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A betulinic acid derivative SH479 inhibits collagen-induced arthritis by modulating T cell differentiation and cytokine balance

Shijie Chen [a,b,1], Yang Bai [a,1], Zhen Li [a,1], Kunhang Jia [a], Yunyun Jin [a], Bei He [a], Wen-Wei Qiu [c], Changsheng Du [d], Stefan Siwko [e], Huaqing Chen [a,*], Mingyao Liu [a,e,*], Jian Luo [a,*]

[a] Shanghai Fengxian District Central Hospital and East China Normal University Joint Center for Translational Medicine, Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China
[b] Department of Orthopaedics, The Third Xiangya Hospital of Central South University, Changsha, Hunan 410013, China
[c] Department of Chemistry, School of Chemistry and Molecular Engineering, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China
[d] Laboratory of Receptor-Based Bio-medicine, Shanghai Key Laboratory of Signaling and Disease Research, School of Life Sciences and Technology, Tongji University, Shanghai 200092, China
[e] Institute of Biosciences and Technology, Department of Molecular and Cellular Medicine, Texas A&M University Health Science Center, Houston, TX, USA

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ABSTRACT

The ideal therapeutic drug for rheumatoid arthritis (RA) should not only inhibit inflammation, but also prevent articular joint damage and particularly inhibit osteoclastogenesis. Betulinic acid (BA) is a natural pentacyclic triterpene that has displayed moderate anti-inflammatory and anti-osteoclastogenesis activities in various experimental systems, suggesting that BA or its derivatives could have an inhibitory effect on RA. In this study, we screened BA derivatives and found a heterocyclic ring-fused BA derivative, SH479, which had greater inhibitory effect than BA on Th17 differentiation. Moreover, we investigated the immune regulatory activity and potential therapeutic effects of SH479 in an experimental model of rheumatoid arthritis, the collagen-induced arthritis (CIA) mouse model. SH479 significantly inhibited Th1 and Th17 polarization, antigen-specific T cell proliferation and splenic lymphocyte-induced osteoclastogenesis. Furthermore, it diminished arthritis scores as well as bone destruction and cartilage depletion in the CIA mouse model. The protective effect of SH479 was accompanied by decreased levels of pro-inflammatory cytokines IL-17 and IFN-γ, together with enhanced anti-inflammatory cytokine expression including IL-10 and IL-4, as well as elevated $CD4^+$ $Foxp3^+$ cell number. At the molecular level, our results indicated that SH479 alleviated CIA through regulation of $CD4^+$ T cell subtypes by JAK-STAT pathways. In conclusion, this study demonstrates that SH479 has therapeutic potential for rheumatoid arthritis through an anti-inflammatory effect by shifting a pathogenic Th17/Th1 response to a Th2/Treg phenotype, and also through an additional articular bone protection effect.

1. Introduction

As a chronic systemic autoimmune disease, human rheumatoid arthritis (RA) is characterized by inflammation, cartilage damage and joint destruction [1], which has a significant influence on quality of life. It is commonly believed that the pathogenesis of this destructive disease involves both inflammation and autoimmunity, with $CD4^+$ T cells, as well as B cells and antigen presenting cells as key cell types responsible [2,3]. The systemic autoimmune response targets the synovial membrane, leading to joint destruction through cartilage degradation and subsequent osteoclast activation [4,5]. Many studies have shown that activated T cells (Th cells) induced by autoantigens play an important role in the pathogenesis of RA. Studies conducted in collagen-induced arthritis (CIA) [6,7], a widely used animal model for RA, have suggested that a possible mechanism involves the activation of inflammatory cells (Th1, Th17), suppression of anti-inflammatory cells (Th2, Treg), and finally the creation of an imbalance of pro-inflammatory cells and anti-inflammatory cells together with a cytokine imbalance [8,9]. This imbalance plays a key role in the pathogenesis of RA. Evaluation of this imbalance leads to the possibility of better understanding the disease mechanism and eventually improving treatment of this disease [10,11].

* Corresponding authors at: Shanghai Key Laboratory of Regulatory Biology, The Institute of Biomedical Sciences, School of Life Sciences, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China.
E-mail addresses: hqchen@bio.ecnu.edu.cn (H. Chen), myliu@bio.ecnu.edu.cn (M. Liu), jluo@bio.ecnu.edu.cn (J. Luo).
[1] These authors contributed equally to this work.

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At present, therapies are available for RA, but most of them have various drawbacks: synthetic disease modifying anti-rheumatic drugs (DMARDs) such as leflunomide and methotrexate [12], leave still a significant number of patients with unsatisfactory treatment effects. Biological agents, on the other hand, usually used in combination with suitable DMARDs, are potent medications but with potentially serious side effects [13–15]. Thus there is a constant need for novel drugs with a better safety and efficacy profile. The ideal therapeutic drug for RA should not only inhibit inflammation, but also prevent articular joint damage and particularly inhibit osteoclastogenesis. Furthermore, small molecules have significant advantages over protein-based biological agents, from the drug development point of view. Developing small compounds which have the capacity to modify pathogenic immunity as well as prevent bone damage remains a priority.

