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ORIGINAL ARTICLE

Anti-inflammatory effects of *Clematis chinensis Osbeck* extract(AR-6) may be associated with NF-κB, TNF-α, and COX-2 in collagen-induced arthritis in rat

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Abstract The root of *Clematis chinensis Osbeck* has been used widely in rheumatoid arthritis in Chinese traditional medicine, and AR-6 is a triterpene saponin isolated from it. In this present study, we investigated the in vivo effects of oral AR-6 in chronic rat with collagen-induced arthritis (CIA) and possible molecular mechanism. CIA was induced by immunizing 56 female Sprague-Dawley (SD) rats with chicken typeIIcollagen (CII). Following eighteen days, the immunization rats with CIA were treated with AR-6 (32, 16, 8 mg/kg), cyclophosphamide (7 mg/kg), and TGP (Total Glucosides of Paeonia) (180 mg/kg) for 7 days, and rats without CIA were given the same volume of purified water. TNF-α and IL-1β levels in peripheral blood will be measured by ELISA, and Western blot analysis will be used to detect the expression of NF-κB p65 subunits, TNF-α and COX-2, in synovial membrane. We found that therapeutic treatment with AR-6 markedly improves the paw swelling and histopathological changes. Moreover, the serum levels of pro-inflammatory cytokines TNF-α and IL-1β were markedly lowered, and the expression of NF-κB p65 subunits, TNF-α and COX-2, in the synovial membrane of CIA rats was significantly inhibited in the AR-6-treated groups. These results enable to prove that AR-6 has a potential anti-inflammatory effect in CIA rats, and its mechanism may relate to the inhibition of the expression of NF-κB p65 subunits, TNF-α and COX-2.

Keywords *Clematis chinensis Osbeck* · Collagen-induced arthritis · NF-κB · TNF-α · COX-2

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Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease with unknown etiology [1]. The main pathological changes of RA include hyperplasia of synovial membrane, infiltration of inflammatory cells, and neovascularization, which ultimately lead to cartilage erosion and articular destruction [2].

The transcription factor NF-κB has been well recognized as a pivotal regulator of inflammation in rheumatoid arthritis [3]. On one hand, NF-κB controls the expression of the pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β), which are expressed at very high levels in peripheral blood and synovial membrane [4]. On the other hand, cytokines TNF-α and IL-1β, which are considered to be the important participants in the histopathology of RA, are potent inducers of NF-κB activation, suggesting an interdependence of persistent NF-κB activation and sustained levels of TNF-α and IL-1β [5]. Active forms of NF-κB, commonly composed of p50/NFKB1 and p65/RelA subunits, are detected in the synovial membrane of rheumatoid arthritis patients, suggesting that NF-κB is involved in the expression of inflammatory genes in the rheumatoid arthritis synovial membrane [6]. Furthermore, NF-κB activation is necessary for the induction of cyclooxygenase-2 and inducible nitric oxide synthetase (iNOS), the

enzymes that catalyze the synthesis of pro-inflammatory prostaglandins and nitric oxide metabolites [5, 7]. Inhibition of NF-κB was shown to block differentiation of the precursor cells of osteoclast through attenuation of the signaling for receptor activator such as TNF-α, IL-1β, and COX-2 [8].

Recently, many cytokine-targeted drugs have been applied to the treatment of RA, for example, TNF-α inhibitors (etanercept, infliximab and adalimumab), IL-1 receptor antagonists and nonsteroidal anti-inflammatory drugs (NSAIDs) [9]. However, NSAIDs still have undesirable side effects such as causing peptic ulcers. AR-6 is a triterpene saponin isolated from the root of *Clematis chinensis Osbeck*. *Clematis chinensis Osbeck* has been frequently used to cure RA and dermatitis glandularis erythematosa in ancient China. Previous study showed that can significantly decrease the clinical signs and inflammatory proteins such as PGE_2, iNOS, TNF-α, and IL-1β in rats with adjuvant-induced arthritis [10]. However, the mechanism of this anti-inflammatory activity remains unclear. In this present study, we investigate in vivo effects and the possible mechanism of oral AR-6 in chronic rats with collagen-induced arthritis (CIA). TNF-α and IL-1β levels in peripheral blood will be measured by enzyme-linked immunosorbent assay (ELISA), and the expression of NF-κB p65 subunits, TNF-α and COX-2, in synovial membrane will be measured by western blot analysis.

