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WN1703 alleviates gout symptoms via inflammatory signaling pathways in an acute gout rat model

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ABSTRACT

We previously synthesized the xanthine oxidoreductase (XOR) inhibitor WN1703. In addition to showing XOR inhibitory effects, WN1703 also showed anti-inflammatory effects in a rat hyperuricemia model. Here, we studied WN1703's anti-inflammatory effects on gout and explored the underlying mechanisms. Tohoku Hospital Pediatrics-1 (THP-1) cells were stimulated by lipopolysaccharide/interferon-γ/monosodium urate (MSU). The levels of inflammatory cytokines in the supernatant and protein expression in THP-1 cells were detected using enzyme-linked immunosorbent assay (ELISA) kits and western blotting, respectively, to verify the inhibitory effects of WN1703 and its mechanism. Potassium oxonate, hypoxanthine, and MSU were administered to establish a hyperuricemia rat model complicated by acute gouty arthritis. At 1–24 h after MSU injection, the degree of ankle swelling was recorded to compare the anti-inflammatory effects at each time point. The potential mechanism was further explored using immunohistochemistry and ELISA. WN1703 significantly downregulated expression of nucleotide-binding oligomerization domain-like receptor thermal protein domain associated protein 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, toll-like receptor-4 (TLR4), myeloid differentiation primary response protein 88 (MyD88), nuclear factor-kappa B (NF-κB), and relevant cytokine levels in THP-1 cells. Identical doses of WN1703 and febuxostat had comparable effects on these proteins and cytokines. In the gout rats, the same dose of WN1703 and febuxostat showed equivalent inhibitory effects on NLRP3, ASC, and NF-κB; however, WN1703 showed weaker impacts on alleviating ankle swelling than febuxostat showed. In conclusion, WN1703 showed significant anti-inflammatory effects in hyperuricemic rats with acute gout. Such effects were related to the inhibition of the NLRP3/ASC/Caspase-1 and TLR4/MyD88/NF-κB signaling pathways, thereby downregulating inflammation-related protein expression and decreasing inflammatory cytokine secretion.

1. Introduction

Gout is caused by the deposition of needle-like monosodium urate (MSU) crystals in joints and soft tissues. The disease is believed to be closely associated with oversaturated uric acid (UA) and hyperuricemia [1]. The *in vivo* synthesis of MSU is derived from xanthine. After a two-step enzyme-catalyzed reaction with xanthine oxidoreductase (XOR), xanthine is oxidized to hypoxanthine before UA is synthesized in the liver [2]. Hyperuricemia is always caused by purine over intake, XOR malfunctioning, decreased UA excretion by the kidney, and other relevant reasons. Subsequently, the over-saturated UA deposits in ankle and other soft tissue as MSU, and further causes gout flares. Gout flares are characterized by redness, warmth, swelling, and intense pain in patient hallux joints or other tissues [3]. These symptoms are common in adults with acute episodes of gout and are essential for the clinical diagnosis of gouty arthritis.

Stimulation of needle-like MSU crystals in the ankle joints may activate various inflammation-associated pathways. Two of the most-reported signaling pathways, which are widely believed to be related to gout, are the toll-like receptor (TLR)4 signaling pathway and nucleotide-binding oligomerization domain-like receptor thermal protein domain associated protein 3 (NLRP3) signaling pathway. The interaction between TLRs and myeloid differentiation primary response protein 88 (MyD88) was enhanced by the stimulation of MSU, followed by the activation of nuclear factor kappa-B (NF-κB) and the increased transcription of tumor necrosis factor-α (TNF-α) [4,5]. In addition, stimulation of needle-like MSU crystals promotes the combination of the

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Abbreviations

MSU	monosodium urate
TLR	toll-like receptor
MyD88	myeloid differentiation primary response protein 88
NLRP3	nucleotide-binding oligomerization domain-like receptor thermal protein domain associated protein 3
ASC	apoptosis-associated speck-like protein containing a CARD
NF-κB	nuclear factor-kappa B
NSAID	nonsteroidal anti-inflammatory drug
IRAK	IL-1 receptor-associated kinase
UA	uric acid
AE	adverse event
XOR	xanthine oxidoreductase
ROS	reactive oxygen species
PMA	phorbol 12-myristate 13-acetate
LPS	lipopolysaccharide
IFN-γ	interferon-γ
THP-M	THP-1-derived macrophage
H&E	hematoxylin and eosin
IL	interleukin
TNF-α	tumor necrosis factor-α
THP-1	Tohoku Hospital Pediatrics-1 cells
ELISA	enzyme-linked immunosorbent assay
IC_{50}	50% inhibitory concentration
RPMI	Roswell Park Memorial Institute
CMC-Na	sodium carboxymethyl cellulose
PMSF	phenylmethylsulfonyl fluoride
CCK	Cell Counting Kit
ECL	electrochemiluminescence
PVDF	polyvinylidene difluoride
IHC	immunohistochemistry

NLRP3 inflammasome, including NLRP3, caspase-1, and apoptosis-associated speck-like protein containing a CARD (ASC). Subsequently, the increased transcription of NLRP3 and pro-interleukin (IL)-1β would further promote the maturity and excretion of IL-1β [6,7].

Gout is primarily treated with anti-inflammatory agents, including non-steroidal anti-inflammatory drugs (NSAID), colchicine, and corticosteroids. Lowering UA levels in the blood using traditional uricosuric agents and novel inhibitors of UA reabsorption are the first-line treatment options for the prevention of recurrent gout [8]. A combination of these two treatments has been proven to be effective in reducing gout flares and relieving acute gout attacks in clinical trials [9–11]. However, treatments have been unsatisfactory because of poor compliance and serious adverse events (AE), such as cardiovascular AEs caused by febuxostat and gastrointestinal AEs due to NSAIDs. Therefore, safe and efficacious drugs are required for the treatment of gout.

Previously, we identified novel XOR inhibitors, WN1703 (previously named 16c [12] and LS087 [13]). The XOR inhibitor showed comparable *in vitro* effects (50% inhibitory concentration [IC_{50}] value = 5.7 nM [12]) with those of febuxostat (IC_{50} = 3.1–5.5 nM from literature [14] and IC_{50} = 5.4 nM in our test [12]). Studies on acute and chronic hyperuricemia in rodents showed that WN1703 not only reduced the serum UA levels, but also reduced XOR expression in the liver. We performed an experiment using rats with hyperuricemia and glucose and lipid disorders. Notably, WN1703 treatment benefitted rats by lowering the triglyceride and low-density lipoprotein levels, increasing the high-density lipoprotein levels, and improving liver function. Moreover, WN1703 could reduce inflammatory factor levels in blood, including IL-1β and TNF-α [12,15].

This study explored the multiple roles of WN1703 in inflammation and gout treatment. WN1703 therapy lowered proinflammatory factor levels and reduced the expression of key proteins: ASC, MyD88, and NF-κB. These data suggest that WN1703 could be used to alleviate inflammation caused by gouty arthritis. Overall, we demonstrated the inhibitory effects of WN1703 *in vitro* and *in vivo*, and we showed that it was more effective than the XOR inhibitory agent was.

2. Materials and methods

2.1. Materials

WN1703 (purity >97%, as determined by high-performance liquid chromatography) was synthesized by our research team [12]. MSU and lipopolysaccharide (LPS, L2630) were purchased from Millipore (Burlington, MA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium and 1% penicillin–streptomycin were obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum was obtained from Newzerum (Christchurch, New Zealand). Mercaptoethanol was purchased from Shanghai Macklin Biochemicals (Shanghai, China). Phorbol 12-myristate 13-acetate (PMA HY-18739) was purchased from MedChemExpress (Monmouth Junction, NJ). Interferon-γ (IFN-γ, H13445) was purchased from Peprotech (Rocky Hill, NJ, USA). Potassium oxonate (98% purity), hypoxanthine (99% purity), and colchicine (98% purity) were obtained from Shanghai Macklin Biochemicals. Febuxostat (purity ≥97%) and sodium carboxymethyl cellulose (CMC-Na) were purchased from Aladdin Biochemical Technology (Shanghai, China). The antibody against ASC (ab309497) was purchased from Abcam (Cambridge, UK). Antibodies against MyD88 (AF5195), NLRP3 (DF7438), NF-κB p65 (AF5006), TLR4 (AF7017), caspase-1 (AF5418), and horseradish peroxidase-conjugated goat anti-rabbit (S0001) were purchased from Affinity Biosciences (Jiangsu, China). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the protein expression in cell lysates, as well as in the supernatant. IL-1β kits (MM-0047R1), TNF-α kits (MM-0180R1), NLRP3 kits (MM-2198H1), caspase-1 kits (MM-13398H1), ASC kits (MM-60433H1), TLR4 kits (MM-13271H1) and MyD88 kits (MM-12937H1) were obtained from Meimian Bioengineering Institute (Jiangsu, China). RIPA lysis buffer, phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitor, and Cell Counting Kit (CCK)8 were purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Electrochemiluminescence (ECL) substrate was purchased from Thermo Scientific (Waltham, MA, USA).

