Pri downregulated the upregulated expression of inflammatory mediators, including iNOS and COX-2, at both the protein and mRNA levels. Cells were treated with IL-1 β (10 ng/mL) with or IL-1 β +Pri (50 nm, 100 nm, and 200 nm) for 24 hours. **(A–C)** Protein expression of iNOS and COX-2 analysis were measured by Western blot. **(D and E)** RT-qPCR was utilized to assess iNOS and COX-2. The results from three independent experiments are presented as mean $\pm$ standard deviation (SD). (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.0001$) indicates statistical significance, $n=3$.

in a more pronounced attenuation of the observed weakening, demonstrating a clear dose-dependent correlation between drug concentration and therapeutic efficacy. Accordingly, our findings indicate that Pri can partially reverse IL-1 β -induced upregulation of inflammatory mediators, a key mechanism in attenuating the inflammatory response underlying osteoarthritis progression.

Pri Regulates Ecm Degradation in IL-1 β -stimulated Chondrocytes

The ECM is an essential component of cartilage, offering essential structural support and mechanical properties that are vital for regulating chondrocyte metabolism and function. By analyzing the protein and mRNA expression levels of MMP-13 and Collagen-II, we assessed the impact of Pri on ECM degradation. After IL-1 β stimulation, there was a notable elevation in both mRNA and protein levels of MMP-13, coupled with the suppression of Collagen-II mRNA and protein expression. In contrast, after treatment with Pri, there was a concentration-dependent reversal of this effect (Figure 3A-E). These alterations are pivotal in preserving the integrity of the ECM, as reduced MMP-13 activity mitigates cartilage degradation, while elevated Collagen-II levels promote ECM restoration. This regulatory mechanism preserves cartilage architecture, preventing further degradation and fostering joint health within the framework of OA. The immunofluorescence results aligned with the aforementioned findings (Figure 3F and G). Therefore, Pri effectively hinders ECM degradation in cartilage cells.

Pri Inhibits the Activation of the NF- κ B Pathway

Our examination aimed to assess the influence of Pri on NF- κ B expression and phosphorylation. Upon IL-1 β stimulation, we observed a significant increase in I κ B α (the α subtype of the inhibitor of nuclear factor κ B) degradation, coupled with noticeable nuclear translocation of p65. On the contrary, the Pri-treated group inhibited the degradation of I κ B α and reduced the nuclear translocation of P65 (Figure 4A-C). Immunofluorescence microscopy further confirmed the protective effect of Pri on NF- κ B translocation into the nucleus (Figure 4D). Collectively, Pri modulates the NF- κ B signaling pathway to regulate the progression of OA, which is of great significance as NF- κ B plays a central role in driving inflammation and catabolic processes in OA.

Analysis of RNA Transcriptome Sequencing Results

To further investigate the potential upstream mechanisms through which Pri alleviates OA, we conducted RNA transcriptome sequencing on chondrocytes from the blank group, IL-1 β -induced group, and Pri-treated group. KEGG pathway analysis revealed that, compared to the blank group, IL-1 β prominently upregulated pathways related to cancer, MAPK signaling pathway and glycolysis, showing higher enrichment levels (Figure 5A). In contrast, the Pri-treated group, relative to the IL-1 β -induced group, exhibited significant downregulation in cancer pathways, MAPK signaling pathway, and cytokine-cytokine receptor interaction, with high enrichment levels (Figure 5B). Therefore, the MAPK signaling pathway might represent a potential upstream mechanism by which Pri mitigates OA, offering a promising therapeutic avenue for slowing the progression of osteoarthritis and preserving joint health.

Molecular Docking Results

We conducted an analysis and classification of the identified interaction forces, revealing the direct formation of multiple interaction forces between small molecules and receptor proteins. Ligands for Pri, P38, JNK and ERK exhibited stable hydrogen bonding interactions. The binding energies for the protein-small molecule complexes were determined to be (−7.6 kcal/mol-9.0 kcal/mol, −8.7 kcal/mol), demonstrating an overall outstanding performance (Figure 5C).

Pri Inhibited MAPK Phosphorylation in Chondrocytes

Early investigations have highlighted the central role of the MAPK pathway in OA. Through our exploration, which involved RNA transcriptome sequencing and molecule docking analysis, we discovered a close interaction between Pri and the MAPK pathway. Therefore, exploring the impact of Pri on the regulation of MAPK-related protein activation becomes crucial. Our analysis using WB revealed that IL-1 β markedly enhances the phosphorylation of MAPK. In contrast, the Pri treatment group exhibited attenuation of MAPK phosphorylation (Figure 6A-D). Then, we employed the