Materials and methods

AR-6: prepared by School of Traditional Chinese Pharmacy, China Pharmaceutical University. AR-6 was suspended in distilled water or PBS before administration. The structure of AR-6 was showed in Fig. 1.

Freund's complete adjuvant and Chicken typeIIcollagen were purchased from Sigma (St. Louis, MO, USA). ELISA kit of rat's TNF-α and IL-1β was obtained from R&D systems (Minneapolis, MN, USA). Capsules of TGP (Total Glucosides of Paeonia) (Moulin) were purchased from Ningbo Li-Hua Pharmaceutical Co., Ltd. Cyclophosphamide was purchased from Jiangsu Hengrui Medicine Co., Ltd. TEMED, SDS, and acrylamide were purchased by Amersham Pharmacia Biotech (Amersham, NJ), and nitrocellulose membrane was purchased from Bio-Rad (Hercules, CA). BCA protein assay kit, Nuclear and Cytoplasmic Protein Extraction Kit, antibodies against NF-κB, TNF-α, COX-2, and β-actin, and secondary antibody IgG_1-HRP were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

Fifty-six female Sprague-Dawley rats, weighed 120–140 g, purchased from the experimental animal center of Zhejiang province, China. Rats were housed under standard laboratory conditions with free access to food and water. The temperature was kept at 18–22°C, and a 12-h light/dark schedule was maintained. On day 1, rats in control group were given an intradermal injection of 0.1 M HAC emulsified in complete Freund's adjuvant (CFA) (1:1, v/v) without CII to the base of tail, while other rats were given 100 μg chicken type II collagen (CII) which had dissolved in 0.1 M HAC and emulsified in complete Freund's adjuvant (CFA) (1:1, v/v). On day 7, rats in control group were given an booster injection of 0.1 M HAC emulsified in incomplete Freund's adjuvant (IFA) (1:1, v/v) without CII, while other rats were given 100 μg CII emulsified in incomplete Freund's adjuvant (IFA) (1:1, v/v) [11, 12]. On day 19, 18 days after primary immunization (day 1), CPA (7 mg/kg), TGP (180 mg/kg), and AR-6(32, 16, 8 mg/kg) were administered once daily from day 19 to day 25, and rats without CIA were given the same volume of purified water [13].

During the 7 days for administration, rats were examined every day for paw volume. The right hind paw volume was measured with plethysmometer chamber (7140 UGO. Basile, Comerio, Italy) before immunization (basic value, day 0) and repeated on day 18th–25th. Paw swelling (ml) was calculated by subtracting the paw volume at day 0 from the related one at days 18th–25th. Swollen ratio was calculated by dividing the paw volume at day 0 from the paw swelling [10, 14].

Hind paws were harvested from all rats on day 26 and fixed in 10% phosphate-buffered formaldehyde, decalcified in 10% EDTA for 8 h, and embedded in paraffin. Tissue sections (7 μm) were prepared and stained with hematoxylin and eosin viewed under a microscope and photographed [15].

On day 26, SD rat blood samples were drawn from orbital plexus for the preparation of serum. We detected tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) levels in peripheral blood on day 27; these two mediators were measured by enzyme-linked immunosorbent assay (ELISA), using appropriate specific ELISA kits.

On day 26, rats were killed, and synovial membrane tissues of the secondary swollen paw in CIA rats were extracted. We used nuclear and cytoplasmic protein extraction kit to separate the nuclear protein and cytoplasmic protein [16, 17]. Then, the concentration of the two kinds of protein was determined by BCA protein assay kit and kept in −70°C.

