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Synthesis, activity and mechanism for double-ring conjugated enones

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ABSTRACT

The relationship between TLR4 and inflammation-related diseases has been paid more and more attention. The studies have shown that TLR4/NF-κB signaling pathway plays an important role in the transmission of inflammatory signals. A large number of pro-inflammatory factors, chemokines, adhesion factors, TLR4 and its ligands interact with each other, and jointly promote the development of diseases. In this work, 8 target compounds were synthesized to screen the inhibitory activity of TLR4 *in vitro*. The results of TLR4 inhibition test *in vitro* showed that the double-ring conjugated enones had a good inhibitory activity, and the IC_{50} value of compound **4f** was 0.56 ± 0.10 μM, and it was superior to the positive control methotrexate. To further study the anti-inflammatory effect and mechanism of double-ring conjugated enones by using LPS induced rat synovial cell inflammation model. The results of the mechanism test showed that compound **4f** could effectively promote the apoptosis of rat synovial cells, and the mechanism might be related to the up-regulation of the expression of apoptosis-related protein Caspase-3. In addition, compound **4f** could significantly inhibit the increase of inflammatory factors TNF-α, IL-1β and IL-6 in rat synovial cells induced by LPS, showing a good anti-inflammatory activity. In the TLR4/NF-κB signaling pathway test of rat synovial cells, compound **4f** can effectively regulate the expression levels of TLR4, MyD88, NF-κB and IκB related proteins in TLR4/NF-κB signaling pathway, which may be due to its inhibition of LPS-induced inflammation in rat synovial cells. At the same time, it inhibits the abnormal proliferation of cells and its important mechanism promoted of apoptosis.

Rheumatoid arthritis (RA) treatment is mainly to relieve joint inflammation and joint pain.[1] Inhibitory the further development of RA and the irreversible destruction of bone, and strive to protect the normal function of joints and muscles.[2–4] So, as to achieve the goal of completely alleviating arthritis or completely being treated, improve the overall function of patients.[5–8] At present, the commonly used for treatment of RA drugs can be divided into five categories: non-steroidal anti-inflammatory drugs, slow-acting anti-rheumatic drugs, glucocorticoids, biological agents targeted therapy, botanical preparation.[9–11] Current, plant drugs was used already appeared much kind in cure RA, wait for on the market medicine such as total glucoside of paeony, sinomenine, tripterygium wilfordii. Plant drugs have certain clinical efficacy in the treatment of RA, but the mechanism of action of the drugs still needs further research.[12–14] Dysdensiol K is a kind of monomer compound with anti-rheumatoid arthritis activity isolated from *Fissistigma oldhamii*. Its inhibitory effect on synovial cells *in vitro* was IC_{50} = 11.8 ± 0.23 μM.[15,16] In the previous work, Dysdensiol K was used as the lead compound to design and synthesize four series of double-ring conjugated enones, and the anti-rheumatoid arthritis activity was evaluated. The results of anti-rheumatoid arthritis activity evaluation showed that double-ring conjugated enones were identified as potential candidates drug for treatment of RA. In the screening of rat synovial cell formation *in vitro*, the double-ring conjugated enones showed ideal inhibitory activity, among which the IC_{50} values of low μM level.[17] Meanwhile, in the CIA rat model, the double-ring conjugated enones showed *in vivo* therapeutic and ameliorative effects on RA compared with methotrexate, achieving the same therapeutic level. The double-ring conjugated enones inhibited the production of IL-6 and appropriately reduced the formation of TNF-α during *in vivo* treatment.[18–20] Molecular docking simulation showed that the target compound had a good match with TLR4 receptor. TLR4/NF-κB plays an important role in the pathogenesis of RA by regulating the expression of cytokines (TNF-α,