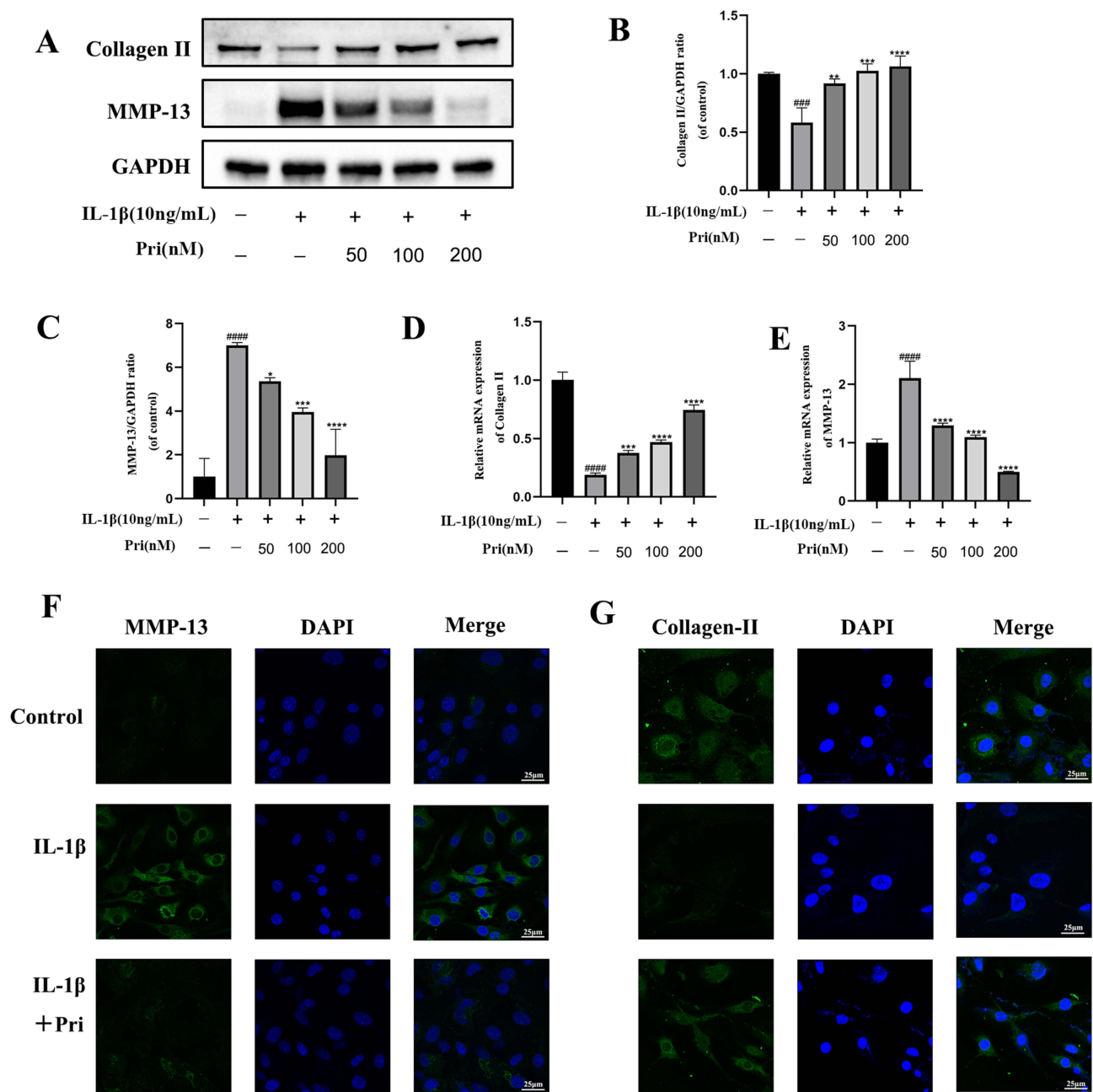


Figure 3 Pri modulated the expression of ECM components, including collagen-II and MMP-13, in IL-1 β -stimulated chondrocytes, partially mitigating ECM degradation. Chondrocytes were treated with IL-1 β (10 ng/mL) with or without Pri (50 nm, 100 nm, and 200 nm) for 24 hours. (A–C) Using Western blot evaluated the protein levels of collagen-II and MMP-13. (D and E) The mRNA expression of MMP-13 and Collagen-II was assessed via RT-qPCR. (F and G) Immunofluorescence staining was utilized to observe the expression of collagen-II and MMP-13. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ##### $p < 0.0001$) indicates statistical significance, $n=3$.

p38 agonist addezmapimod to stimulate the MAPK pathway, phosphorylation of P-P38 was assessed by Western blot, with subsequent Western blot analyses to measure inflammatory markers and cartilage synthesis-related factors. Results indicated that addezmapimod effectively restored p38 phosphorylation levels previously reduced by pri treatment (Figure 6E-F). Additional analyses revealed increased protein levels of COX-2, iNOS and MMP-13, alongside a reduction in Collagen-II expression. Therefore addezmapimod-mediated p38 activation successfully partially counteracted pri-induced changes in inflammatory and cartilage synthesis markers (Figure 6G-K). This suggests that Pri shows promise in mitigating the advancement of OA by reducing the Excitation of the IL-1 β -stimulated MAPK pathway.