We use extracted nuclear protein to determine NF-κB p65 subunits and extracted cytoplasmic protein to determine TNF-α and COX-2. Fifty micrograms of protein was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel (12%) and blotted onto nitrocellulose membrane. After blocking with 5% skim milk in PBST, the membrane was probed with the relevant antibodies: anti-NF-κB, anti-

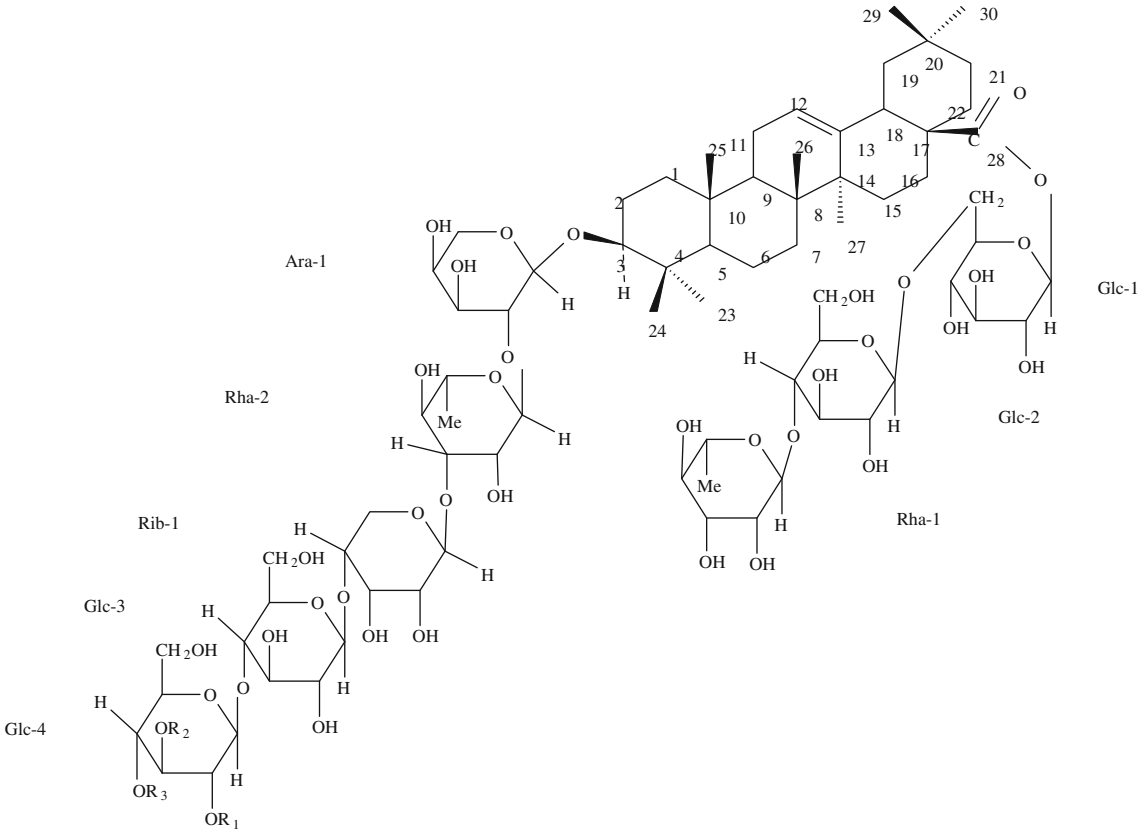

Fig. 1 Chemical structure of AR-6

COX-2, anti-TNF-α, and β-actin; then, the blotted and washed membrane was visualized by enhanced chemiluminescence (ECL), according to the Gel Imaging System (BIO-RAD, NJ, USA) [18, 19]. Assays were performed in triplicate independent trials.

Statistical analysis

Experiments were performed three times independently, and the results were expressed as the mean ± S.D. Statistically significant values were compared using ANOVA and Dunnett's post hoc test, and P values of less than 0.05 were considered statistically significant.

Results

During the 7 days for administration, the right hind paw swollen ratio was significantly increased in model group rats compared with normal group, AR-6 (32 and 16 mg/kg) apparently diminished the swollen ratio after the administration. The results are better than the CPA (cyclophosphamide, 7 mg/kg) group and TGP (Total Glucosides of Paeonia, 180 mg/kg) group (Table 1).

