SH479, a Betulinic Acid Derivative, Ameliorates Experimental Autoimmune Encephalomyelitis by Regulating the Th17/Treg Balance

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Abbreviations:
MS: multiple sclerosis
EAE: Experimental Autoimmune Encephalomyelitis
BA: Betulinic acid
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
STAT: Signal transducer and activator of transcription
CNS: central nervous system
ROR-γt: Retinoid orphan receptor γt
SWH: Sambucus williamsii Hance
i.p.: intraperitoneally
SD: standard deviation
LPS: Lipopolysaccharides
MOG: myelin oligodendrocyte glycoprotein
ELISA: enzyme-linked immunosorbent assay
Abstract

CD4+ T helper cells, especially Th17 cells, combined with immune regulatory network dysfunction, play key roles in autoimmune diseases including multiple sclerosis (MS). Betulinic acid (BA), a natural pentacyclic triterpenoid, has been reported to be involved in anti-inflammation, in particular having an inhibitory effect on pro-inflammatory cytokine IL-17 and IFN-γ production. In this study, we screened BA derivatives and found a BA derivative, SH479, which had a greater inhibitory effect on Th17 differentiation. Our further analysis showed that SH479 had a greater inhibitory effect on Th17 and Th1, and a more stimulatory effect on Tregs. To evaluate the effects of SH479 on autoimmune diseases in vivo, we employed the extensively used MS mouse model experimental autoimmune encephalomyelitis (EAE). Our results showed that SH479 ameliorated clinical and histological signs of EAE in both prevention and therapeutic protocols by regulating the Th17/Treg balance. SH479 dose-dependently reduced splenic lymphocyte pro-inflammatory factors and increased anti-inflammatory factors. Moreover, SH479 specifically inhibited splenic lymphocyte viability from EAE mice but not normal splenic lymphocyte viability. At the molecular level, SH479 inhibited Th17 differentiation by regulating STAT3 phosphorylation, DNA binding activity, and recruitment to the Il-17a promoter in CD4+ T cells. Furthermore, SH479 promoted the STAT5 signaling pathway and inhibited the NF-κB signaling pathway. Together, our data demonstrated that SH479 ameliorated EAE by regulating the Th17/Treg balance through inhibiting the STAT3 and NF-κB pathways while activating the STAT5 pathway, suggesting that
SH479 is a potential novel drug candidate for autoimmune diseases including multiple sclerosis.
Introduction

Immune regulatory network dysfunction, together with CD4+ T cell abnormalities, play key roles in autoimmune diseases including multiple sclerosis (MS), which is a chronic inflammatory and demyelinating disease of the central nervous system (CNS) (Lucchinetti et al., 2000; Noseworthy et al., 2000). The most widely used autoimmune model in mice, experimental autoimmune encephalomyelitis (EAE), shares many similarities with MS, and has been a major vehicle in the development of new therapeutic targets for MS (Benson et al., 2015; Dang et al., 2015). CD4+ T helper cells, especially Th17 cells, are critically involved in the disease pathogenesis of MS and EAE (McWilliams et al., 2015; Yang et al., 2015a). Th17 cells promote the inflammatory process by secreting IL-17 (Cho et al., 2012; Hundorfean et al., 2012; Roeleveld and Koenders, 2015). Retinoid orphan receptor γt (ROR-γt), a master regulator of Th17, plays an important role in maintaining IL-17 production (Yang et al., 2015b). Both IL-17 KO mice and ROR-γt KO mice exhibited reduced EAE disease severity (Ivanov et al., 2006; Komiyama et al., 2006; Yang et al., 2008a).