2.2. Cell culture

Tohoku Hospital Pediatrics-1 (THP-1) cells were obtained from EK Bioscience and Biotechnology (Shanghai, China). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin–streptomycin, and mercaptoethanol (0.05 mM) at 37 °C in a 5% CO_2 atmosphere.

2.3. Evaluation of cell viability

THP-1 cells (5×10^5 cells/mL) were inoculated in 96-well plates (100 μL/well). THP-1 cells were induced by PMA (100 ng/mL) to differentiate into macrophages for 24 h [16]. THP-1 macrophages were cultured for 48 h with LPS (100 ng/mL) and IFN-γ (20 ng/mL) [17]. IFN-γ was diluted according to the manufacturer's instructions. The culture medium was then replaced with fresh culture medium containing WN1703 (2.5, 5, 10, 20, 40, and 80 μg/mL), febuxostat (5 μg/mL), or colchicine (0.1 μg/mL), respectively, for 0.5 h before application of a MSU suspension (500 μg/mL). CCK8 solution (10 μL) was added to each well at 37 °C in a 5% CO_2 atmosphere and left for 2 h. Blank wells were filled with an equal amount of dimethyl sulfoxide. Duplicate wells were included in the

analysis. The absorbance of each well was measured at 450 nm using a multi-functional microplate analysis system (Ensipre-2300; PerkinElmer). Untreated (control) cells were used to determine the cell viability.

$$\text{Cell viability} = \frac{A_{Treated} - A_{Blank}}{A_{Control} - A_{Blank}} \times 100\%$$

2.4. THP-1 cell stimulation with LPS/IFN-γ/MSU

After stimulation with PMA (100 ng/mL) for 24 h, THP-1-derived macrophages (THP-M) were obtained. Stimulation of THP-Ms with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for 48 h resulted in M1 macrophage formation. After removal of the culture medium, cells were pretreated with WN1703 (2.5, 5, and 10 μg/mL), febuxostat (5 μg/mL), or colchicine (0.1 μg/mL), respectively, for 0.5 h before addition of MSU suspension for 23.5 h. At the end of the experiment, supernatants were collected to measure f IL-1β and TNF-α levels. Cells were collected before digestion with lysis buffer (RIPA buffer: PMSF: phosphatase inhibitor = 50:1:1) to measure protein expression.

2.5. Western blotting (WB)

WB experiments were undertaken to evaluate the NF-κB p65 levels in THP-1 cells. All samples were diluted to the same concentration loading buffer (5 ×) and phosphate-buffered saline, and heated at 95–100 °C for 10 min. Recovered proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes for 1 h. PVDF membranes were blocked with 5% non-fat milk for 2 h at 25 °C and then exposed to rabbit *anti*–NF–κB antibody (1:1000 dilution) overnight at 4 °C, followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2000) for 2 h. After washing three times with 1 × Tris-buffered saline and Tween-20) for 10 min, reactive bands were visualized with the ECL substrate using a smart imaging system (Vilber Bio Imaging, Paris, France). Densitometric quantification of each band was performed using ImageJ (US National Institutes of Health, Bethesda, MD, USA).

2.6. ELISA

The ELISA kits were used according to the manufacturer's instructions. All supernatants and cell lysates were diluted 1:5 with the sample diluent. The standard samples were diluted with a standard diluent. Plates were incubated for 30 min at 37 °C and washed with washing buffer five times after the samples and enzyme reagents were added to each well. Color-rendering agents were added to the plate before incubation in the dark for 10 min at 37 °C. Measurements were undertaken using a multi-functional microplate analysis system at 450 nm followed by the addition of stop agents to each well.

2.7. Animal experiments

The animal experiment complied with the ARRIVE guidelines and was carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The study protocol was approved (AEC number: 2021003) by the Ethics Committee of South China University of Technology (Guangzhou, China). Seventy specific pathogen-free male Sprague–Dawley rats (160–200 g) were purchased from Hunan Sleke Jingda Experimental Animals (SYXK (Guangdong) 2017–0178; Guangzhou, China). The cage temperature was 20–26 °C and the relative humidity of the room was 40–70%. Rats were adaptively provided with standard feed (Laboratory Animal Center of South China University of Technology, Guangzhou, China) for one week. Food was withheld for 12 h before blood was collected from the abdominal aorta; water was freely available.

2.8. Hyperuricemia and gout models

The rats were randomly divided into seven groups of 10 rats each. All drugs, hypoxanthine, and potassium oxonate used in the rat experiments were dissolved in 0.5% CMC-Na solution. Potassium oxonate (250 mg/kg, subcutaneous) and hypoxanthine (150 mg/kg, intraperitoneal) were injected for seven days to create hyperuricemia rat models [12,18]. Rats in the MSU group (gavaged 0.5% CMC-Na solution), 5 mg/kg febuxostat, 2.5 mg/kg WN1703, 5 mg/kg WN1703, 10 mg/kg WN1703, and 0.5 mg/kg colchicine were administered agents via the oral route 1 h after the daily treatments to induce the hyperuricemia model [12]. Control rats were injected with the same volume of 0.5% CMC-Na via subcutaneous or intraperitoneal routes or by gavage.

Rats were injected with a sterile MSU suspension (0.05 mL) into their ankle joints on day 8, except for the control group (injected with the same volume of sterile saline). Changes in ankle joint swelling were measured within 24 h of MSU administration. The ankle diameter was measured using a Vernier caliper before and 1, 2, 4, 6, and 24 h after MSU injection to calculate ankle swelling. On day 9, rats were anesthetized with 20% urethane. Blood was collected from the abdominal aorta. After the rats were sacrificed, the ankle joint cavities were opened to remove the light-yellow synovial tissue, which was then washed with pre-cooled saline and placed in 10% neutral formalin. After fixing for 24 h, the collected tissues were dehydrated and embedded in paraffin. Blood samples were analyzed using an automatic biochemical analyzer (AU5811, Beckman Coulter, U.S.), according to the manufacturer's instructions. The percentage ankle swelling was calculated using the following formula:

$$\text{Ankle swelling} = (D_t - D_0) / D_0 \times 100\%$$

where D_t is the diameter of the ankle joint t h after MSU injection, and D_0 is the diameter of the ankle joint before MSU injection.

2.9. Hematoxylin and eosin (H&E) staining

The synovial tissue was immersed in 10% neutral formalin for 24 h, dehydrated using a series of graded ethanol solutions (70–100%), defatted in xylene, and embedded in paraffin. The embedded wax was cut into slices of 4-μm thickness and then stained with H&E. Images of the stained slices were captured using a microscope (Eclipse CI, Nikon, Japan).

2.10. Immunohistochemistry

The tissues were fixed in a 10% neutral formalin solution for 24 h to prepare tissue slices, which were then dewaxed and hydrated. Antigens were removed by placing the tissue slices in a microwave oven for 30 min at 25 °C. Hydrogen peroxide (3%) was added to the sections to block endogenous peroxidase activity. After the addition of 3% bovine serum albumin, antibodies were added to the slices overnight at 4 °C before incubation at 25 °C for 1 h witha horseradish peroxidase-conjugated goat anti-rabbit antibody. The 3, 3′-diaminobenzidine dye was used as a chromogenic reagent and hematoxylin was used to re-dye slices. Finally, the treated slices were dehydrated and sealed with neutral gum prior to analysis. All immunohistochemical images were analyzed using Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) to compare the relative expression of each protein in the seven groups.

2.11. Statistical analyses

Data are presented as the mean ± standard deviation. One-way analysis of variance was used to compare and calculate the *P* values. Data were analyzed using Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1. Cell viability according to the CCK8 assay

To evaluate cell viability at various doses of medication, the CCK8 assay was used in the *in vitro* experiment. The effect of WN1703 (2.5–80 μg/mL) administration on cell viability was dose-dependent. The viability of THP-1 cells was >90% at 2.5, 5, and 10 μg/mL of WN1703, 5 μg/mL of febuxostat, and 0.1 μg/mL of colchicine. Therefore, WN1703, febuxostat, and colchicine were considered suitable for the subsequent experiments (see Fig. 1).