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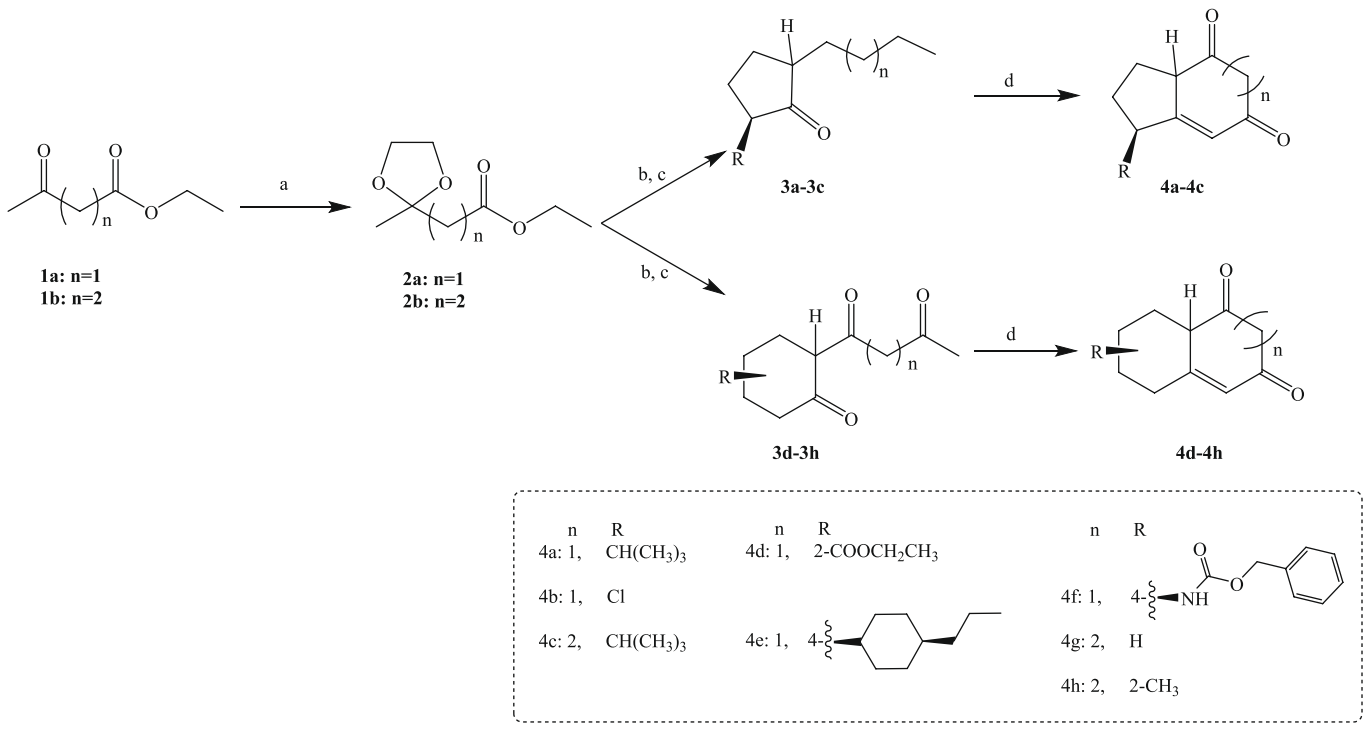


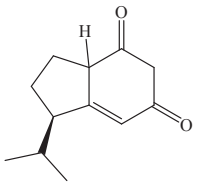
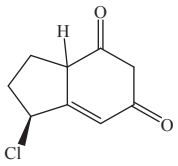
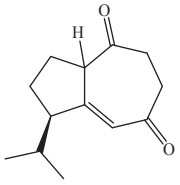
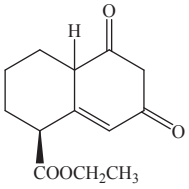
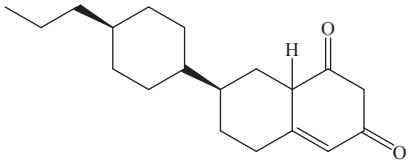
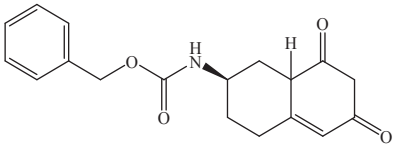
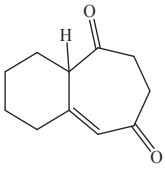
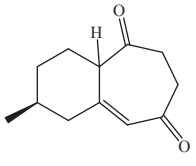
Scheme 1. The synthetic route of the double-ring conjugated enones **4a-4h**. Reagents and conditions: (a) ethanediol, benzene, *p*-TsOH, reflux for 48 h; (b) substituted cyclopentanone or cyclohexanone, C_2H_5OH, C_2H_5ONa, reflux for 12 h; (c) HCl, H_2O, 60 °C, 2 h, (d) KOH, C_2H_5OH, reflux for 12 h.

IL-1β), matrix metalloproteinases (MMP-3, MMP-9), angiogenic factors (VEGF), inducible enzymes (COX-2, iNOS), and other genes.[21,22] Involved in the pathological process of arthroinflammatory response and injury response.[23] Based on the existing activity evaluation, this work we synthesized eight double-ring conjugated enones, and further studied the effect target and the mechanism of action against RA.[24–26] The TLR4 receptor inhibitory test was used *in vitro* to determine the receptor of action.[27] The effects on the apoptosis of primary rat synovial cells, the expression of apoptotic-related proteins in primary rat synovial cells, the effects on the inflammatory factors in primary rat synovial cells induced by LPS and the effects on the TLR4/NF-κB signaling pathway in primary rat synovial cells induced by LPS were determined to study the mechanism of action.[28–30] The mechanism of anti-rheumatoid arthritis was studied to provide theoretical guidance for the drug pharmacodynamics research, and provides a certain experimental basis for further research and development of anti-rheumatoid arthritis drugs in the future.