Compared with normal rats (Fig. 2a), CIA rats showed hyperplastic synovium, inflammatory cell infiltration, and pannus formation (Fig. 2b). These symptoms were significantly alleviated in CIA rats after the administration of AR-6(32 and 16 mg/kg); the proliferation and infiltration of mononuclear cells and pannus were partly inhibited, and the destruction of articular cartilages was alleviated (Fig. 2e, f); the results are similar with the CPA and TGP groups (Fig. 2c, d).

Consistent with the joint swelling result, significant increase in the levels of TNF-α and IL-1β was observed in the serum of model group rats compared with control group ($P < 0.01$). Significant decrease in the levels of two cytokines was observed in the serum of CIA rats treated with treated with AR-6 (32 mg/kg) compared with the model group ($P < 0.05$) (Fig. 3). These results suggest that AR-6 can inhibit the production of two pro-inflammatory cytokines as TNF-α and IL-1β in the serum of CIA rats.

NF-κB is an upstream regulator of inflammation which can regulate the expression of TNF-α and COX-2, with the activation of NF-κBp65 subunits in nuclear protein, while TNF-α can also induce the activation of NF-κB. We detect NF-κBp65 subunits, COX-2 and TNF-α protein expression levels by Western blot analysis. Results (Fig. 4a) showed

Table 1 The effect of AR-6 on the swollen ratio of secondary adjuvant-induced arthritis in mice ($\overline{X} \pm S$, $n = 8$)

Group	Dose (mg/kg)	Swollen ratio						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control		0.34 ± 2.14**	0.60 ± 1.33**	0.52 ± 1.73**	0.65 ± 2.20**	0.90 ± 2.34**	0.17 ± 2.25**	0.80 ± 1.93**
Model		31.92 ± 9.31	33.17 ± 9.18	34.60 ± 8.93	35.71 ± 9.24	36.79 ± 9.18	37.80 ± 9.55	38.88 ± 9.52
CPA	7	31.60 ± 7.97	25.62 ± 7.86	24.21 ± 7.82*	22.98 ± 7.61**	24.91 ± 7.55**	20.36 ± 7.77**	18.87 ± 7.79**
TGP	180	32.04 ± 8.30	27.94 ± 7.69	26.57 ± 7.47	24.94 ± 6.98*	23.58 ± 6.87**	22.14 ± 6.92**	20.95 ± 6.68**
AR-6	32	30.83 ± 8.64	22.03 ± 7.69*	20.16 ± 7.50**	18.39 ± 7.26**	16.70 ± 6.96**	14.73 ± 6.60**	12.87 ± 6.31**
	16	28.42 ± 5.72	23.38 ± 5.59*	21.77 ± 5.47**	20.16 ± 5.32**	18.72 ± 5.18**	17.37 ± 5.17**	16.01 ± 5.01**
	8	30.55 ± 9.51	31.41 ± 9.41	30.14 ± 9.15	28.61 ± 8.93	27.16 ± 8.85*	25.90 ± 9.08**	24.46 ± 8.86**