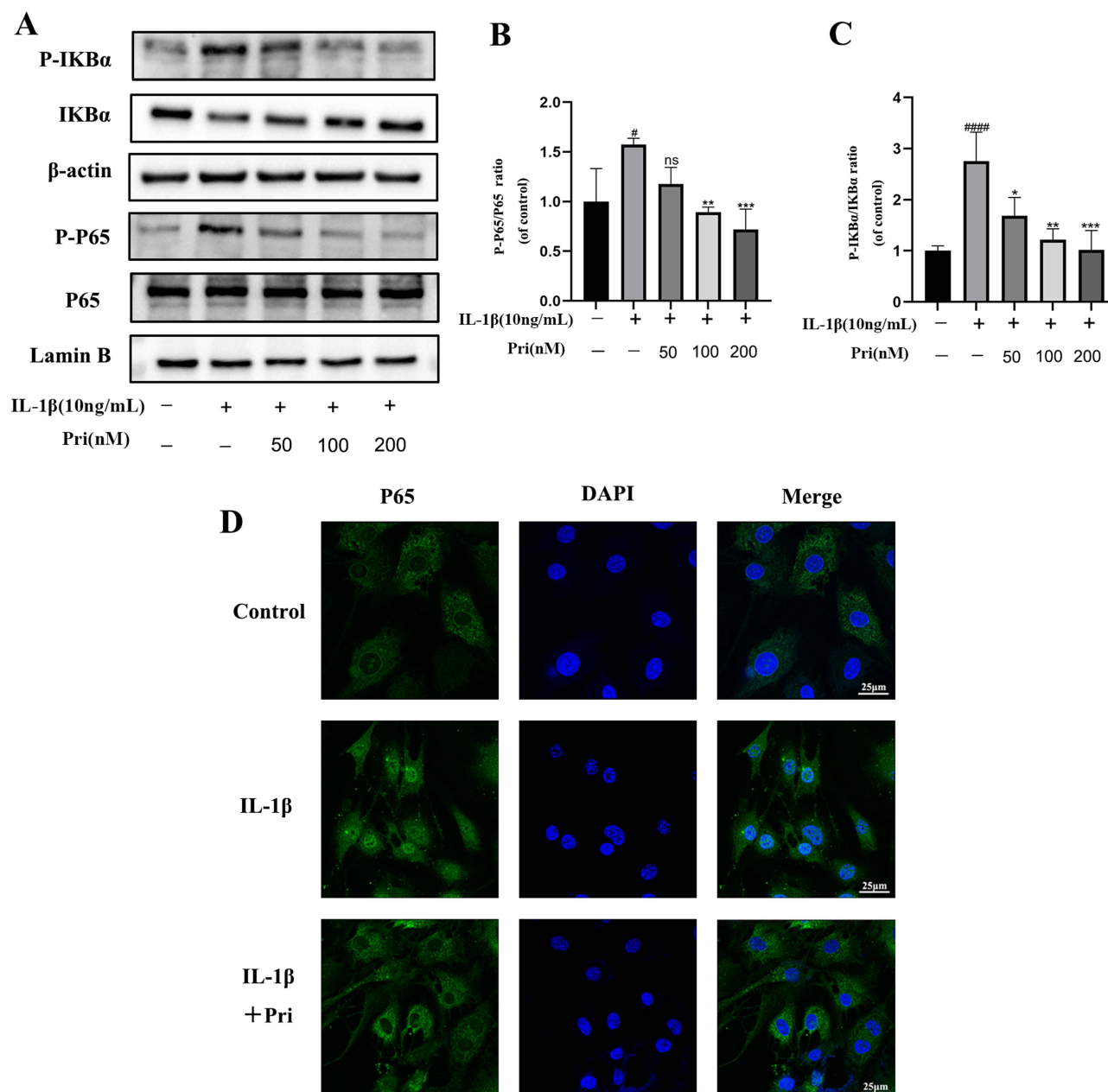


Figure 4 Pri inhibits activation of the NF- κ B pathway, this regulation of NF- κ B signaling highlights Pri's potential therapeutic role in controlling chronic inflammation and preserving joint health. Cells were treated with IL-1 β (10 ng/mL) with or without Pri (50 nm, 100 nm, and 200 nm) for 6 hours. (A–C) The expression of NF- κ B and I κ B α were investigated using Western blotting. (D) Immunofluorescence staining was utilized to observe p65 nuclear translocation. (* p < 0.05, ** p < 0.01, *** p < 0.001, # p < 0.05, #### p < 0.001, ##### p < 0.0001) indicates statistical significance, (ns p > 0.05) indicates no significant difference, n =3.

Histopathological Analysis and Morphological Evaluation of Tissues

By employing the DMM model in mice, we assessed the *in vivo* efficacy of Pri in attenuating the progression of OA. Safranin-O/Fast Green and Hematoxylin-Eosin staining revealed distinct morphological differences, where the sham-operated rats exhibited a smooth articular cartilage surface, contrasting with the OA rats characterized by evident roughness, cartilage destruction, and diminished cartilage thickness. Conversely, the mice in the treatment group displayed mitigated OA symptoms, demonstrating significantly lower severity than the modeling group (Figure 7A and B). Meanwhile, OARSI scores revealed a significant increase in the OA group. On the other hand, the scores in the Pri group were markedly lower than those in the OA group (Figure 7C). Additionally, compared with the control group, the modeling group displayed a decline in collagen-II-positive cells and an increase in MMP-13-positive cells. This trend

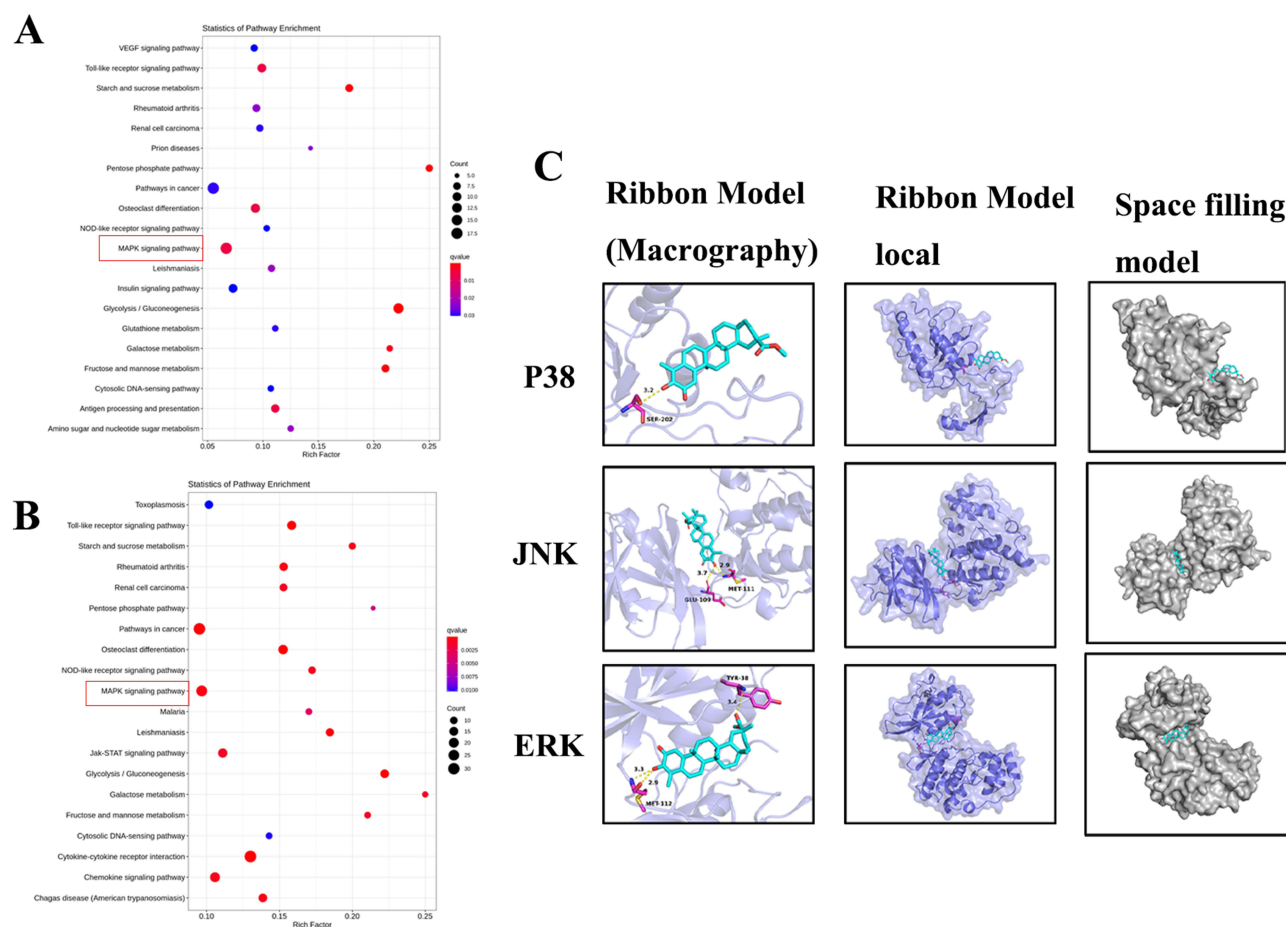


Figure 5 RNA transcriptome sequencing combined with molecular docking analysis indicates that the MAPK pathway is a potential upstream mechanism through which Pri exerts its therapeutic effects in alleviating OA. **(A and B)** The KEGG pathway analysis was conducted on differentially expressed genes. **(C)** The molecular docking results demonstrate that Pri tightly binds to p38, JNK and ERK proteins. Pri formed hydrogen bonds with SER-202, GLU-109, MET-111, MET-112 and other molecules.

was effectively reversed by Pri treatment (Figure 7D and E). These collective findings indicate that Pri treatment exerts a certain degree of inhibition on ECM degradation, providing protection to knee joint tissues against damage and inflammation. Consequently, it alleviates the progression of OA.