Betulinic acid (BA) is a natural pentacyclic triterpenoid available from tropical plants and is particularly abundant in Sambucus williamsii Hance (SWH) [16–18]. In China, SWH is a folk medicine with a long history of being used for treatment of joint diseases, bone fractures and inflammation. Recent studies have shown that BA has anti-tumor and anti-HIV activities [18–20], has an inhibitory effect on pro-inflammatory cytokine IL-17 and IFN-γ production and anti-oxidant activities [16,21–23]. Importantly, BA has moderate cartilage protective effects [22] and inhibits osteoclast differentiation and bone resorption [24]. As RA is an autoimmune disease that eventually results in articular cartilage and bone destruction [25–28], it is reasonable to speculate that BA derivatives have a greater inhibitory effect on RA, through modulating immune responses that relieve inflammation, as well as by protecting bone. In this study, we screened more than 40 BA derivatives using a Th17 differentiation assay and found that a heterocyclic ring-fused BA derivative, SH479, was the most potent compound to inhibit Th17 differentiation. Interestingly, SH479 has been reported as a strong inhibitor of osteoclastogenesis and bone resorption (identical to compound 20 in Ref. [24]). Furthermore, we investigated effects of SH479 on T cell differentiation and proliferation, as well as its potential immune-regulatory properties in mouse CIA. Results presented here provide new insight into the novel regulatory mechanism of the BA derivative SH479 in RA treatment.

2. Results

2.1. Effects of SH479 on $CD4^+$ T cell differentiation and antigen specific T cell proliferation

Betulinic acid (BA) is a natural pentacyclic triterpene that displays moderate anti-inflammatory and anti-osteoclastogenic activities in various experimental systems, suggesting that BA or its derivatives could have an inhibitory effect on rheumatoid arthritis (RA). Th1 and Th17 play important roles in development and pathogenesis of RA. Therefore, we screened BA derivatives for inhibition of Th17 differentiation and found a heterocyclic ring-fused BA derivative, SH479, which had a greater inhibitory effect on Th17 differentiation than BA (data not shown, Fig. 1A). To evaluate differential effects on T cell differentiation, $CD4^+$ T cells were purified and different T cell differentiation factors were added. Cells were incubated for 72 h in the presence of 10 μM BA or SH479, then subjected to intracellular staining and flow cytometric analysis. Results showed that SH479 but not BA significantly inhibited Th1 differentiation. Furthermore, for Th17 differentiation, BA showed 50% inhibition, whereas SH479 inhibited differentiation by 90% as compared with the control group (Fig. 1B). These data indicated that SH479 had a greater inhibitory effect than BA on Th1 and Th17 differentiation.

We then evaluated the effect of SH479 on collagen-specific T cell proliferation which is related to the development of autoimmunity in CIA. Splenic lymphocytes were isolated from CIA mice on day 35 after primary immunization and cultured for 48 h in medium containing 20 μg/ml chicken type II collagen (CII). Then SH479 at different concentrations was added. We found that CII-stimulated proliferation was dose-dependently suppressed by SH479 (Fig. 1C). At the same time, SH479 up to 25 μM showed no obvious effect on the viability of splenic lymphocytes from both normal mice and CIA mice (Fig. 1D and E).

2.2. Amelioration of CIA by SH479 treatment in the mouse model

To further investigate the effect of SH479 on CIA, we treated CIA mice with SH479 every day starting from day 23, and assessed the clinical disease activity daily. Disease onset was evident around day 23 and the mean arthritis score reached 6.88 in CIA control mice on day 41. The severity of CIA in SH479-treated mice was significantly attenuated, with a mean score of 2.63 on day 41 (Fig. 2A). The paw volume in SH479-treated mice was significantly decreased as well, as shown in Fig. 2B. On day 41, the paw volume peaked at 2.74 mm in the CIA control mice compared with 2.37 mm in SH479-treated mice. The normal group mice did not exhibit any edema.

When we assessed articular bone damage, X-ray imaging of CIA control mouse hind paws showed typical signs of articular destruction. However, radiographic findings in SH479-treated CIA mice (Fig. 2C and D) showed significantly less articular damage, consistent with the clinical score and paw volume measurement. There were no apparent differences in body weight among normal mice, CIA mice and SH479-treated mice (data not shown).

2.3. SH479 treatment reduced histologic synovitis and cartilage destruction in the CIA mouse model

We next assessed histopathologic changes. In normal joints, the synovial membrane usually contains a monolayer of synoviocytes, whereas in CIA mice, synoviocytes over-proliferated and grew into multiple layers that were infiltrated with various inflammatory cells. As shown in Fig. 3A, inflammation induced by CIA was associated with cellular infiltration, synoviocyte proliferation, pannus formation and cartilage damage. After treating with SH479, arthritis cartilage destruction and inflammatory cell infiltration were both significantly suppressed (Fig. 3A). Joint sections were stained with Safranin O and semiserial sections were scored for the degree of cartilage surface erosion. As shown in Fig. 3B, there was marked proteoglycan depletion and obvious cartilage damage in the CIA group, whereas in the SH479-treated group cartilage was well preserved. These findings suggest that SH479 can greatly reduce joint synovitis and preserve cartilage.

To further analyze expression of inflammatory cytokines, MMPs (matrix metalloproteinases) and the osteoclast regulator RANKL (receptor activator for NF-κB ligand) in synovium, total RNA was extracted from the synovial membrane and quantitative PCR was conducted. Data demonstrated that IL-1β, IL-6, MMP3, MMP9 and RANKL were all over-expressed in CIA mice but significantly lower (often to levels in normal mouse controls) in SH479-treated mice (Fig. 3C). These data suggest that SH479 can significantly inhibit expression of inflammatory factors, reverse synovitis and cartilage erosion.

2.4. SH479 ameliorated CIA through regulating pro-inflammatory and anti-inflammatory cytokine production

In order to gain insight into the SH479 mechanism of action, we specifically analyzed serum cytokines of different groups of mice