* $P < 0.05$, ** $P < 0.01$ versus model group

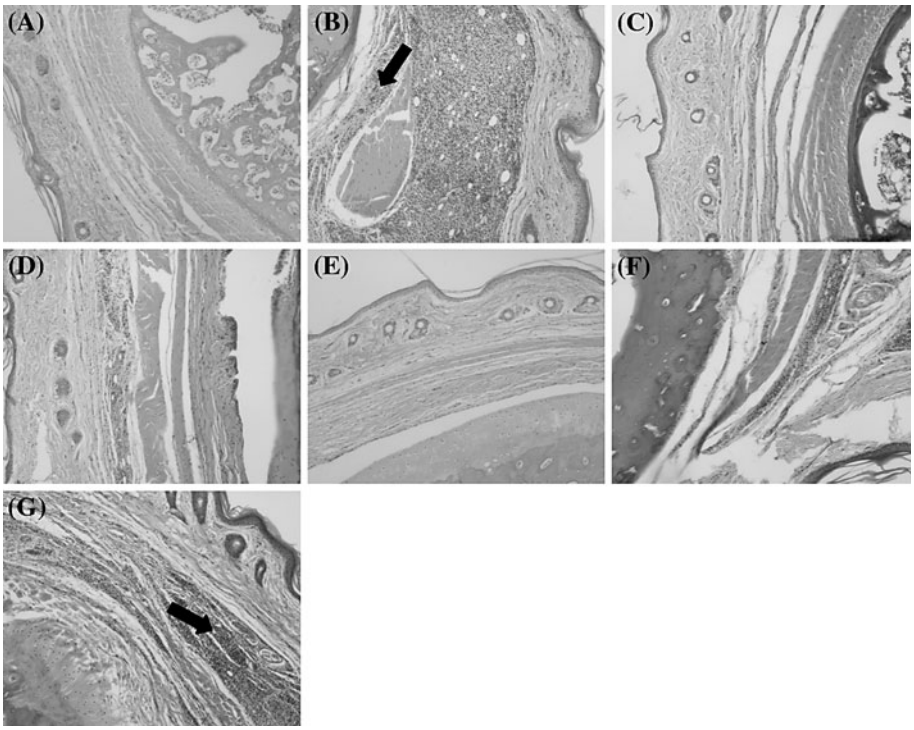

Fig. 2 Photographs of articulation slices of rats. **a** normal group; **b** CIA model group; **c** CPA group (7 mg/kg); **d** TGP group (180 mg/kg). **e** AR-6 high-dose group (32 mg/kg), **f** AR-6 middle-dose group (16 mg/kg). **g** AR-6 low-dose group (8 mg/kg). The *black arrowhead* indicates the location of inflammatory cells infiltration and pannus formation. All the magnification is 200X

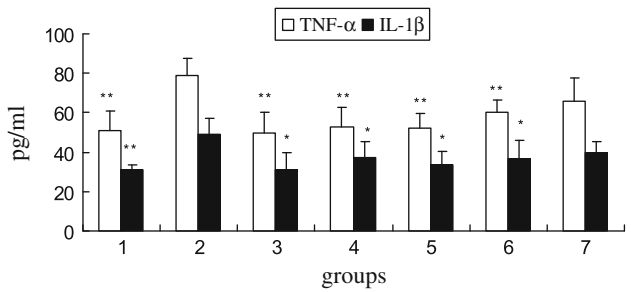

Fig. 3 Effect of AR-6 on the levels of TNF-α and IL-1β in serum of CIA rats ($\overline{X} \pm S$, $n = 8$). *1* normal group, *2* CIA model group, *3* CPA group (7 mg/kg), *4* TGP group (180 mg/kg), *5* AR-6 high-dose group (32 mg/kg), *6* AR-6 middle-dose group (16 mg/kg), *7* AR-6 low-dose group (8 mg/kg). *$P < 0.05$, **$P < 0.01$ versus model group

that AR-6 can markedly inhibit the expression of NF-κBp65 subunits, COX-2 and TNF-α, demonstrating apparently dose correlation of the expression of NF-κBp65 subunits and TNF-α. However, the dose correlation of COX-2 is not very clear (Fig. 4b).

Discussion

Rheumatoid arthritis (RA) is a chronic, cytokine-mediated destructive inflammatory polyarticular joint disease. It is characterized by massive synovial proliferation and systemic and local inflammation resulting in cartilage and bone destruction [20]. Previous studies in our laboratory