Discussion

OA, a degenerative condition common among the elderly, is characterized by joint cartilage damage and inflammation.²⁸ Although NSAIDs are extensively utilized in managing OA, their long-term usage may lead to a range of side effects, particularly adverse effects on the digestive system. These medications can potentially induce gastric ulcers, bleeding and other gastrointestinal issues.²⁹ Joint replacement surgery remains the sole effective option for advanced OA cases, albeit carrying inherent invasiveness and surgical risks.³⁰ Consequently, there exists a necessity to investigate novel therapeutic modalities for alleviating the onset and progression of OA. In recent times, there has been extensive exploration of traditional plant-derived remedies for their potential in addressing OA.³¹ Pri, a natural triterpenoid compound derived from *Tripterygium wilfordii*, has demonstrated notable efficacy in conditions such as colitis, autoimmune hepatitis, and autoimmune arthritis.^{32,33} However, the potential therapeutic benefits of Pri in the context of OA and its specific mechanisms remain uncharted. This research aims to comprehensively reveal the role and possible mechanisms of Pri in slowing down the advancement of OA, employing a combination of in vitro and in vivo experiments and bioinformatics analysis. Through cellular experiments and bioinformatics analysis, we investigate the impact of Pri on key signaling pathway and its regulatory effects on associated phenotypic changes. Simultaneously, in vivo experiments

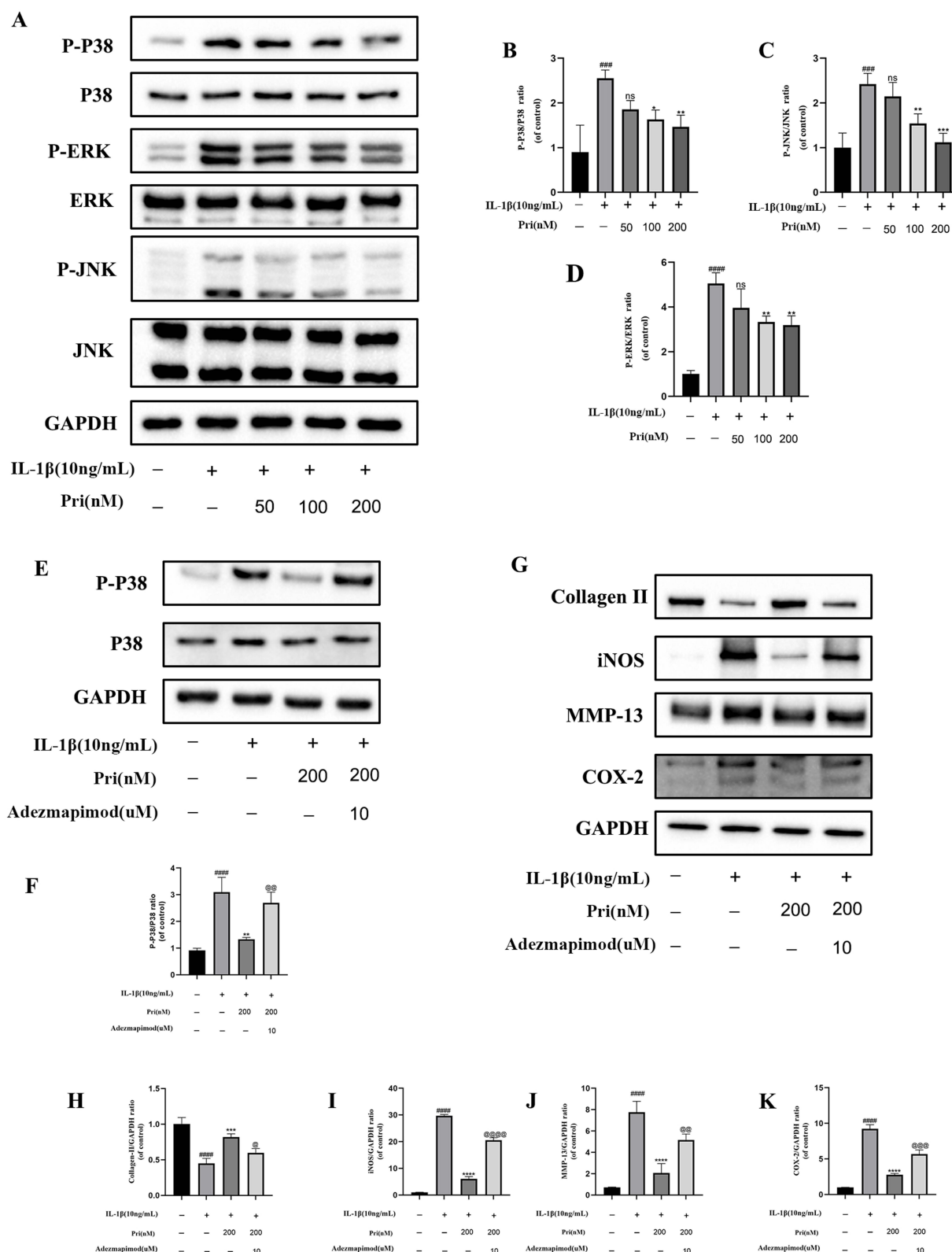


Figure 6 Pri inhibits the phosphorylation of key proteins in the MAPK pathway, thereby attenuating pro-inflammatory and catabolic signaling responses. Chondrocytes were treated with IL-1 β (10 ng/mL), IL-1 β +Pri (50 nm, 100 nm, and 200 nm) and atezmapimod (10 μ m) for 12 hours. **(A–K)** Western blot was used to detect the expression of P38, P-P38, ERK, p-ERK, JNK, p-JNK, Collagen II, iNOS, MMP-13, COX-2. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, @ $p < 0.05$, @@ $p < 0.01$, @@@ $p < 0.001$, @@@@ $p < 0.0001$) indicates statistical significance, (ns $p > 0.05$) indicates no significant difference, $n=3$.