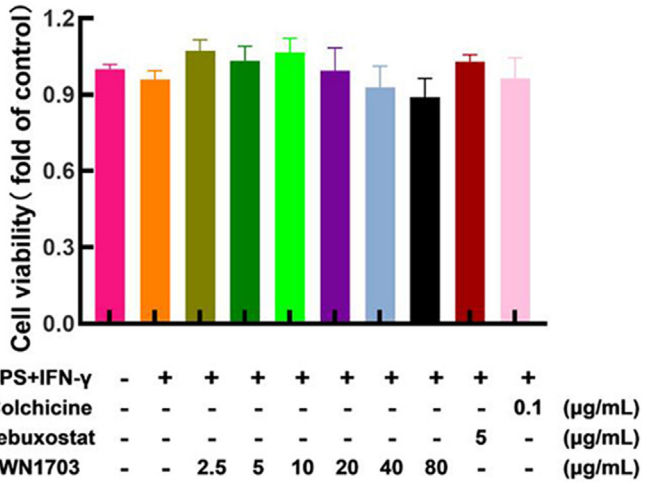


Fig. 1. Activity of THP-1 cells induced by LPS/IFN-γ/MSU after administration of febuxostat, colchicine, and WN1703 (2.5–80 μg/mL) respectively. Abbreviations: THP-1, Tohoku Hospital Pediatrics-1 cells; LPS, lipopolysaccharide; IFN-γ, interferon-γ; MSU, monosodium urate.

3.2. WN1703 downregulated protein expression of the NLRP3/ASC/caspase-1 signaling pathway in vitro

ELISA kits were used to detect protein expression in the cell lysate as well as the inflammatory cytokine concentration in the supernatant. After THP-1 macrophages were stimulated with MSU and produced proinflammatory cytokines, the protein expression of NLRP3, ASC, caspase-1, and IL-1β secretion in the MSU group were enhanced significantly compared with that in the control group (Fig. 2). These results suggested that stimulation with MSU successfully activated the NLRP3 signaling pathway in THP-1 macrophages.

After administration of colchicine (0.1 μg/mL), protein expression of NLRP3 ($P < 0.05$), ASC ($P < 0.01$), caspase-1 ($P < 0.05$), and the IL-1β levels ($P < 0.05$) were downregulated markedly. Administration of 5 μg/mL febuxostat reduced protein expression of ASC ($P < 0.01$) and caspase-1 ($P < 0.05$). WN1703 (10 μg/mL) reduced expression of NLRP3 ($P < 0.05$), ASC ($P < 0.05$), caspase-1 ($P < 0.01$), and IL-1β ($P < 0.05$), whereas 2.5 μg/mL WN1703 and 5 μg/mL WN1703 showed a significant downregulating effect on ASC ($P < 0.05$) only. No significance difference was found between 5 μg/mL febuxostat and the same dose of WN1703 in terms of reducing the expression of the above-mentioned proteins ($P > 0.05$). However, the inhibitory effects of 5 μg/mL WN1703 on decreasing the level of IL-1β was inferior to that of colchicine (0.1 μg/mL colchicine vs. 5 μg/mL WN1703, 39.51 ± 3.51 vs. 47.66 ± 2.35, $P < 0.05$). Therefore, WN1703 attenuated the maturing and secretion of IL-1β by downregulating the NLRP3/ASC/caspase-1 signaling pathway in THP-1 macrophages.

3.3. WN1703 downregulated protein expression of the TLR4/MyD88/NF-κB signaling pathway in vitro

ELISA kits were used to detect protein expression in the cell lysate as well as the inflammatory cytokine concentration in the supernatant. In addition, Western blot was conducted to evaluate the expression of NF-κB. After THP-1 macrophages were stimulated with MSU and proinflammatory cytokines were produced, protein expression of TLR4, MyD88, NF-κB, and TNF-α secretion in THP-1 cells increased significantly compared with that in the control group (Fig. 3). These results suggest that MSU successfully activated the TLR4 signaling pathway in THP-1 macrophages.

After administration of colchicine (0.1 μg/mL), expression of TLR4, MyD88, and NF-κB, as well as the secretion of TNF-α ($P < 0.05$) were reduced remarkedly. Febuxostat (5 μg/mL) showed its effects by reducing the protein expression of TLR4 and MyD88 and the secretion of TNF-α ($P < 0.05$) *in vitro*. In particular, 5 μg/mL WN1703 reduced the relative expression of MyD88, TLR4, and NF-κB and TNF-α secretion ($P < 0.05$) in THP-1 cells. No significant difference was found between febuxostat and WN1703 treatment at the same dose in terms of reducing the expression of MyD88, TLR4, and NF-κB and TNF-α secretion ($P > 0.05$). Therefore, WN1703 attenuated the maturing and secretion of TNF-α by downregulating the TLR4/MyD88/NF-κB signaling pathway in THP-1 macrophages.

3.4. WN1703 attenuated hyperuricemia induced by potassium oxonate and hypoxanthine in rats

To compare the hyperuricemia status of the rats in each group, the levels of all biochemical indicators were determined using automatic biochemical analysis equipment (Beckman Coulter, U.S.). Potassium oxonate and hypoxanthine markedly increased serum UA levels in the MSU group ($P < 0.01$) (Fig. 4), indicating that hyperuricemia modeling in rats was successful.

Treatment with febuxostat (5 mg/kg) significantly reduced serum UA levels in rats with hyperuricemia ($P < 0.01$). Additionally, creatinine and urea nitrogen levels were markedly reduced in the febuxostat group. Compared with that of MSU, WN1703 significantly reduced the serum UA levels at all three doses ($P < 0.01$); however, only a high WN1073 dose downregulated the level of urea nitrogen in rats with hyperuricemia.

3.5. WN1703 alleviated MSU-induced ankle swelling and synovial inflammation in rats

To compare MSU-induced ankle swelling and synovial inflammation in each group, the degree of ankle swelling at each time point and H&E staining images of synovial tissue in each group were recorded. The ankle diameters of the rats in each group were continuously measured using a Vernier caliper from 0 to 24 h after MSU injection to evaluate the alleviating effects of WN1703 on rat joints. After MSU injection, the ankle joints of the rats were swollen, red, and hot. Ankle swelling in rats in the control group peaked 1 h after injection and decreased gradually thereafter. The percentage of ankle swelling in the MSU group was significantly higher than that in the control group at 2–24 h after MSU injection (Table 1). These observations suggested that hyperuricemia was induced in rats with acute gouty arthritis.

The degree of ankle swelling in rats in each administration group was alleviated at 2–24 h compared with that in the MSU group. However, only the administration of 5 mg/kg febuxostat and 10 mg/kg WN1703 maintained this effect on MSU-induced ankle swelling for 24 h ($P < 0.05$). From 1 to 4 h, no significant difference in ankle swelling degree was found between 5 mg/kg febuxostat administration and the three doses of WN1703 administration ($P > 0.05$). However, the ankle swelling effect of 5 mg/kg febuxostat was more pronounced at 6 and 24 h than that of 2.5–5 mg/kg WN1703 (all $P < 0.05$).

Rat synovial tissue samples were stained with H&E to compare the pathological status of the rats. The ratio of rats with infiltration of