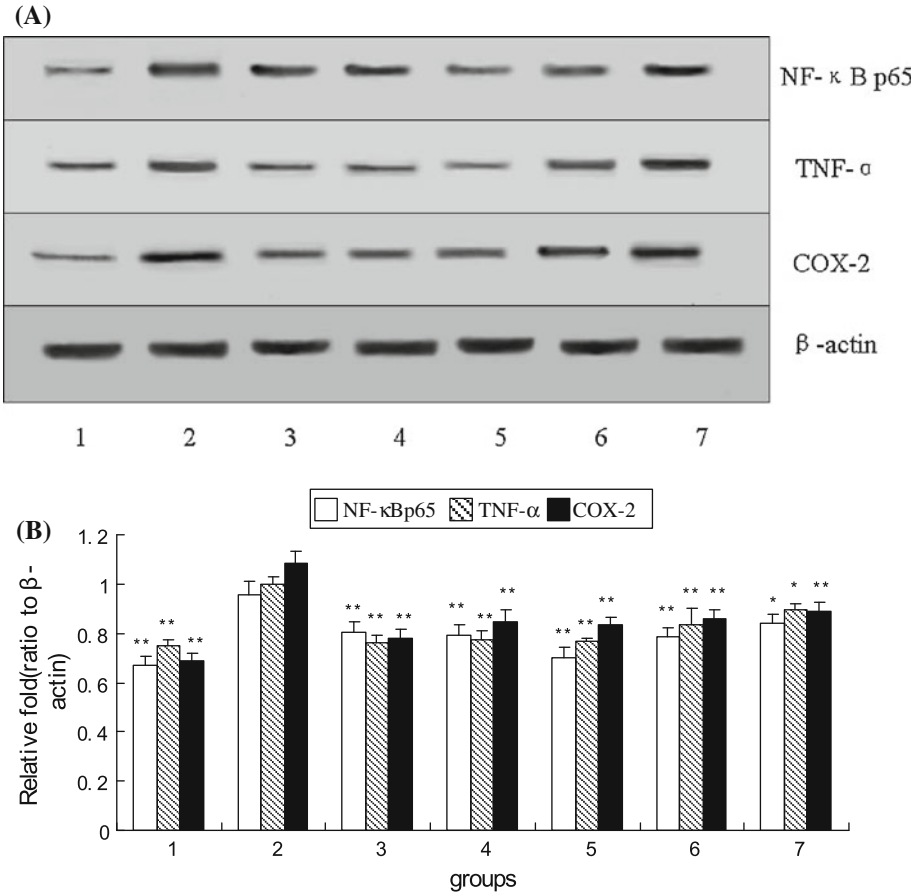

Fig. 4 Effect of AR-6 on the expression of NF-κBp65 subunits, TNF-α and COX-2, in the synovial membrane of the secondary swollen paw of CIA rats ($\overline{X} \pm S$, $n = 3$). **a** *Lane 1* control *2* model *3* CPA (7 mg/kg) *4* TGP (180 mg/kg) *5* AR-6 high dose (32 mg/kg) *6* AR-6 middle dose (16 mg/kg) *7* AR-6 low dose (8 mg/kg). **b** *1* normal group; *2* CIA model group; *3* CPA (cyclophosphamide, 7 mg/kg) group; *4* TGP (180 mg/kg) group. *5* AR-6 high-dose group (32 mg/kg), *6* AR-6 middle-dose group (16 mg/kg), *7* AR-6 low-dose group (8 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$ versus model group

had demonstrated that Clematis chinensis Osbeck (AR-6) significantly inhibited the serum inflammatory mediators (TNF-α, IL-1β, IL-6,iNOS) in adjuvant arthritis(AA) rats and downregulated the production of TNF-α and NO in LPS-stimulated macrophages [10].

Collagen-induced arthritis is similar to AA both in pathological and in serological changes, such as paw swelling, joint erosions, and the involvement of inflammatory mediators in the arthritic etiology, but CIA in rats resembles human RA in more respects. One of the most important features of CIA is chronic synovitis, including inflammatory cell infiltration, pannus formation, and destruction of cartilage/bone erosion. The similarities in the joint pathology between CIA and RA are most widely used to elucidating the pathogenesis of RA and for screening new drugs for treatments of rheumatoid diseases [20, 21]. Moreover, previous study of AR-6 was based on the level of inflammatory factors in serum, while RA is a kind of autoimmune disease which focuses on synovial membrane as a target organ, and relative pro-inflammatory process would be activated in synovial membrane in the initial stage of RA. For these reasons, we examined the therapeutic effects and detected the expression of several inflammatory proteins in synovial membrane to investigate possible molecular mechanism of AR-6 on CIA rats in vivo in the present study.