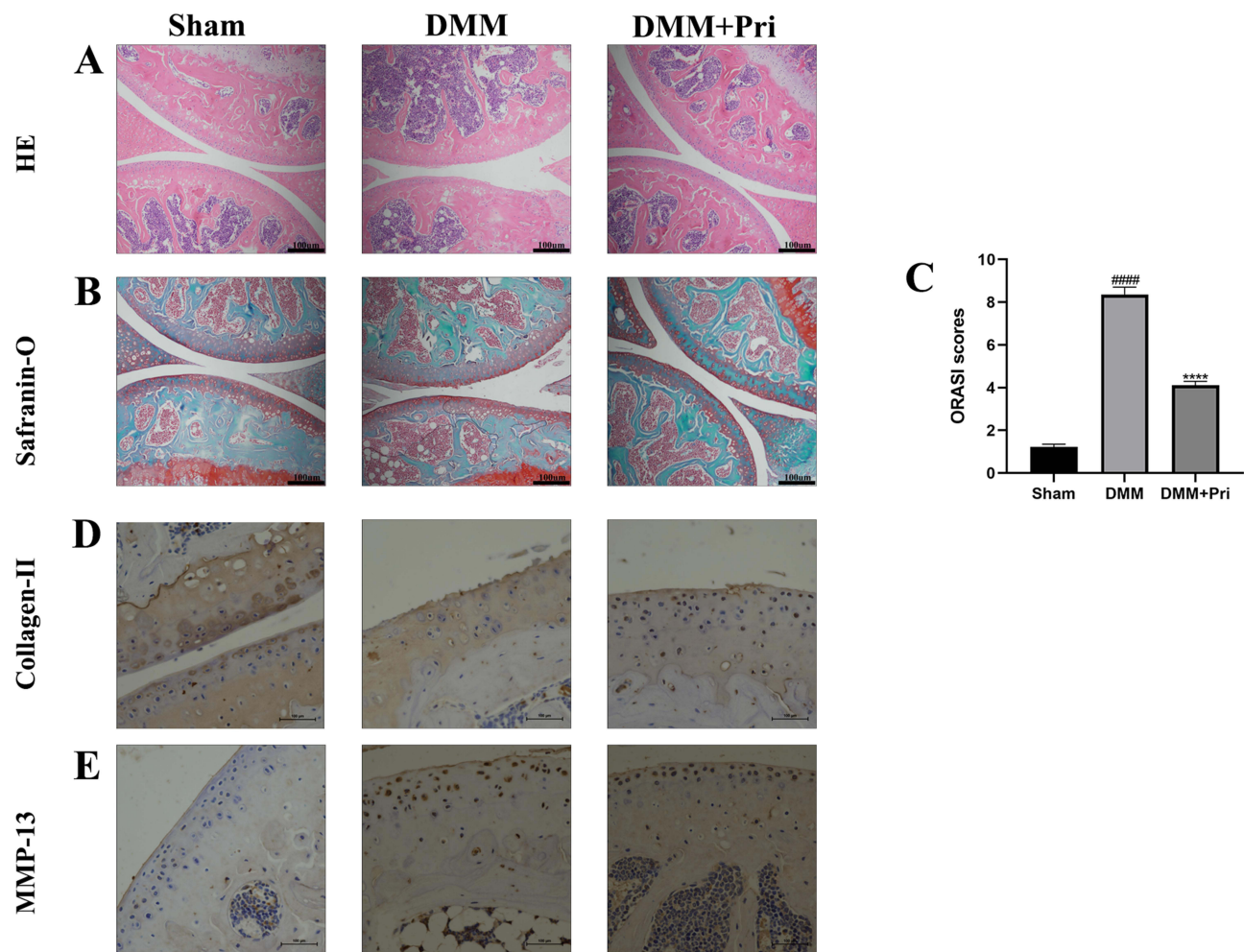


Figure 7 In the mouse DMM model, histological analysis and immunohistochemistry demonstrated that Pri mitigated the progression of OA in vivo. (A and B) After 8 weeks post-surgery, this figure displays typical Safranin-O/Fast Green and Hematoxylin-Eosin staining of cartilage in three groups, $n=6$. (C) Sham surgery, DMM, and DMM +Pri groups were evaluated according to the scoring system established by OARSI. (D and E) Immunohistochemical staining was performed to detect the expression of MMP-13 and Collagen-II in the cartilage samples, $n = 6$. (**** $p < 0.0001$, #### means $p < 0.0001$) indicates statistical significance.

utilizing an animal model mimicking OA, we explore the tissue-level impacts of Pri to attain a more comprehensive comprehension of its therapeutic potential.

At the outset of the experiment, we conducted a cytotoxicity assay to establish the non-toxic concentration range of Pri for chondrocytes. It was ultimately determined that Pri at concentrations of 50 nM, 100 nM, and 200 nM did not impair chondrocyte viability. Among the myriad pathogenic factors contributing to OA, chronic low-grade inflammation stands out as a prominent instigator. In the array of pro-inflammatory cytokines implicated in OA progression, IL-1 β assumes a pivotal role.³⁴ On one front, the heightened expression of IL-1 β triggers chondrocytes to unleash a substantial quantity of MMP-13.³⁵ This excessive release of MMP-13 disrupts the delicate equilibrium between ECM degradation and synthesis, ultimately resulting in diminished expression of collagen-II.³⁶ On the other hand, IL-1 β , through the induction of heightened COX-2 gene expression, concurrently amplifies the upregulation of iNOS, resulting in an augmented production of iNOS. Elevated NO levels can to a certain extent, impede the synthesis of the ECM and foster ECM degradation.³⁷ Our experimental results demonstrate that Pri effectively inhibits the upregulation of iNOS, COX-2, MMP-13 and the downregulation of collagen-II. This suggests that Pri plays a protective role in preserving the integrity of the ECM, thereby mitigating cartilage degradation in OA.

NF- κ B is a transcription factor group that intricately regulates various biological processes, including inflammation, immune response, and apoptosis.³⁸ Research indicates that different signaling pathways can activate NF- κ B, leading to

an increased expression of pro-inflammatory and catabolic factors, thereby exacerbating the progression of OA.^{39,40} It actively participates in cellular survival, proliferation, and stress responses, playing a pivotal role in diverse biological pathways. NF- κ B commonly associates with the inhibitory protein I κ B α , existing in an inactive state within the cytoplasm. IL-1 β can induce the degradation of I κ B α , subsequently leading to an elevation in NF- κ B nuclear translocation. In this experiment, Pri mitigates the progression of OA by attenuating I κ B α degradation and reversing p65 Nuclear migration.⁴¹ By disrupting this key inflammatory signaling pathway, Pri effectively limits the activation of pro-inflammatory and catabolic factors, highlighting its potential as a therapeutic agent in controlling inflammation and preserving cartilage integrity in OA.