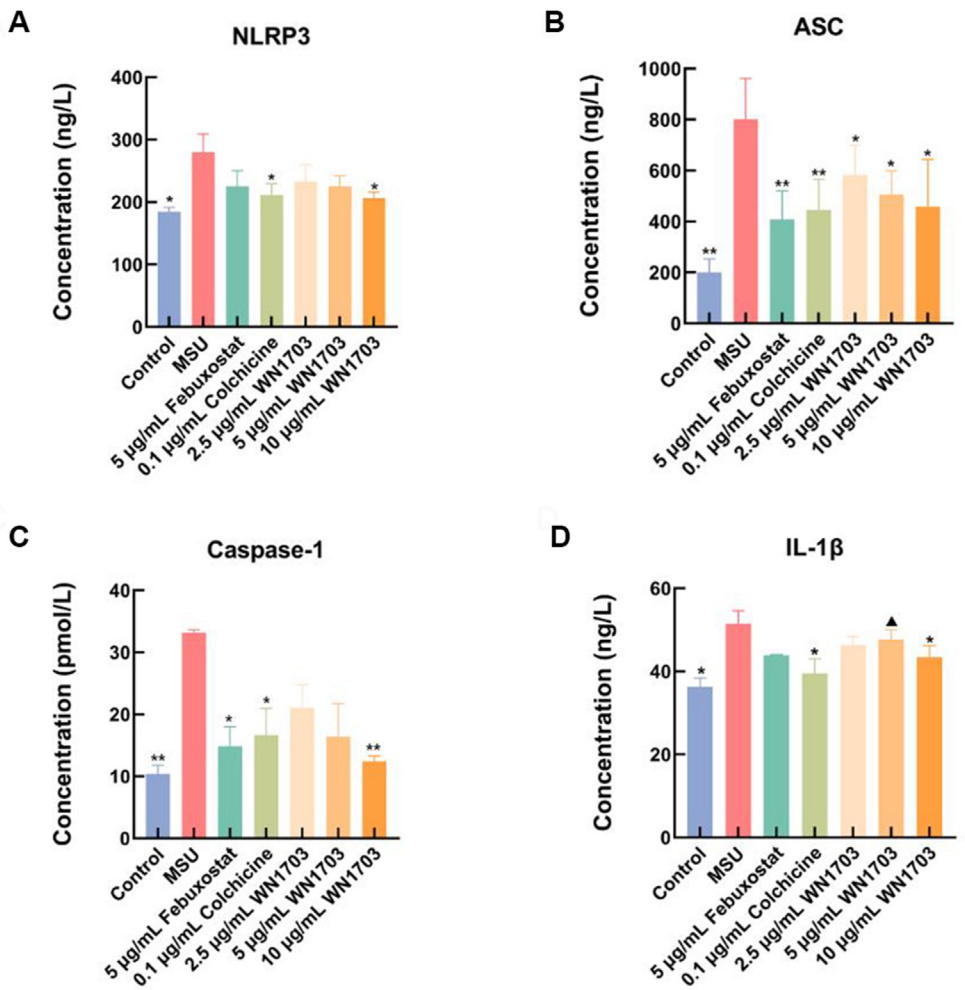


Fig. 2. Protein expression and proinflammatory factor levels associated with NLRP3 signaling pathway in THP-1 cells 24 h after LPS/IFN-γ/MSU induction and drug administration (Control: THP-1 cells without LPS/IFN-γ/MSU induction and treated with an equal volume of blank solvent containing DMSO; MSU: THP-1 cells inducted by LPS/IFN-γ/MSU and treated with an equal volume of blank solvent containing DMSO; 5 μg/mL Febuxostat: THP-1 cells inducted by LPS/IFN-γ/MSU and treated with febuxostat (5 μg/mL); 0.1 μg/mL Colchicine: THP-1 cells inducted by LPS/IFN-γ/MSU and treated with colchicine (0.1 μg/mL); 2.5, 5, and 10 μg/mL WN1703: THP-1 cells inducted with LPS/IFN-γ/MSU and treated with 2.5, 5, and 10 μg/mL WN1703. A: NLRP3; B: ASC; C: Caspase-1; D: IL-1β. One-way analysis of variance was used to compare and calculate the P values. Compared with MSU, $^{*}P < 0.05$, $^{**}P < 0.01$; 0.1 μg/mL Colchicine vs. WN1703, $^{▲}P < 0.05$, $^{▲▲}P < 0.01$). Abbreviations: NLRP3: nucleotide-binding oligomerization domain-like receptor thermal protein domain associated protein 3; THP-1, Tohoku Hospital Pediatrics-1 cells; LPS, lipopolysaccharide; IFN-γ, interferon-γ; MSU, monosodium urate; DMSO, dimethyl sulfoxide; ASC: apoptosis-associated speck-like protein containing a CARD; IL: interleukin.

inflammatory cells in synovial tissue in the MSU, 5 mg/kg febuxostat, 0.5 mg/kg colchicine and three doses of WN1703 groups were 9/10, 2/10, 2/10, 8/10, 4/10, and 1/10 respectively. Staining of ankle tissues with severe lesions in each group is shown in Fig. 5. After MSU injection, inflammation was observed in the synovial tissue of rats in the MSU and the low-dose WN1703 group, and the number of inflammatory cells in the joints in these groups increased significantly. Therefore, these pathological changes and ankle swelling were alleviated compared with those in the MSU group after the administration of WN1703, febuxostat, or colchicine.

3.6. WN1703 improved synovial inflammation in rats with acute gouty arthritis by inhibiting the NLRP3 signaling pathway

To preliminarily explore the potential anti-inflammatory mechanism of WN1703, NLRP3 inflammasome-associated protein expression in synovial tissue was assessed using immunohistochemistry (IHC) and the concentration of IL-1β was detected using ELISA kits. The relative protein expression levels of NLRP3, ASC, and caspase-1 in the synovial tissues of the rats in the MSU group were significantly higher than those in the control group (Fig. 6). These results suggest that the NLRP3 signaling pathway in synovial tissue is activated during the induction of inflammation in rat joints.

The administration of febuxostat (5 mg/kg) and colchicine (0.5 mg/kg) helped to downregulate relative protein expression of the key proteins NLRP3, ASC and caspase-1, and decrease the serum IL-1β levels ($P < 0.01$). Administration of WN1703 (5 mg/kg) lowered the relative expression of NLRP3, ASC ($P < 0.01$), and caspase-1 ($P < 0.05$), as well as the level of IL-1β ($P < 0.05$). After administration of WN1703 (10 mg/kg), relative expression of NLRP3 ($P < 0.05$), ASC, caspase-1 and blood IL-1β levels decreased ($P < 0.01$). The IL-1β level in synovial-tissue homogenates of rats was also reduced, but not significantly. No significant difference was found between treatment with 5 mg/kg febuxostat and the same dose of WN1703 in terms of reducing the relative expression of ASC and NLRP3 ($P > 0.05$). Regarding caspase-1 and serum IL-1β, 5 mg/kg febuxostat was more effective at downregulating their levels than the same dosage of WN1703 was ($P < 0.01$). Therefore, WN1703 attenuated the expression of proteins in the NLRP3/ASC/Caspase-1 signaling pathway to reduce the secretion of serum IL-1β.

3.7. WN1703 improved synovial inflammation in rats with acute gouty arthritis by inhibiting the TLR4 signaling pathway

To preliminarily explore the potential anti-inflammatory mechanism of WN1703, TLR4 pathway-associated protein expression in synovial tissue was assessed using IHC and the concentration of TNF-α was detected using ELISA kits. The protein expression of MyD88 and NF-κB in the synovial tissues of rats in the MSU group was significantly higher than that in the control group ($P < 0.01$) (Fig. 7). These observations suggested that inflammation was induced in rat joints and that the TLR4 signaling pathway in the synovial tissue was partly activated.

Oral administration of febuxostat (5 mg/kg) and colchicine (0.5 mg/kg) downregulated relative protein expression of MyD88 and NF-κB and serum TNF-α levels ($P < 0.01$). Administration of WN1703 (5 mg/kg) could aid downregulation of the relative protein expression of NF-κB ($P < 0.05$) and serum TNF-α ($P < 0.01$). The TNF-α level in synovial-