The Dysodensiol K which has an anti-rheumatoid arthritis activity ($IC_{50} = 11.8 \pm 0.23$ μM) and it was isolated from the *Fissistigma oldhamii*. The chemical structures of the lead compound was analyzed and it was found that all of them had the double-ring structures. Through the analysis of SAR, it was found that the double-ring structure was the parent nuclear structure of the compound, and the activity of the compound against rheumatoid arthritis was determined by the substituents on the ring. On the basis of this, the Dysodensiol K was used as the lead compound to design the double-ring conjugated enones with similar parent nucleus structure. From the chemical structure analysis, it found that the target compounds and the Dysodensiol K also have the structure of double-ring conjugated enone. In such a structural design, the target compounds should have a similar spatial structure of the lead compound, which may also show a good anti-rheumatoid arthritis activity in the biological activity. Even after the chemical structure modification of the lead compound, some of the target compounds have a better biological activity than the lead compound. During the structural modification of the lead compound, we introduced two different substituents of electron-absorbing group and electron-donating group at position arcus adiposus to change the log*P* and pK_a of the target compounds, so as to achieve the goal of changing the anti-rheumatoid arthritis activity of the target compounds **4a-4h**. In the synthesis of the target compounds **4a-4h** we chose an efficient and simple synthesis method. According to the pathway described in Scheme 1, a series of double-ring conjugated enones were synthesized. We used ethyl acetoacetate or ethyl levulinate as the starting material, and synthesize the target compounds through four steps: acetylcarbonyl protection, condensation reaction, hydrolysis reaction and ring formation reaction. During the protection of acetylcarbonyl group, ethyl acetoacetate (compound **1a**) and ethylene glycol were used for condensation reaction in benzene. In this step, *p*-methylbenzene sulfonic acid (*p*-TsOH) was used as reaction catalyst, and the reduced ketals (compound **2a**) could be obtained within 48 h after reflux (yield 99%). The general method were used to synthesis compounds **2b**. After the acquisition of compound **2a** and **2b**, the key intermediates (compounds **3a-3h**) were obtained by condensation reaction and hydrolysis reaction with substituted cyclopentanone and cyclohexanone. Anhydrous ethanol was used as the reaction solvent, and solid sodium hydroxide (NaOH) was used as the basic substance. After reflux reaction for 12 h, the key intermediates compounds **3a-3h** were obtained by hydrolysis and removal of the carbonyl protection. Finally, the compounds **3a-3h** were dissolved in anhydrous ethanol to generate a ring reaction. The solid potassium hydroxide (KOH) was used as the basic material, and the target compounds (compounds 4**a-4h**) could be obtained after reflux reaction for 12 h. The structures of all target compounds were characterized by ^{1}H NMR, ^{13}C NMR and HR-ESI-MS spectra. The synthesis route was characterized by simple operation, mild reaction conditions, cheap reagents and raw materials, and the total yield was moderate to good.

Toll-like receptor 4 (TLR4) was belong to the pattern receptor family. They were highly conserved receptor families that recognize conserved pathogen associated submodes and therefore represent the first line of defense. TLR4 was thought to be a recognition receptor for lipopolysaccharide in gram-negative bacteria. In addition, TLR4 has been widely shown to be involved in the recognition of endogenous molecules that were released by damaged tissues and necrotic cells, and these damage-

Table 1
TLR4 *in vitro* inhibition data.

Compounds	Structure	TLR4 IC_{50} (μM)[a] ± *SD*
4a		2.53 ± 0.31
4b		2.71 ± 0.30
4c		2.82 ± 0.28
4d		1.54 ± 0.21
4e		0.73 ± 0.15
4f		0.56 ± 0.10
4g		1.85 ± 0.26
4h		2.66 ± 0.26
methotrexate[b]		1.04 ± 0.22

[a] Values are the average of 3 independent experiments run in triplicate.
[b] Methotrexate is use as positive control.

related molecular pattern molecules induce and promote inflammatory response activation by interacting with TLR4.[31] Thus, TLR4 was a key receptor that was induced by exogenous and endogenous ligand proinflammatory responses in infectious stimulation-mediated interactions. TLR4 test kits was a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). TLR4 was placed in the coated micropores of the captured antibody, and the detection antibodies to be tested and HRP-labeled was added successively. The antibodies were incubated and thoroughly washed. The substrate TMB, catalyzed by peroxidase, was transformed into blue and the final yellow under the action of acid. The shade of the color was positively correlated with the TLR4 in the sample. The absorbance was measured at 450 nm with a microplate analyzer and the concentration of the sample was calculated.[31] To further investigate the *in vitro* anti-rheumatoid arthritis activity of these target compounds, we tested compounds **4a-4h** for TLR4 inhibition *in vitro*. As could be seen from the test data in Table 1, compounds **4a-4h** showed good inhibitory activity against TLR4. The IC_{50} values of these compounds inhibited TLR4 *in vitro* were at the level of μM, among which compounds **4e** and **4f** were the most prominent, and the IC_{50} values of TLR4 inhibition activities were 0.73 ± 0.15 and 0.56 ± 0.10 μM, respectively, which were better than the positive control methotrexate (1.04 ± 0.22 μM).These results suggest that the target compound can bind well to TLR4, inhibit its activity and reduce the release of inflammatory factors, and ultimately improve or treatment RA.