To further elucidate the potential pathways by which Pri alleviates the progression of OA, RNA transcriptome sequencing reveals that Pri primarily reduces the expression of proteins linked to tumor-related and mitogen-activated protein kinase signaling pathways. Molecular docking analysis concurrently indicates a stable interaction between Pri and P38, JNK, and ERK. MAPK primarily consists of the P38, JNK and ERK signaling pathways. It transduces extracellular stimuli intracellularly, regulating gene expression, and plays a pivotal role in preserving cellular function and adapting to environmental changes.^{42,43} Earlier investigations have indicated that intervention in the MAPK pathway expression can alleviate the progression of OA.^{44,45} In this research, Western blotting results indicate that Pri exhibits a concentration-dependent inhibition of the phosphorylation of JNK, ERK and P38. Subsequently, we used P38 phosphorylation agonist to stimulate MAPK activation, and protein blotting experiments were used to detect the phosphorylation level of P38 as well as the levels of osteoarthritis inflammatory factors and cartilage synthesis-related factors, and found that P38 agonists were successful in partially reversing the protective effect of pri. Building upon the aforementioned in vitro experiments, we conducted further assessments of Pri's protective effect on OA through in vivo experiments. The DMM model was employed to induce the progression of OA, and subsequent intra-articular drug injections were administered periodically to evaluate the therapeutic efficacy of Pri. Safranin-O/Fast Green and H-E staining results revealed

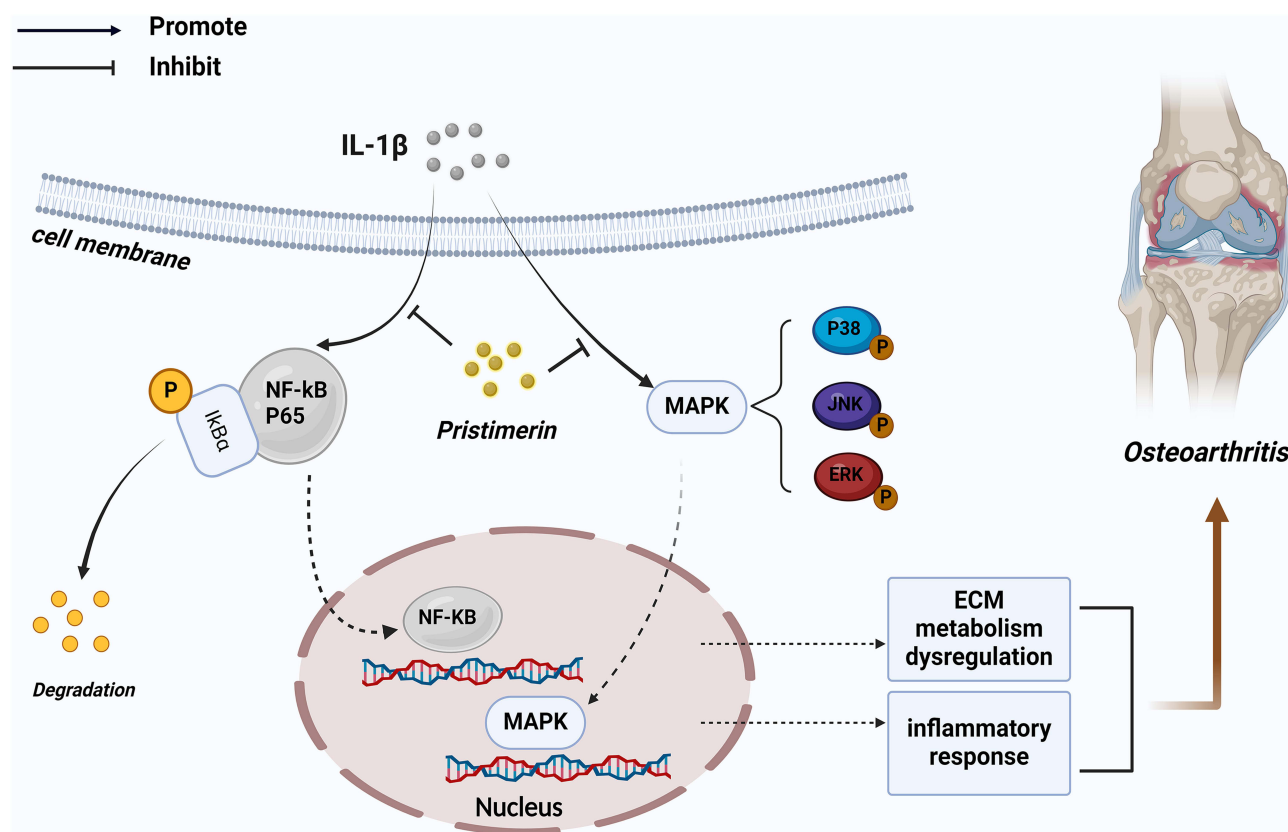


Figure 8 Molecular mechanism of Pristimerin on IL-1 β -induced mouse chondrocytes.

a noteworthy reduction in the severity of cartilage erosion in the treatment group in contrast to the model group. Additionally, immunohistochemical analysis was performed to evaluate the expression of ECM-related genes and inflammatory factors, confirming the protective outcome of Pri, consistent with the observed *in vitro* effects of Pri. In summary, Pri alleviates osteoarthritis progression by inhibiting MAPK pathway activation, specifically through concentration-dependent suppression of JNK, ERK and P38 phosphorylation. Both *in vitro* and *in vivo* experiments, including RNA transcriptome sequencing, molecular docking, and histological analyses, confirm Pri's protective effect on cartilage and ECM integrity in OA models.

Pri is widely distributed and accessible, so it has certain significance in reducing medical costs. This study demonstrated through *in vivo* experiments that Pri targets the MAPK signaling pathway to alleviate the progression of OA. Animal experiments further supported the efficacy of Pri. We further summarize the molecular mechanism of Pri in Figure 8. Given the complexity and challenging nature of OA, the development and clinical application of Pri drugs could offer new treatment options for patients, aiming to alleviate symptoms and enhance quality of life. Therefore, Pri shows promise as a potentially effective drug for treating OA.

In the future, further clinical trials and extended investigations will be crucial to comprehensively assess the efficacy and safety profile of Pri in humans. Optimization of dosage, delivery methods, and overcoming potential challenges in large-scale production will be pivotal steps toward translating these experimental findings into clinical applications. If Pri demonstrates efficacy in larger trials, its integration into OA management could offer a more accessible and cost-effective therapeutic option, particularly for populations with limited access to advanced medical care.