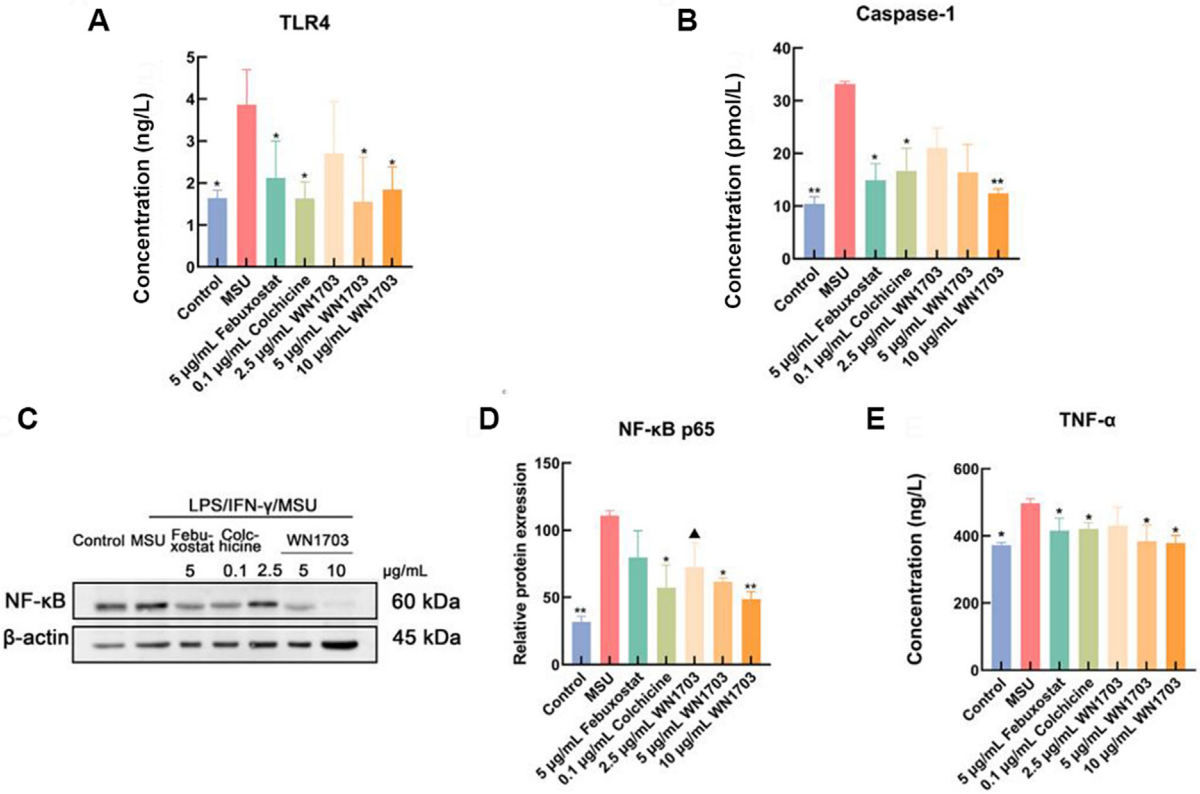


Fig. 3. Protein expression and levels of proinflammatory factors associated with the TLR4/MyD88/NF-κB pathway in the supernatant of THP-1 cells 24 h after LPS/IFN-γ/MSU inductions and drug administration (Control: THP-1 cells without LPS/IFN-γ/MSU induction and treated with an equal volume of blank solvent containing DMSO; MSU: THP-1 cells inducted by LPS/IFN-γ/MSU and treated with an equal volume of blank solvent containing DMSO; 5 μg/mL Febuxostat: THP-1 cells inducted by LPS/IFN-γ/MSU and treated with febuxostat (5 μg/mL); 0.1 μg/mL Colchicine: THP-1 cells inducted by LPS/IFN-γ/MSU and treated with colchicine (0.1 μg/mL); 2.5, 5, and 10 μg/mL WN1703: THP-1 cells inducted by LPS/IFN-γ/MSU and treated with 2.5, 5, and 10 μg/mL WN1703. A: TLR4 measured using ELISA kits; B: MyD88 measured using ELISA kits; C: NF-κB p65 measured using western blotting; D: statistical analysis of NF-κB expression; E: TNF-α measured using ELISA kits. One-way analysis of variance was used to compare and calculate the P values. Compared with MSU, $^{*}P < 0.05$, $^{**}P < 0.01$; 0.1 μg/mL Colchicine vs. WN1703, $^{▲}P < 0.05$, $^{▲▲}P < 0.01$). Abbreviations: TLR: toll-like receptor; MyD88, myeloid differentiation primary response protein 88; NF-κB: nuclear factor-kappa B; THP-1, Tohoku Hospital Pediatrics-1 cells; LPS, lipopolysaccharide; IFN-γ, interferon-γ; MSU, monosodium urate; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; TNF-α: tumor necrosis factor-α.

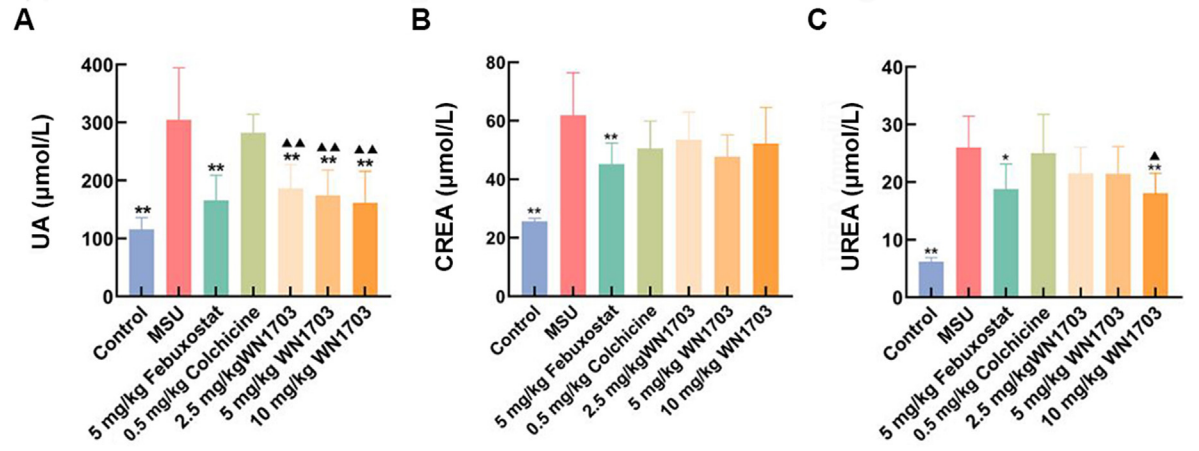


Fig. 4. Biochemical index of rats in each group after hyperuricemia was induced with potassium oxonate and hypoxanthine (Control: rats injected with CMC-Na and treated with CMC-Na; MSU: rats injected with potassium oxonate/hypoxanthine/MSU and treated with CMC-Na; 5 mg/kg Febuxostat: rats injected with potassium oxonate/hypoxanthine/MSU and treated with febuxostat (5 mg/kg); 0.5 mg/kg Colchicine: rats injected with potassium oxonate/hypoxanthine/MSU and treated with colchicine (0.5 mg/kg); 2.5, 5, and 10 mg/kg WN1703: rats injected with potassium oxonate/hypoxanthine/MSU and treated with 2.5, 5, and 10 mg/kg WN1703, respectively. A: UA; B: CREA; C: UREA. One-way analysis of variance was used to compare and calculate the P values. Compared with MSU, $^{*}P < 0.05$, $^{**}P < 0.01$; 0.5 mg/kg Colchicine vs. WN1703, $^{▲}P < 0.05$, $^{▲▲}P < 0.01$). Abbreviations: CMC-Na, sodium carboxymethyl cellulose; MSU, monosodium urate; UA, uric acid; CREA: creatinine; UREA: urea nitrogen.

Table 1
Ankle joint swelling degree (%) of rats with hyperuricemia 1–24 h after MSU injection to create severe gouty arthritis rat model.

	1 h	2 h	4 h	6 h	24 h
Control	21.77 ± 8.80	12.64 ± 6.73**	11.13 ± 5.11**	8.62 ± 2.89**	8.58 ± 4.59**
MSU	26.97 ± 9.10	40.58 ± 9.53	44.43 ± 9.73	39.29 ± 6.61	32.62 ± 12.14
5 mg/kg Febuxostat	23.54 ± 6.42	27.31 ± 8.58**	31.41 ± 9.49**	21.08 ± 10.54**	20.38 ± 4.16*
0.5 mg/kg Colchicine	26.75 ± 6.91	31.68 ± 4.68*	32.83 ± 4.49*	27.74 ± 3.18**	29.95 ± 7.73
2.5 mg/kg WN1703	26.65 ± 10.87	32.42 ± 5.25*	33.03 ± 8.37*	29.17 ± 4.32**,#	30.12 ± 10.69#
5 mg/kg WN1703	27.17 ± 6.45	28.34 ± 3.90**	30.65 ± 5.74**	29.00 ± 6.78**,#	27.74 ± 3.78#
10 mg/kg WN1703	26.17 ± 8.64	28.42 ± 5.75**	29.34 ± 8.17**	26.42 ± 9.77**	21.64 ± 7.35*

Control: rats injected with sodium carboxymethyl cellulose (CMC-Na) and treated with CMC-Na; MSU: rats injected with potassium oxonate/hypoxanthine/monosodium urate (MSU) and treated with CMC-Na; 5 mg/kg Febuxostat: rats injected with potassium oxonate/hypoxanthine/MSU and treated with febuxostat (5 mg/kg); 0.5 mg/kg Colchicine: rats injected with potassium oxonate/hypoxanthine/MSU and treated with colchicine (0.5 mg/kg); 2.5, 5, and 10 mg/kg WN1703: rats injected with potassium oxonate/hypoxanthine/MSU and treated with 2.5, 5, and 10 mg/kg WN1703, respectively. One-way analysis of variance was used to compare and calculate the P values. Compared with MSU, $^{*}P < 0.05$, $^{**}P < 0.01$; 5 mg/kg Febuxostat vs. WN1703, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