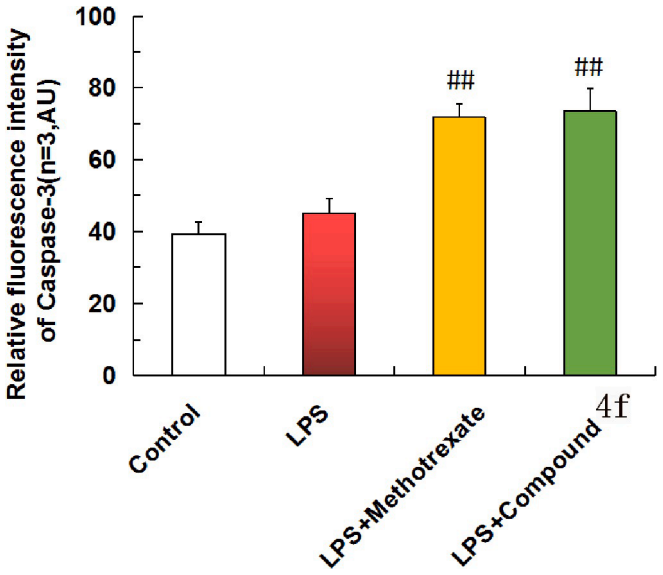


Fig. 1. The changes in the morphology of the synovial cells in rats (Tunel staining, 100×).

Cytomorphology, also known as cytological examination. It is a traditional and classic clinical diagnosis method with a long history. Cytomorphology is a science that studies the microstructure and submicrostructure of cells and their components, including the structure of biological macromolecules that represent the phenomena of cell life.[32] Because of its simplicity, practicality and the fact that seeing directly is better than hearing, cytomorphology is often use in testing the activity of compounds. Based on the results of LTR4 inhibition test *in vitro*, we selected the target compound **4f**for the morphological observation of rat synovial cells (Fig. 1). The results showed that, compared with the normal group, the synovial cells of rats induced by LPS were significantly proliferated, and the cell bodies were significantly enlarged, and were round, oval or irregular spindle shaped. However, after treatment with 1 μM methotrexate and 1 μM compound 6, the number of cells was significantly reduced, the refractive ability was reduced, and the cell body was irregular spindle or round, suggesting that compound **4f** could significantly inhibit the abnormal proliferation of rat synovial cells induced by LPS.[32]

Apoptosis is a basic phenomenon that exists widely in the biological world. It is plays a very important role as well as cell growth, development and proliferation. Currently, it is believe that the extracellular stimulation to induce apoptosis must be transmitted through a series of

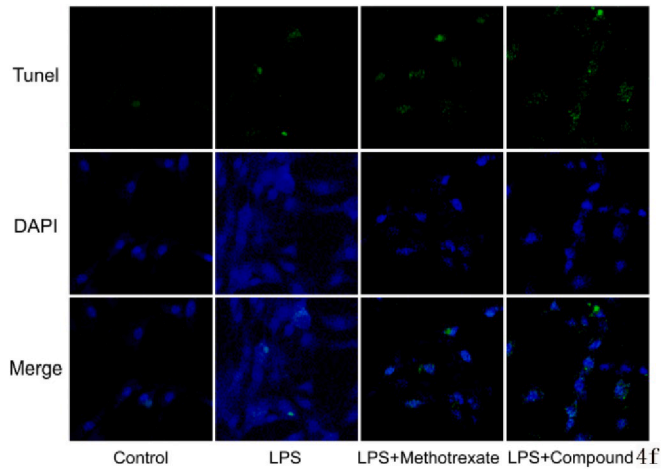


Fig. 2. Apoptosis of rat synovial cells (Tunel staining, 200×).

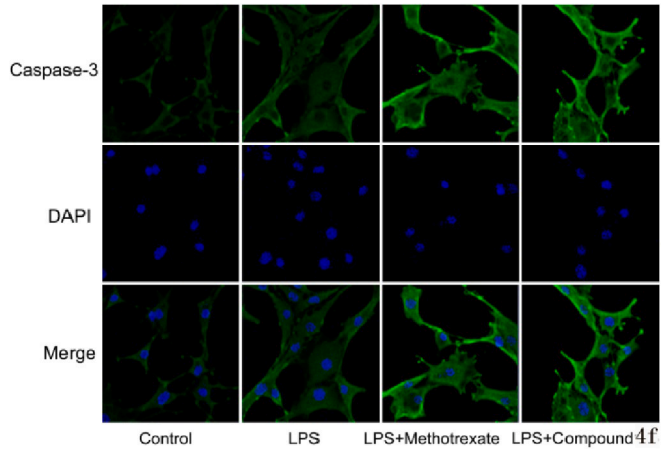


Fig. 3. Expression of apoptosis-related protein Caspase-3 in rat synovial cells (Immunofluorescence, 200×).

intracellular signals.[33] Apoptosis was induced by endogenous and exogenous factors in the induction stage. Endogenous factors include the activation of apoptosis inducing mechanisms (such as Fas ligands, tumor necrosis factor, etc.) and the inactivation of inhibition mechanisms (such as growth factors, hormones, receptor factors and other proliferative factors). Exogenous factors include physical factors such as radiation and heat shock, chemical factors such as drugs and poisons, biological factors such as viruses and bacteria. The reason of apoptosis has become a hot spot of research is largely determined by the close relationship between apoptosis and clinical virus. This relationship is not only manifest in the study of apoptosis and its mechanism, which elucidates the pathogenesis of a large class of immune diseases, but also leads to the emergence of new therapies for diseases. In particular, the close relationship between apoptosis and tumor, AIDS and RA has attracted much attention.[33] The clinical manifestation of RA was synovitis, so inhibiting the growth of synovial cells and promoting their apoptosis could be used as an evaluation index. In the process of rat synovial cell apoptosis induced by compound **4f**, the positive apoptotic cells were stained green after Tunel staining (Fig. 2). The results of cell apoptosis showed that there were almost no positive apoptotic cells in the normal group. After LPS (1 μg/mL) induction, the number of apoptotic cells increased, but there was no significant difference compared with the normal group. However, after treatment with 1 μM methotrexate and 1 μM compound **4f**, the number of apoptotic cells increased significantly, showing typical apoptotic characteristics such as