Conclusions

Our *in vitro* studies reveal that Pri potently suppresses the expression of pivotal inflammatory mediators, including iNOS, COX-2, and MMP-13, in IL-1 β -activated mouse chondrocytes. Simultaneously, Pri markedly attenuates ECM degradation. Additionally, our *in vivo* studies demonstrate that Pri delays OA progression by modulating key signaling pathways, notably the MAPK pathway. These findings underscore Pri's therapeutic potential in the treatment of OA and position it as a promising candidate for further drug development targeting OA therapy.

Abbreviations

OA, osteoarthritis; Pri, pristimerin; ECM, extracellular matrix; IL-1 β , interleukin-1 β ; DMM, destabilization of the Medial Meniscus; NF- κ B, nuclear factor kappa-B; MAPK, mitogen-activated protein kinase; MMP-13, matrix metalloproteinase-13; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; NSAIDs, non-steroidal anti-inflammatory drugs; RT-qPCR, real-time quantitative polymerase chain reaction; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; OARSI, osteoarthritis Research Society International; NO, nitric oxide.

Data Sharing Statement

Data will be made available on request.

Ethics Approval and Informed Consent

All experimental procedures adhered to the standards outlined by the Ethics and Animal Use Committee of Zhejiang Provincial People's Hospital. The reference number for animal ethics approval is (20240107111105464218).

Acknowledgment

This study was supported by the State Administration of Traditional Chinese Medicine Science and Technology Department-Zhejiang Provincial Administration of Traditional Chinese Medicine co-construction of science and technology program (2024035416) and (2022ZB034), Zhejiang public welfare technology application research program (LGF22H060029), Zhejiang medicine and health science and technology project (2023KY045), National High-tech Research and Development Program (YCZYPT[2023]03), Zhejiang Provincial Science and Technology Department key research and development program (2021C03078) and Medical and Health Science and Technology Project of Zhejiang Province (2020KY029) and (2022KY500).

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

- Abramoff B, Caldera FE. Osteoarthritis: pathology, diagnosis, and treatment options. *Med Clin North Am*. 2020;104(2):293–311. doi:10.1016/j.mcna.2019.10.007
- Mandl LA. Osteoarthritis year in review 2018: clinical. *Osteoarthritis Cartilage*. 2019;27(3):359–364. doi:10.1016/j.joca.2018.11.001
- Barnett R. Osteoarthritis. *Lancet*. 2018;391(10134):1985. doi:10.1016/s0140-6736(18)31064-x
- Litwic A, Edwards MH, Dennison EM, Cooper C. Epidemiology and burden of osteoarthritis. *Br Med Bull*. 2013;105(1):185–199. doi:10.1093/bmb/lds038
- Prieto-Alhambra D, Judge A, Javaid MK, Cooper C, Diez-Perez A, Arden NK. Incidence and risk factors for clinically diagnosed knee, Hip and hand osteoarthritis: influences of age, gender and osteoarthritis affecting other joints. *Ann Rheum Dis*. 2014;73(9):1659–1664. doi:10.1136/annrheumdis-2013-203355
- Bennell K, Hinman RS, Wrigley TV, Creaby MW, Hodges P. Exercise and osteoarthritis: cause and effects. *Compr Physiol*. 2011;1(4):1943–2008. doi:10.1002/cphy.c100057
- Thomas AC, Hubbard-Turner T, Wikstrom EA, Palmieri-Smith RM. Epidemiology of posttraumatic osteoarthritis. *J Athl Train*. 2017;52(6):491–496. doi:10.4085/1062-6050-51.5.08
- Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. *Lancet*. 2019;393(10182):1745–1759. doi:10.1016/s0140-6736(19)30417-9
- Berenbaum F, Wallace IJ, Lieberman DE, Felson DT. Modern-day environmental factors in the pathogenesis of osteoarthritis. *Nat Rev Rheumatol*. 2018;14(11):674–681. doi:10.1038/s41584-018-0073-x
- O'Neill TW, McCabe PS, McBeth J. Update on the epidemiology, risk factors and disease outcomes of osteoarthritis. *Best Pract Res Clin Rheumatol*. 2018;32(2):312–326. doi:10.1016/j.berh.2018.10.007
- Molnar V, Matisić V, Kodvanj I, et al. Cytokines and chemokines involved in osteoarthritis pathogenesis. *Int J Mol Sci*. 2021;22(17):9208. doi:10.3390/ijms22179208
- Motta F, Barone E, Sica A, Selmi C. Inflammaging and Osteoarthritis. *Clin Rev Allergy Immunol*. 2023;64(2):222–238. doi:10.1007/s12016-022-08941-1
- Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol*. 2011;7(1):33–42. doi:10.1038/nrrheum.2010.196
- Al-Kofahi M, Omura S, Tsunoda I, et al. IL-1 β reduces cardiac lymphatic muscle contraction via COX-2 and PGE(2) induction: potential role in myocarditis. *Biomed Pharmacother*. 2018;107:1591–1600. doi:10.1016/j.biopha.2018.08.004
- Cho Y, Jeong S, Kim H, et al. Disease-modifying therapeutic strategies in osteoarthritis: current status and future directions. *Exp Mol Med*. 2021;53(11):1689–1696. doi:10.1038/s12276-021-00710-y
- Martel-Pelletier J, Barr AJ, Cicuttini FM, et al. Osteoarthritis. *Nat Rev Dis Primers*. 2016;2(1):16072. doi:10.1038/nrdp.2016.72
- Sidhu VS, Kelly TL, Pratt N, et al. Effect of aspirin vs enoxaparin on symptomatic venous thromboembolism in patients undergoing hip or knee arthroplasty: the CRISTAL randomized trial. *JAMA*. 2022;328(8):719–727. doi:10.1001/jama.2022.13416
- Ferguson RJ, Palmer AJ, Taylor A, Porter ML, Malchau H, Glyn-Jones S. Hip replacement. *Lancet*. 2018;392(10158):1662–1671. doi:10.1016/s0140-6736(18)31777-x
- Wan Y, Shen K, Yu H, Fan W. Baicalein limits osteoarthritis development by inhibiting chondrocyte ferroptosis. *Free Radic Biol Med*. 2023;196:108–120. doi:10.1016/j.freeradbiomed.2023.01.006
- Huang X, Pei W, Ni B, Zhang R, You H. Chondroprotective and antiarthritic effects of galangin in osteoarthritis: an in vitro and in vivo study. *Eur J Pharmacol*. 2021;906:174232. doi:10.1016/j.ejphar.2021.174232
- Sharma A, Goel A. Pathogenesis of rheumatoid arthritis and its treatment with anti-inflammatory natural products. *Mol Biol Rep*. 2023;50(5):4687–4706. doi:10.1007/s11033-023-08406-4
- Sharma A, Goel A. Inflammatory cytokines in rheumatoid arthritis: diagnostic challenges, pathogenic mechanisms and their role in depression and management. *Curr Top Med Chem*. 2023;23(27):2535–2551. doi:10.2174/1568026623666230915095151
- Wang Y, Feng W, Wang X, et al. The multifaceted mechanisms of pristimerin in the treatment of tumors state-of-The-art. *Biomed Pharmacother*. 2022;154:113575. doi:10.1016/j.biopha.2022.113575
- Liang J, Yuan S, Wang X, et al. Attenuation of pristimerin on TNF- α -induced endothelial inflammation. *Int Immunopharmacol*. 2020;82:106326. doi:10.1016/j.intimp.2020.106326
- Al-Tamimi M, Khan AQ, Anver R, et al. Pristimerin mediated anticancer effects and sensitization of human skin cancer cells through modulation of MAPK signaling pathways. *Biomed Pharmacother*. 2022;156:113950. doi:10.1016/j.biopha.2022.113950
- Jiang H, Xie Y, Lu J, et al. Pristimerin suppresses AIM2 inflammasome by modulating AIM2-PYCARD/ASC stability via selective autophagy to alleviate tendinopathy. *Autophagy*. 2024;20(1):76–93. doi:10.1080/15548627.2023.2249392
- Sharma A, Goel A, Lin Z. Analysis of anti-rheumatic activity of Nyctanthes arbor-tristis via in vivo and pharmacovigilance approaches. *Front Pharmacol*. 2023;14:1307799. doi:10.3389/fphar.2023.1307799
- Zhou Y, Ni J, Wen C, Lai P. Light on osteoarthritic joint: from bench to bed. *Theranostics*. 2022;12(2):542–557. doi:10.7150/thno.64340
- Scanzello CR, Moskowitz NK, Gibofsky A. The post-NSAID era: what to use now for the pharmacologic treatment of pain and inflammation in osteoarthritis. *Curr Rheumatol Rep*. 2008;10(1):49–56. doi:10.1007/s11926-008-0009-6
- Zeng C, Bennell K, Yang Z, et al. Risk of venous thromboembolism in knee, Hip and hand osteoarthritis: a general population-based cohort study. *Ann Rheum Dis*. 2020;79(12):1616–1624. doi:10.1136/annrheumdis-2020-217782
- Yang H, Wang Z, Wang L, et al. Scutellarin ameliorates osteoarthritis by protecting chondrocytes and subchondral bone microstructure by inactivating NF- κ B/MAPK signal transduction. *Biomed Pharmacother*. 2022;155:113781. doi:10.1016/j.biopha.2022.113781