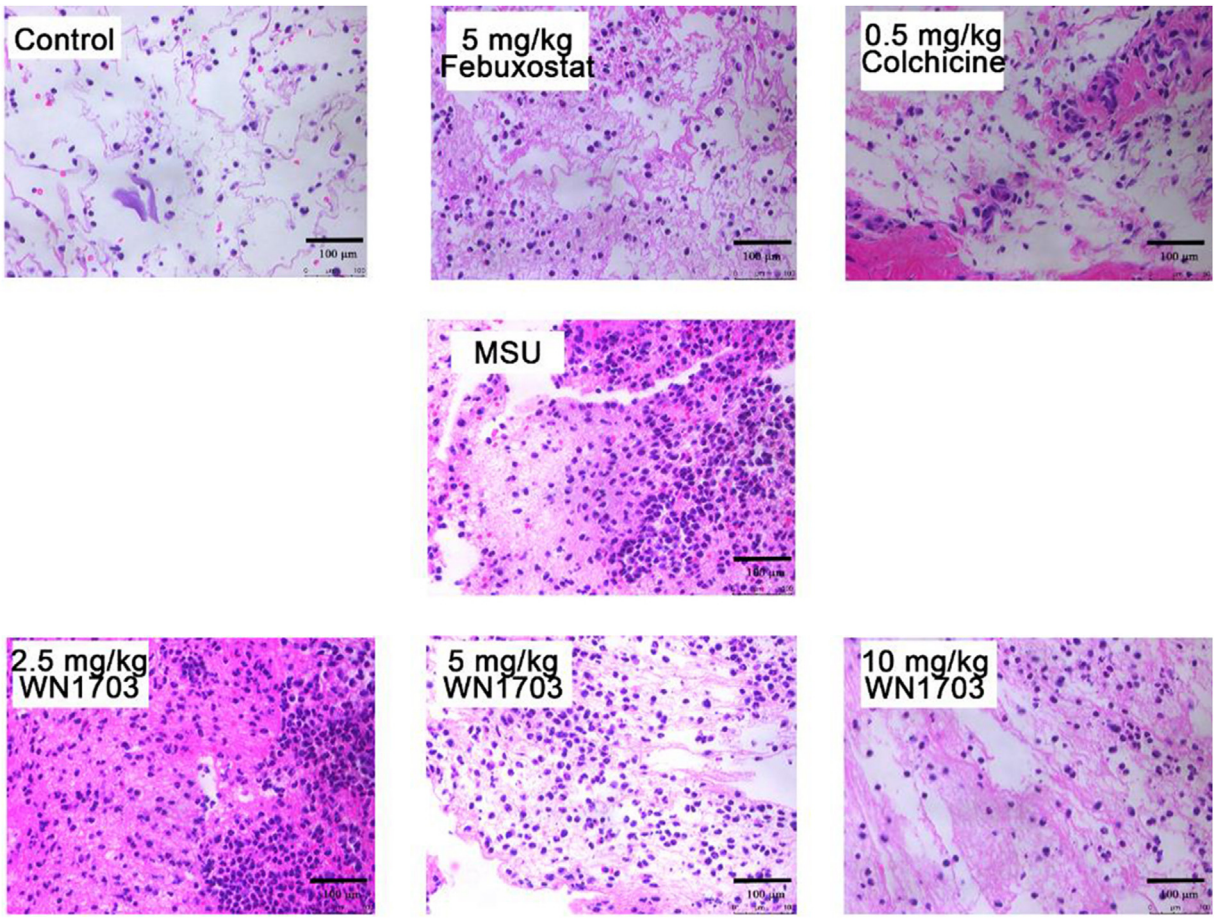


Fig. 5. H&E staining of the synovial tissue of rats suffering from hyperuricemia after MSU was injected in ankle joints to create a model of severe gouty arthritis (400 × magnification. Control: rats injected with CMC-Na and treated with CMC-Na; MSU: rats injected with potassium oxonate/hypoxanthine/MSU and treated with CMC-Na; 5 mg/kg Febuxostat: rats injected with potassium oxonate/hypoxanthine/MSU and treated with febuxostat (5 mg/kg); 0.5 mg/kg Colchicine: rats injected with potassium oxonate/hypoxanthine/MSU and treated with colchicine (0.5 mg/kg); 2.5, 5, and 10 mg/kg WN1703: rats injected with potassium oxonate/hypoxanthine/MSU and treated with 2.5, 5, and 10 mg/kg WN1703, respectively. Abbreviations: H&E, hematoxylin and eosin; MSU, monosodium urate; CMC-Na, sodium carboxymethyl cellulose.

tissue homogenates of rats was also reduced, but not significantly. At the same dose, febuxostat was superior to WN1703 in terms of down-regulating relative expression of MyD88 (5 mg/kg febuxostat vs. 5 mg/kg WN1703, 0.036 ± 0.007 vs. 0.045 ± 0.009, $P < 0.05$). Moreover, no significant difference in their ability to lower NF-κB and TNF-α levels was found at the same 5 mg/kg dose ($P > 0.05$). Therefore, WN1703 attenuated the expression of proteins in the TLR4/MyD88/NF-κB signaling pathway to reduce the secretion of serum TNF-α.

4. Discussion

In our previous study [15], WN1703 and febuxostat were found to reduce the levels of inflammatory factors. Thus, we studied the effects of WN1703 on inflammation using a rat gout model and explored the underlying anti-inflammatory mechanism in THP-1 cells.

MSU is commonly used to stimulate macrophages and build gout models *in vivo*, and THP-1 is a human leukemia monocytic cell line that can be induced by PMA to become a macrophage cell line [19] and

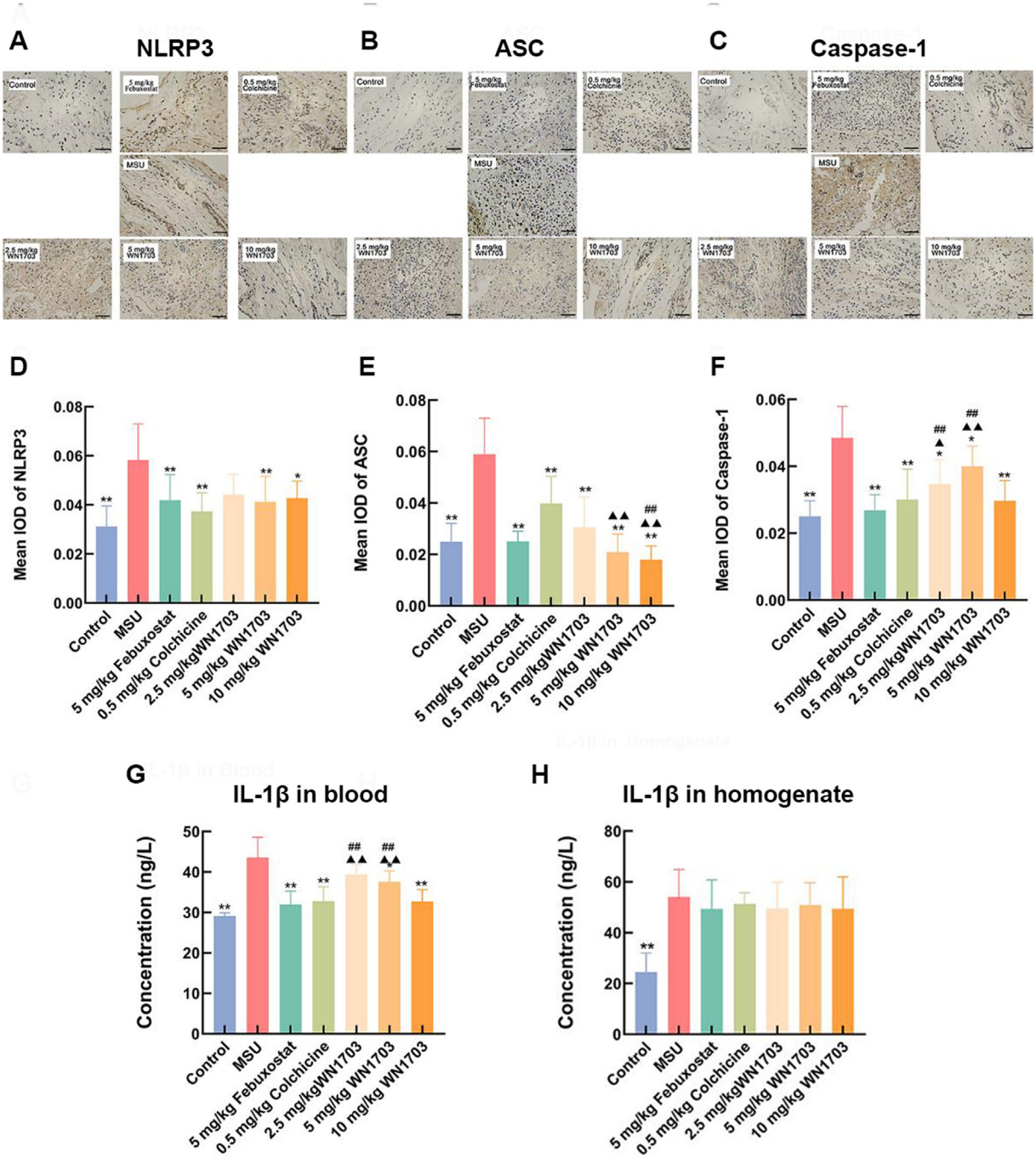
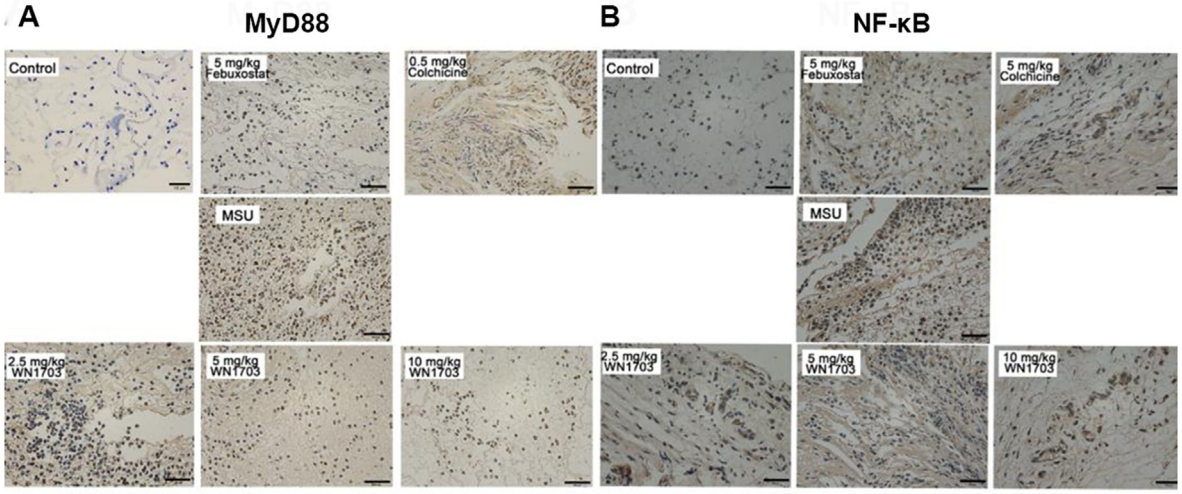
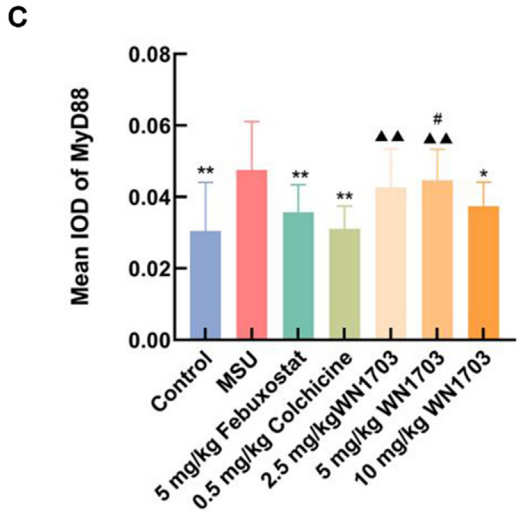