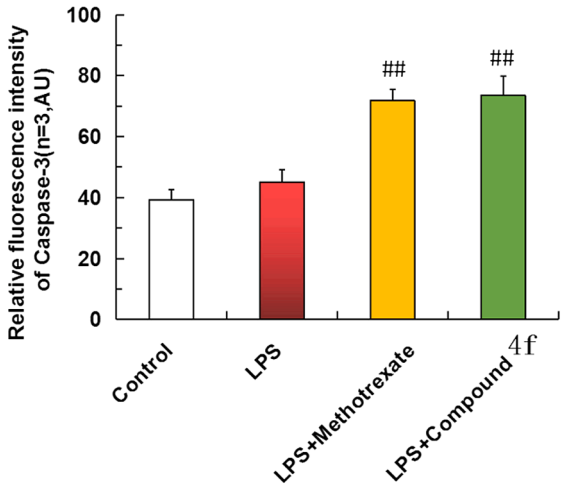


Fig. 4. Comparison of the expression levels of apoptosis-related protein Caspase-3 in rat synovial cells (n = 3). Compared with the LPS group, the significance symbols is ## = $p < 0.01$.

nuclear pyknosis and chromatin concentration, suggesting that compound **4f** can effectively promote the apoptosis of rat synovial cells.[33]

Caspase-3 is a protease, a member of the Caspase family which plays an irreplaceable role in cell apoptosis. Caspase-3 can be activate by a variety of factors. In CTL cell killing, Caspase-3 can be activate by both the Fas/FasL pathway and the granulosase B pathway. Granulosase B is a serine esterase in CTL cell granules, and it is the only mammalian protease that cleaves after Asp except the Caspase protease. Caspase-3 normally exists in the cytoplasm in the form of proenzyme (32 kDa). In the early stage of apoptosis, it is activate.[34] The activate Caspase-3 consists of two large subunits (17 kDa) and two small subunits (12 kDa), cleavages the corresponding cytoplasmic and nuclear substrates, and eventually leads to apoptosis. However, the activity of caspase-3 decrease significantly in the late stage of apoptosis and in the dead cells. In order to verify the mechanism of compound **4f** promoting the apoptosis of rat synovial cells, we further studied the effect of compound **4f**on the expression level of apoptosis-related protein Caspase-3 (Fig. 3 and Fig. 4). The results of Caspase-3 expression test showed that the expression of apoptosis-related protein Caspase-3 was increased in the LPS group compared with the normal group, but there was no significant difference[34]. Compared with LPS group, 1 μM methotrexate and 1 μM compound group **4f** significantly increased the expression level of apoptosis-related protein Caspase-3 in synovial cells ($p < 0.01$), which suggested that compound **4f**group could effectively promote the apoptosis of rat synovial cells, and the mechanism of promoting apoptosis might be related to the up-regulation of apoptosis-related protein Caspase-3.

RA is an autosystemic immune disease with symmetrically and multiarticular synovitis as the main clinical manifestation. With progression of synovitis, the synovial cells proliferate abnormally and form pannus of synovial vessels.[35] There is a large number of neovascularization and infiltration of various inflammatory cells inside, which erode cartilage and bone and lead to joint destruction. In RA patients, synovial cells are significantly increase, including a large number of fibroblast synovial cells and inflammatory cells are infiltrate around proliferative FLS. The produce a large number of inflammatory factors, such as TNF-α, IL-1β, IL-6, etc., leading to synovial inflammation and cartilage and bone destruction.[35] In this experiment, LPS induced synovial cell inflammation model, and the content of factors (TNF-α, IL-1β, IL-6) in the supernatant of cell culture was detected by ELISA (Fig. 5a-5c). Compared with the normal group, the contents of TNF-α, IL-1β and IL-6 in the synovial cell supernatant of rats in LPS group were significantly increased ($p < 0.01$). Compared with LPS group, the contents of inflammatory