32. Park JH, Kim JK. Pristimerin, a naturally occurring triterpenoid, attenuates tumorigenesis in experimental colitis-associated colon cancer. *Phytomedicine*. 2018;42:164–171. doi:10.1016/j.phymed.2018.03.033
33. El-Agamy DS, Shaaban AA, Almaramhy HH, Elkablawy S, Elkablawy MA. Pristimerin as a novel hepatoprotective agent against experimental autoimmune hepatitis. *Front Pharmacol*. 2018;9:292. doi:10.3389/fphar.2018.00292
34. Robinson WH, Lepus CM, Wang Q, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. *Nat Rev Rheumatol*. 2016;12(10):580–592. doi:10.1038/nrrheum.2016.136
35. Tabeian H, Betti BF, Dos Santos Cirqueira C, et al. IL-1 β damages fibrocartilage and upregulates MMP-13 expression in fibrochondrocytes in the condyle of the temporomandibular joint. *Int J Mol Sci*. 2019;20(9). doi:10.3390/ijms20092260
36. Tchétina EV, Kobayashi M, Yasuda T, Meijers T, Pidoux I, Poole AR. Chondrocyte hypertrophy can be induced by a cryptic sequence of type II collagen and is accompanied by the induction of MMP-13 and collagenase activity: implications for development and arthritis. *Matrix Biol*. 2007;26(4):247–258. doi:10.1016/j.matbio.2007.01.006
37. Aguado A, Rodríguez C, Martínez-Revelles S, et al. HuR mediates the synergistic effects of angiotensin II and IL-1 β on vascular COX-2 expression and cell migration. *Br J Pharmacol*. 2015;172(12):3028–3042. doi:10.1111/bph.13103
38. Guldenpfennig C, Teixeira E, Daniels M. NF- κ B's contribution to B cell fate decisions. *Front Immunol*. 2023;14:1214095. doi:10.3389/fimmu.2023.1214095
39. Yao Q, Wu X, Tao C, et al. Osteoarthritis: pathogenic signaling pathways and therapeutic targets. *Signal Transduct Target Ther*. 2023;8(1):56. doi:10.1038/s41392-023-01330-w
40. Lepetsos P, Papavassiliou KA, Papavassiliou AG. Redox and NF- κ B signaling in osteoarthritis. *Free Radic Biol Med*. 2019;132:90–100. doi:10.1016/j.freeradbiomed.2018.09.025
41. Wang T, Zhang X, Li JJ. The role of NF- κ B in the regulation of cell stress responses. *Int Immunopharmacol*. 2002;2(11):1509–1520. doi:10.1016/s1567-5769(02)00058-9
42. Meng X, Zhang S. MAPK cascades in plant disease resistance signaling. *Annu Rev Phytopathol*. 2013;51(1):245–266. doi:10.1146/annurev-phyto-082712-102314
43. Lee Y, Kim YJ, Kim MH, Kwak JM. MAPK cascades in guard cell signal transduction. *Front Plant Sci*. 2016;7:80. doi:10.3389/fpls.2016.00080
44. Qiu J, Jiang T, Yang G, et al. Neratinib exerts dual effects on cartilage degradation and osteoclast production in Osteoarthritis by inhibiting the activation of the MAPK/NF- κ B signaling pathways. *Biochem Pharmacol*. 2022;205:115155. doi:10.1016/j.bcp.2022.115155
45. Mao Y, Xie X, Jiang T, et al. XI019, a novel JAK inhibitor, suppressed osteoclasts differentiation induced by RANKL through MAPK signaling pathway. *Biochem Pharmacol*. 2023;215:115704. doi:10.1016/j.bcp.2023.115704

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