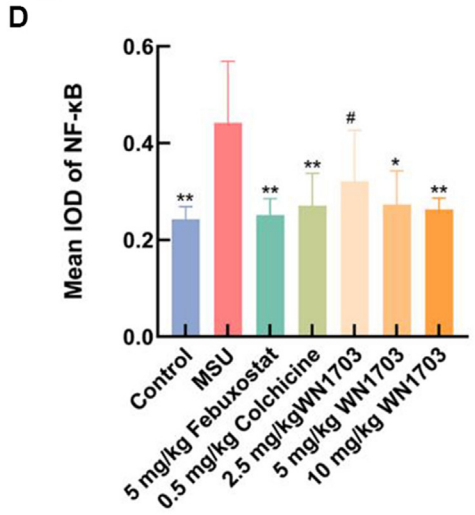
Fig. 6. Relative protein expression of NLRP3, ASC, and caspase-1 in the synovial tissue of rats suffering from hyperuricemia after MSU was injected in ankle joints to create a model of severe gouty arthritis (Control: rats injected with CMC-Na and treated with CMC-Na; MSU: rats injected with potassium oxonate/hypoxanthine/MSU and treated with CMC-Na; 5 mg/kg Febuxostat: rats injected with potassium oxonate/hypoxanthine/MSU and treated with febuxostat (5 mg/kg); 0.5 mg/kg Colchicine: rats injected with potassium oxonate/hypoxanthine/MSU and treated with colchicine (0.5 mg/kg); 2.5, 5, and 10 mg/kg WN1703: rats injected with potassium oxonate/hypoxanthine/MSU and treated with 2.5, 5, and 10 mg/kg WN1703, respectively. A, B, C: comparison of the IHC results of synovial tissue in each group of rats, 400 × magnification; D, E, F: statistical analysis of protein expression in synovial tissue of rats in each group; G, H: level of IL-1β in the serum and synovial tissue of rats in each group. One-way analysis of variance was used to compare and calculate the P values. Compared with MSU, $^{*}P < 0.05$, $^{**}P < 0.01$; 5 mg/kg Febuxostat vs. WN1703, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; 0.5 mg/kg Colchicine vs. WN1703, $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$). Abbreviations: NLRP3, nucleotide-binding oligomerization domain-like receptor thermal protein domain associated protein 3; ASC, apoptosis-associated speck-like protein containing a CARD; MSU, monosodium urate; CMC-Na, sodium carboxymethyl cellulose; IHC, immunohistochemistry; IL: interleukin.

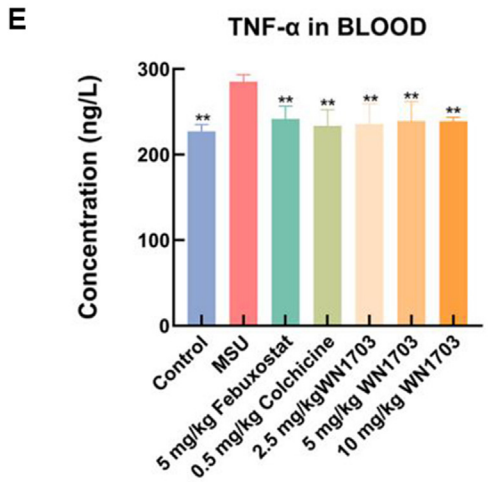
express TLRs and NLRP3 [20,21]. Therefore, this cell line has been used in inflammation research, including acute gouty arthritis. Hence, we selected THP-1 cells to study the inhibitory effect of WN1703 and compared it with that of febuxostat and colchicine. To explore the mechanism of action of WN1703 on gouty arthritis, expression of key proteins in the NLRP3/ASC/caspase-1 and TLR4/MyD88/NF-κB signaling pathways was investigated.

MSU mediates inflammation in an inflammasome-dependent manner [7]. MSU triggers an autoimmune response closely related to the activation and assembly of the NLRP3 inflammasome (a multiprotein complex composed of NLRP3, ASC, and caspase-1) [22]. After enhancement of the molecular interactions between ASC and NLRP3, inactive









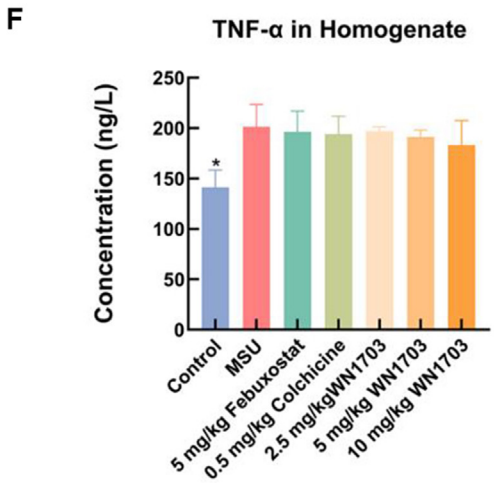


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Fig. 7. Relative protein expression of MyD88 and NF-κB in the synovial tissue of rats suffering from hyperuricemia at the experimental endpoint after MSU injection in ankle joints to create a model of severe gouty arthritis (Control: rats injected with CMC-Na and treated with CMC-Na; MSU: rats injected with potassium oxonate/hypoxanthine/MSU and treated with CMC-Na; 5 mg/kg Febuxostat: rats injected with potassium oxonate/hypoxanthine/MSU and treated with febuxostat (5 mg/kg); 0.5 mg/kg Colchicine: rats injected with potassium oxonate/hypoxanthine/MSU and treated with colchicine (0.5 mg/kg); 2.5, 5, and 10 mg/kg WN1703: rats injected with potassium oxonate/hypoxanthine/MSU and treated with 2.5, 5, and 10 mg/kg WN1703, respectively. A, B: comparison of the IHC results of synovial tissue in each group of rats, 400 × magnification; C, D: statistical analysis of protein expression in the synovial tissue of rats in each group; E, F: levels of TNF-α in the serum and synovial tissue of rats in each group. One-way analysis of variance was used to compare and calculate the P values. Compared with MSU, $^{*}P < 0.05$, $^{**}P < 0.01$; 5 mg/kg Febuxostat vs. WN1703, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; 0.5 mg/kg Colchicine vs. WN1703, $^{▲}P < 0.05$, $^{▲▲}P < 0.01$). Abbreviations: MyD88: myeloid differentiation primary response protein 88; NF-κB: nuclear factor-kappa B; MSU, monosodium urate; CMC-Na, sodium carboxymethyl cellulose; IHC, immunohistochemistry; TNF-α: tumor necrosis factor-α.

pro-caspase-1 and pro-IL-1β are cleaved and mature IL-1β is secreted [23,24]. Alternatively, stimulation by MSU activates the NLRP3 inflammasome to mediate neutrophil recruitment and increases the release of IL-1β and other cytokines. One of the postulated pathogenic mechanisms of gout is that the activated NLRP3 inflammasome upregulates transcriptional expression of NLRP3 and pro-IL-1β, promotes inflammasome assembly, and mediates pro-IL-1β cleavage and IL-1β maturation [7,25–27].