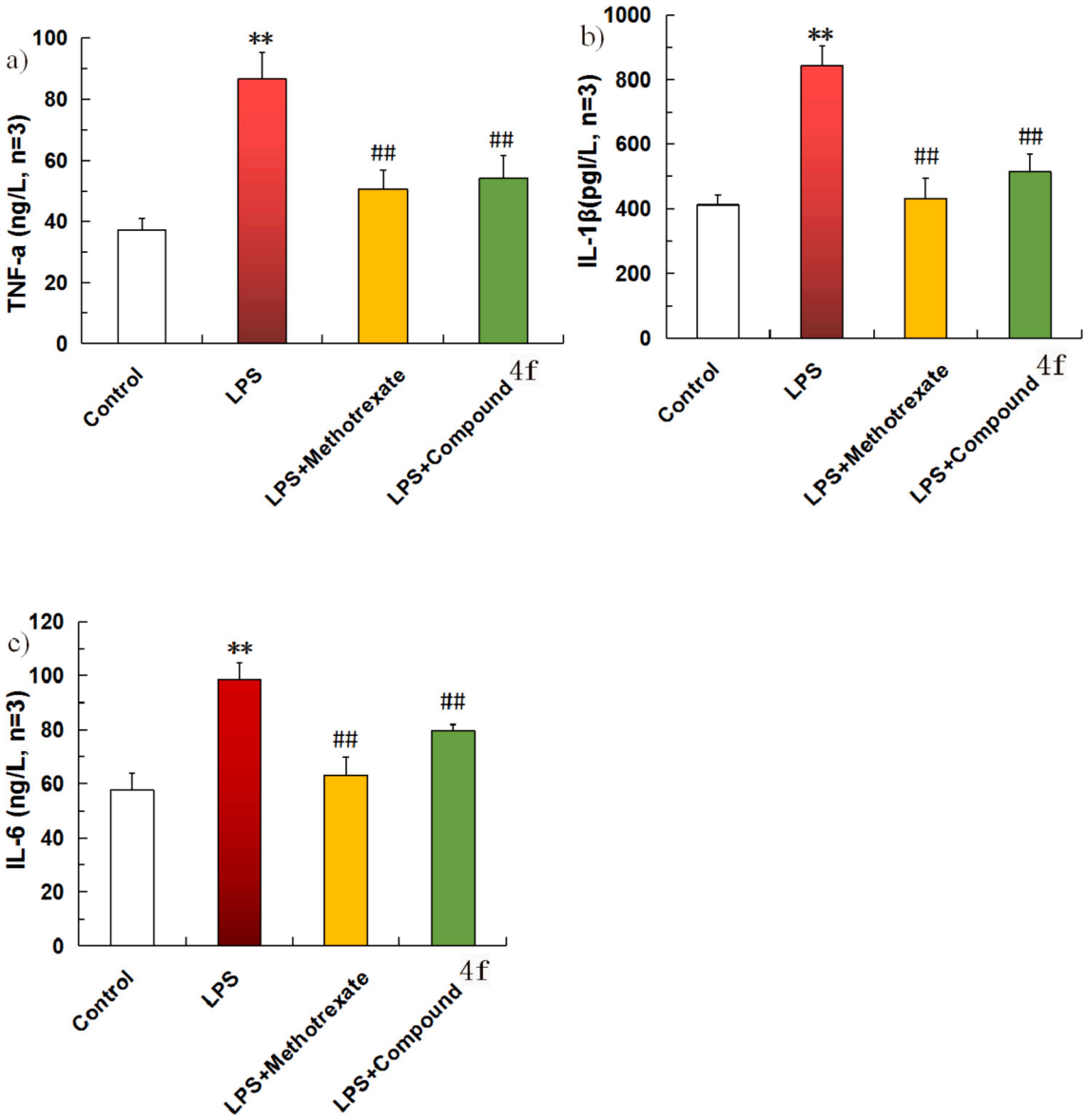


Fig. 5. The content of inflammatory factors TNF-α, IL-1βand IL-6 in rat synovial cells were determined (n = 3). Compared with the normal group, the significance symbols is ** = $p < 0.01$; compared with the LPS group, the significance symbols is ## = $p < 0.01$.

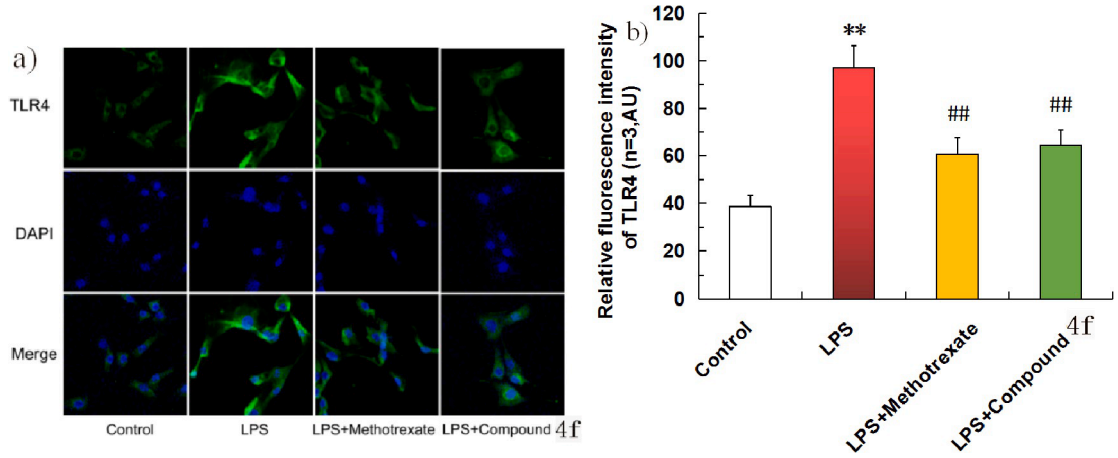


Fig. 6. The TLR4 protein expression and content in rat synovial cells (Immunofluorescence, 200×, n = 3). Compared with the normal group, the significance symbols is ** = $p < 0.01$; compared with the LPS group, the significance symbols is ## = $p < 0.01$.