During the process of experiment, CIA rats treated by AR-6 demonstrate faster onset of action than TGP and CPA groups (Table 1). During the 7 days of treatment with AR-6, the swollen ratio of secondary sides significantly decreased gradually, comparing to the model group. According to the analysis of histopathological change of ankle joint, we determined that was one of the principal factors contributing to the lead joint damage in RA. AR-6 can inhibited anomalous hyperplasia of synovial membranes and the proliferation and infiltration of mononuclear cells and pannus and alleviated the destruction of articular cartilages, which indicated that AR-6 possessed certain anti-inflammatory effect [22, 23].

The NF-κB pathway is a therapeutic target in inflammatory diseases because NF-κB plays an important role in the transcriptional activation of TNF-α, IL-1β, COX-2, IL-2, IL-6, IL-8, and iNOS [24, 25]. Inappropriate regulation of NF-κB is directly involved in a wide range of human

disorders, including a variety of cancers, neurodegenerative diseases, arthritis, asthma, inflammatory bowel disease, sepsis, and numerous other inflammatory conditions. Therefore, agents that inhibit NF-κB activation would have anti-inflammatory effects [26]. The previous study had demonstrated that AR-6 can decrease the serum inflammatory mediator (TNF-α, IL-1β, IL-6, iNOS) levels in AA rats, and we also proved that AR-6 can decrease the levels of TNF-α and IL-1β in the serum of CIA rats (Fig. 2), two cytokines that were considered as the most representative pro-inflammatory cytokines in RA [27]. These results guide us to prove whether AR-6 can impact the expression of NF-κB, which is the upstream regulating factor to control the release of those cytokines. Our results revealed that anti-inflammatory activities of AR-6 are mediated through the inhibition of nuclear translocation of the NF-κB p65 subunit, since it was one part of the active form of NF-κB which can be detected in nuclear protein of many inflammatory models. This conclusion demonstrated that NF-κB p65 subunit may be an upstream target of AR-6. The results also show that AR-6 also can inhibit the expression of TNF-α in synovial membrane tissues of CIA rats, and it is consistent with the result of serological changes. The expression of these two proteins shows significant dose correlation on CIA rats treated by AR-6(32, 16, and 8 mg/kg), which indicates that AR-6 may inhibit the expression of NF-κB and TNF-α through the same mechanism in the inflammatory process.

Cyclooxygenase(COX) has two isoforms; COX-1 has been suggested to provide a physiologic level of PGE_2 for normal platelet, stomach, and kidney function, but COX-2 has been found to be highly induced at inflammatory sites in animals as well as patients with inflammatory diseases [28, 29]. Overexpression of COX-2 leads to increased levels of PGE_2, a central mediator of inflammation, which we had detected abundant expression in secondary inflammatory hind paws of AA rats [10]. We determined the expression of COX-2 in synovial membrane tissues of CIA rats to elucidate the upstream mechanism. The results showed that AR-6 can markedly inhibit the expression of COX-2 in AR-6-treated groups, but we cannot see significant dose correlation like NF-κB p65 and TNF-α. Besides NF-κB and TNF-α, many inflammatory proteins, such as MCP-1, ICAM-1 and phospholipase had been reported to be the inducer of COX-2 [30]. In that case, we had better investigate the effects of AR-6 on the expression of those proteins and elucidate the relationship of them in further research in the future.

In summary, AR-6 significantly inhibited the symptoms (swollen ratio and histopathological change) and serum inflammatory mediators (IL-1β and TNF-α) in CIA rats. And AR-6 also downregulated the expression of NF-κB p65 subunits, TNF-α and COX-2, in synovial membrane tissues of CIA rats. While previous study in our laboratory had proved the absence of apparent toxicity and the availability of therapeutic of AR-6 in AA rats, our results supplement the evaluation of the therapeutic effect of CIA rats and suggest that AR-6 may be effectively applied to rheumatoid arthritis at the level of pro-inflammatory cytokine and mediator regulation; its effective target may be correlate with NF-κB, TNF-α, and COX-2, but its precise mechanism is not clear. Therefore, further basic investigation of AR-6 at cellular level and molecular level is imperative for its application to RA in the future.

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