MSU is recognized by TLR4 in macrophages, which initiates a signaling cascade and releases pro-inflammatory transcription factors such as MyD88 and NF-κB to produce IL-1β and other factors [28,29]. MSU promotes the interaction between the cytoplasmic Toll/IL-1 receptor domain and MyD88 contained in this domain [19]. MyD88 attracts IL-1 receptor-associated kinase (IRAK)-4 to TLRs. IRAK-1 may be activated by phosphorylation and interact with tumor necrosis factor receptor associated factor 6, which subsequently activates the IκB kinases complex, activates mitogen-activated protein kinase and NF-κB, and upregulate the transcription of proinflammatory factors such as IL-1β and TNF-α [5]. Furthermore, the combination of IL-1β and the IL-1 receptor aids the recruitment of MyD88, and these factors in turn promote this proinflammatory response in a MyD88-dependent manner [22].

The therapeutic effect of colchicine on gout was thought to be closely associated with its inhibitory effects on microtubule polymerization [30,31]. The minimum concentration of colchicine needed to alleviate inflammation in bone-marrow-derived macrophages was 10 nM, and 10 nM of colchicine effectively reduced activation of NLRP3, caspase-1, and release of IL-1β [32]. In our study, colchicine (0.1 ng/mL) reduced protein expression of the NLRP3 and TLR4 signaling pathways in THP-1 cells. Febuxostat elicited remarkable anti-inflammatory effects on the downregulation of expression of NLRP3, caspase-1, and NF-κB [33–35]. Pretreatment with febuxostat (30 μM) was proved to relieve the abnormal increase in NLRP3 and caspase-1 expression and cytokine levels in LPS-induced THP-1 cell models [33]. Similarly, in our study, febuxostat and WN1703 showed remarkable inhibitory effects on the NLRP3 signaling pathway. Febuxostat showed effects on mitigating activation of the NF-κB signaling pathway and reducing the TNF-α level in primary astrocytes [34,36]. WN1703 and febuxostat also showed similar effects in terms of downregulating expression of TLR4, MyD88, and NF-κB. At the same dose, febuxostat and WN1703 had a comparable ability to reduce the release of IL-1β and TNF-α; however, statistical analysis revealed that their downregulating effects on key proteins in the NLRP3 and TLR4 signaling pathways were not completely consistent, which suggested that they may mediate similar mechanisms in the process of MSU-related stimulation, but with different strength effects.

Coderre et al. injected MSU (0.2 mL) into rat ankles to simulate an MSU-associated inflammatory response in gout [26,37,38]. Needle-like MSU crystals stimulate synovial tissue and induce inflammation in ankle joints of rats. The anti-inflammatory effects of these XOR inhibitors were verified in rats with MSU-induced gouty arthritis.

In the present study, H&E staining demonstrated neutrophil recruitment caused by MSU, and the results were consistent with the degree of ankle swelling in rats. The 5 mg/kg WN1703 had comparable effects with 0.5 mg/kg colchicine in terms of relieving inflammation and ankle swelling with no statistical difference, and the effect of 5 mg/kg febuxostat and 10 mg/kg WN703 was superior to that elicited by colchicine. No statistical difference was found between the same dose of WN1703 and febuxostat in the *in vitro* experiment, as well as in the *in vivo* expression of NLRP3, ASC, and NF-κB. Administrations of colchicine, febuxostat, or WN1703 reduced relative expression of ASC, MyD88, and NF-κB, etc. in synovial tissue, with IL-1β and TNF-α levels decreasing simultaneously. Hence, treatment with febuxostat or WN1703 alleviated inflammation in gouty arthritis in rats through downregulation of key proteins expression in the NLRP3/ASC/caspase-1 and TLR4/MyD88/NF-κB signaling pathways to decrease the level of proinflammatory factors. XOR was reported to catalyze UA production, accompanied by the release of reactive oxygen species (ROS) [2]. The inhibition of XOR would impair IL-1β and reduce the level of caspase-1 and ROS [39,40]. Soluble UA, independent of MSU, is a pro-inflammatory agent [41], and prophylactic treatment with febuxostat could lessen acute gout flares [11]. Moreover, a clinical trial showed that gradually increasing the dose of febuxostat is a viable alternative to low-dose colchicine treatment [42]. Thus, the superior anti-inflammatory effects of febuxostat and high-dose WN1703 may be related not only to their impact on proinflammatory proteins, but also to their downregulating effects on the level of soluble serum UA and the prevention of acute attacks of gout.

Compared with that in the *in vitro* experiment, WN1703 and febuxostat showed stronger inhibitory effects on proteins in the NLRP3/ASC/caspase-1 and TLR4/MyD88/NF-κB pathways in the *in vivo* experiment. This improved activity may be because WN1703 and febuxostat not only interfered with key proteins in the NLRP3/ASC/caspase-1 and TLR4/MyD88/NF-κB inflammatory pathways in rats, but also reduced the UA levels to alleviate the UA-induced peripheral inflammatory response in rats. Febuxostat reduces intracellular inflammation by inhibiting XOR activity, reducing ROS release in cellular mitochondria, and hindering NLRP3 aggregation [43]. However, the intrinsic association between the anti-inflammatory effects of WN1703 and its XOR-inhibitory activity remains unclear. No significant upregulation in TLR4 protein expression was detected in the synovial tissue of MSU rats (IHC results on TLR4 are listed in the Supplementary File). TLR4 is essential for innate immunity during MSU-induced inflammation [44]. However, some *in vivo* experiments in mice or rats have revealed that TLR4 deficiency does not influence MyD88 activation or the production of proinflammatory factors through an IL-1 receptor-mediated signaling pathway [22,28]. Hence, the inflammation induced by the MSU injection in the present study may also have been activated in a TLR4-independent manner. However, in this experiment, we failed to observe increased expression of TLR4 based on the IHC results, and the underlying mechanism related to the anti-inflammatory effects of WN1703 remains unclear. Thus, in our future research, we will attempt to develop additional animal models with TLR4 expression upregulated and explore additional receptors, proteins, and cytokines that play key roles in the anti-inflammatory process associated with WN1703.

In our previous 35-day study [15], febuxostat and WN1703 showed strong downregulating effects on IL-1β; however, the downregulating effects of WN1703 on serum TNF-α was not significant. This phenomenon may be due to the complex pathological changes in rats during long-term hyperuricemia, and WN1703 may be best at relieving inflammation in the early stages of hyperuricemia.

5. Conclusions

In this study, we first examined the inhibitory effects of WN1703 on inflammatory cytokine production in THP-1 cells stimulated with MSU and its anti-inflammatory effects in acute gout rats. The results revealed that such effects were related to reductions in the expression of key proteins in the NLRP3/ASC/caspase-1 and TLR4/MyD88/NF-κB signaling pathways both *in vivo* and *in vitro*. Therefore, the anti-inflammatory activity of WN1703 may aid in the treatment of gouty arthritis.

Author contributions

Fuyao Liu: Investigation, visualization, methodology, writing (original draft), and conceptualization.

Xiaodan Lu: Investigation and methodology.

Lei Zhang: Visualization and validation.

Jing Li: Conceptualization and supervision.

All the authors have read and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Ethics approval

The study protocol was approved (AEC: 2021003) by the Ethics Committee of South China University of Technology (Guangzhou, China).

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Declaration of competing interest

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.

Acknowledgments

Images of rats and THP-1 cell clusters in the Graphical Abstract were obtained from the Biorender website (https://www.biorender.com/). DNA and other signals that were observed in this study were drawn using Adobe Illustrator 2020 (Adobe, U.S.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pscia.2024.100039.

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