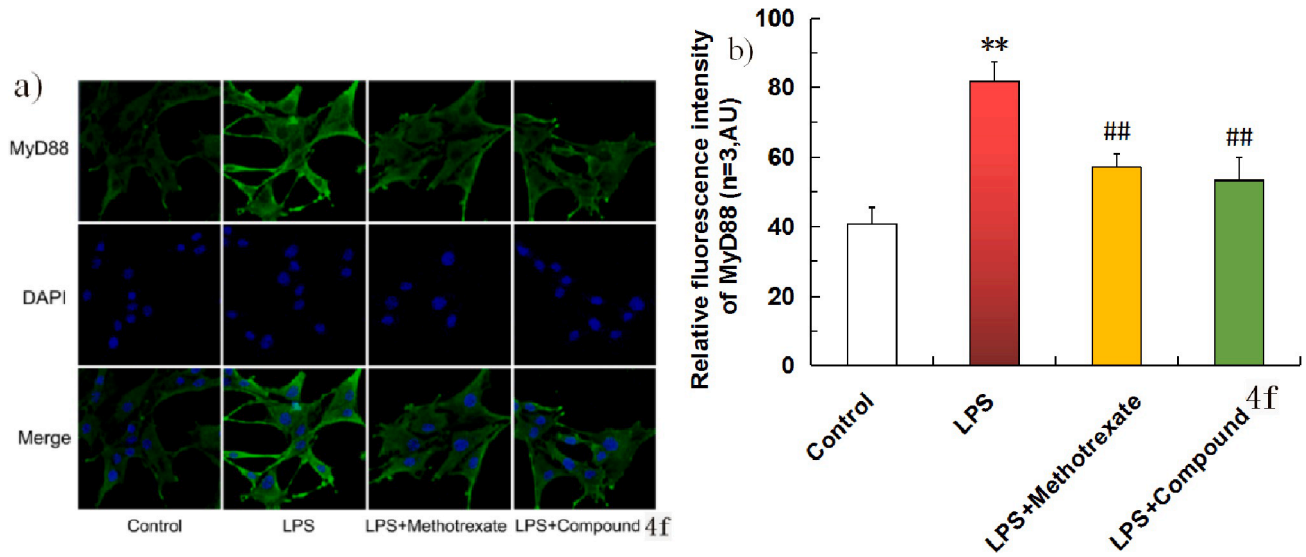


Fig. 7. The MyD88 protein expression and content in rat synovial cells (Immunofluorescence, 200×, n = 3). Compared with the normal group, the significance symbols is ** = $p < 0.01$; compared with the LPS group, the significance symbols is ## = $p < 0.01$.

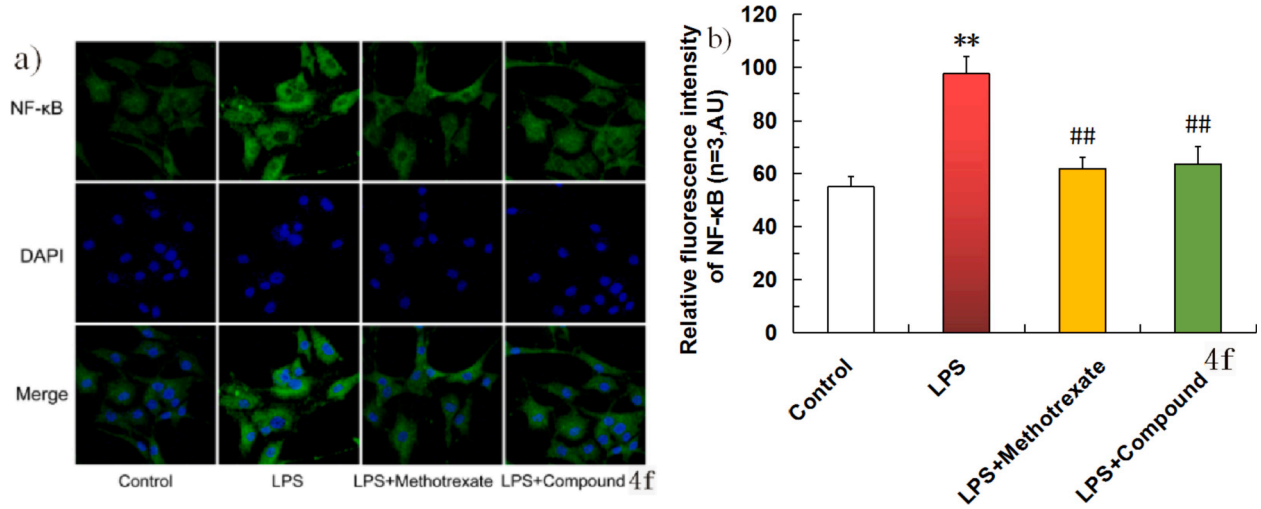


Fig. 8. The NF-κB protein expression and content in rat synovial cells (Immunofluorescence, 200×, n = 3). Compared with the normal group, the significance symbols is ** = $p < 0.01$; compared with the LPS group, the significance symbols is ## = $p < 0.01$.

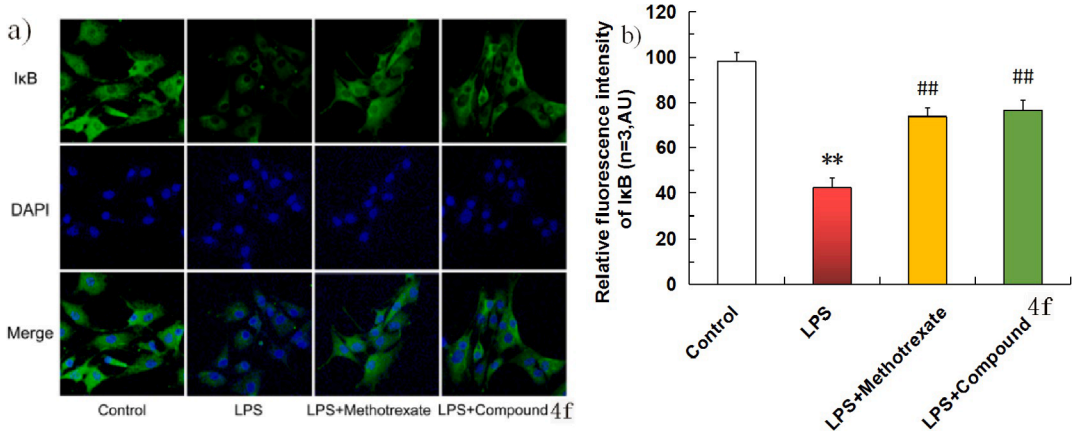


Fig. 9. The IκB protein expression and content in rat synovial cells (Immunofluorescence, 200×, n = 3). Compared with the normal group, the significance symbols is ** = $p < 0.01$; compared with the LPS group, the significance symbols is ## = $p < 0.01$.

factors TNF-α, IL-1β and IL-6 in cell supernatant of 1μM methotrexate and 1μM compound **4f** group were significantly decreased ($p < 0.01$), which indicated that compound **4f** group could significantly inhibit the LPS-induced increase of inflammatory factors TNF-α, IL-1β and IL-6 in rat synovial cells.[35]

The expression levels of TLR4/NF-κB signaling pathway (includeTLR4, MyD88, NF-κB and IF-κB) were determined by immunofluorescence method. The intervention effect of compound **4f** on LPS-induced rat synovial cell inflammation model was observed.[36] The anti-inflammatory effect and mechanism of compound **4f** were investigated *in vitro* (Fig. 6–9). The results showed that compared with the normal group, the expressions of TLR4, MyD88 and NF-κB related proteins in TLR4/NF-κB signaling pathway were significantly increased in the LPS group ($p < 0.01$), the expression of IκB protein was significantly decreased ($p < 0.01$). Compared with LPS group, the expressions of TLR4, MyD88 and NF-κB signaling pathway related proteins TLR4, MyD88 and NF-κB in synovial cells of rats in 1 μM methotrexate and 1 μM compound **4f**groups were significantly down-regulated ($p < 0.01$). The expression of IκB protein was significantly up-regulated ($p < 0.01$), which suggested that compound **4f** group could effectively regulate the expression levels of TLR4, MyD88, NF-κB and IκB related proteins in TLR4/NF-κB signaling pathway. It might be an important mechanism to inhibit LPS-induced inflammatory response of rat synovial cells, and inhibit abnormal cell proliferation and promote apoptosis.[36] TLR4/NF-κB regulates apoptosis signaling networks and related regulatory molecules, and how various environmental factors influence the regulation of TLR4/NF-κB on apoptosis activity. In addition, TLR4/NF-κB activity was abnormally upregulated in RA, and the ability of compound **4f** to target active inhibitors of TLR4/NF-κB may help prevent the occurrence of this disease.

The main clinical manifestation of RA was synovitis. The double-ring conjugated enones have been proved to have anti-rheumatoid arthritis activity. Based on the strong anti-rheumatoid arthritis activity of double-ring conjugated enones, this study conducted a further study on their anti-inflammatory effect and mechanism. The target of double-ring conjugated enones and the possible mechanism of anti-inflammatory action were preliminarily expounded. *In vitro* TLR4 inhibition test results showed that compound **4f** showed a good inhibitory activity, $IC_{50} = 0.56 \pm 0.10$ μM, which was better than the positive methotrexate ($IC_{50} = 1.04 \pm 0.22$ μM). The results of the study on the mechanism of action showed that compound **4f** could up-regulate the expression of apoptosis-related protein Caspase-3, which may be the mechanism of promoting the apoptosis of rat synovial cells. In addition, compound **4f** could significantly inhibit the inflammatory factors TNF-α, IL-1β and IL-6 induced by LPS in rat synovial cells, and effectively regulate the expression levels of TLR4, MyD88, NF-κB and IκB related proteins in TLR4/NF-κB signaling pathway. These tests data suggest that this may be an important molecular mechanism to improve the abnormal proliferation of synovial cells in rats with rheumatoid arthritis, as well as anti-inflammatory and pro-apoptotic effects. *In vitro* TLR4 inhibition test and mechanism study, for further study of double-ring conjugated enones anti-rheumatoid arthritis efficacy to provide certain theoretical guidance.

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.

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