Both Th17 and Treg cells are CD4+ T cell subsets, but they have remarkably differing biological activities. Th17 cells are pro-inflammatory and Treg cells are anti-inflammatory. However, TGF-β drives differentiation of both Th17 and Treg cells (Bettelli et al., 2006; Dong, 2008; Littman and Rudensky, 2010; O'Quinn et al., 2008; Veldhoen and Stockinger, 2006). In response to TGF-β, many T cells express both ROR-γt and Foxp3 (Fontenot et al., 2003; Hori et al., 2003; Veldhoen and Stockinger,
In the absence of IL-6, TGF-β induces Foxp3 and represses IL-23R. Foxp3 then interacts with ROR-γt and prevents ROR-γt binding to DNA, preventing Th17 associated gene transcription. Under the condition CD4+ T-cells will be more inclined to differentiate into Tregs. In contrast, when IL-6 is present together with low concentrations of TGF-β, STAT3 will be activated, overcoming the inhibitory effect of Foxp3 on ROR-γt transcription (Burchill et al., 2007; Chang et al., 2015; Cohen et al., 2006; Ivanov et al., 2006; Yang et al., 2007; Yao et al., 2007). STAT5A and B are crucial transcription factors for Treg development, as they can directly bind the Foxp3 gene to promote the development and maintenance of Tregs (Burchill et al., 2007; Cohen et al., 2006; Murawski et al., 2006; Yao et al., 2007; Zorn et al., 2006). STAT5A/B can also directly bind the IL-17 gene to repress Th17 differentiation gene transcription (Basu et al., 2015; Kozela et al., 2015; Pandiyan et al., 2012; Stockinger, 2007; Zheng et al., 2015). Therefore, STAT5A/B play important roles in maintaining the Th17/Treg balance (Basu et al., 2015; Kozela et al., 2015; Laurence et al., 2007; Zheng et al., 2015). Nuclear factor kappa B (NF-κB) signaling also plays a critical role in the regulation of immune responses. NF-κB (p65) deficient mice are significantly compromised in Th17 differentiation and responses and are resistant to EAE (Ruan and Chen, 2012; Ruan et al., 2011).

Betulinic acid (BA) is a natural pentacyclic triterpenoid, which is found in the bark of many species of plants, and is particularly abundant in plants of the genus Sambucus, for example, Sambucus williamsii Hance (SWH) (Mukherjee et al., 1997). In China, SWH is a folk medicine with a long history of being used for treatment of
inflammation and bone fractures. A recent study suggests that BA has an anti-inflammatory effect, especially inhibiting pro-inflammatory IL-17 and IFN-γ production (Blazevski et al., 2013). Considering the inhibitory effects of SWH and BA on inflammation, BA is very likely to inhibit T cell differentiation. Thus, we initially examined the effect of BA on Th17 cell differentiation and found that BA is a moderate Th17 differentiation inhibitor. Therefore, we screened BA derivatives and found a heterocyclic ring-fused BA derivative, SH479, which had a greater inhibitory effect on Th17 differentiation. Furthermore, SH479 had a greater inhibitory effect on Th1 cells, and stimulated Treg differentiation. SH479 has been reported to inhibit osteoclast differentiation and bone resorption (Xu et al., 2012). The anti-inflammatory effect of SH479 remains to be defined. In this study, we found that SH479 can prevent and treat EAE by inhibiting T cell differentiation into Th17 cells while promoting Treg differentiation. SH479 had a more significant effect on Th17 and Treg cells than BA. Furthermore, SH479 treatment could ameliorate the clinical signs of EAE in both prevention and therapeutic protocols. Mechanistic analysis indicated that SH479 affected Th17 and Treg cells by blocking the STAT3 and NF-κB pathways and activating the STAT5 pathway.
Materials and Methods

Reagents and Mice

C57BL/6 mice were purchased from Shanghai Slac Laboratory Animal Limited Company, and were bred and maintained at the SPF Laboratory Animal Center of East China Normal University. All animal procedures were approved by the institutional Animal Ethics Committee. All of the antibodies were purchased from Cell Signaling Technology except when otherwise indicated, including CD4, p-JAK1, JAK1, p-JAK2, JAK2, p-STAT5, STAT5, p-STAT3, STAT3, p-p65, p65, p-IκBα, IκBα, RORγ, and RORα.

Stability and pharmacokinetic properties

The human liver microsomal stability assay was performed by WuXi AppTec (Shanghai, China) according to the standard operating protocol. The plasma pharmacokinetics of SH479 in rats were determined by Shanghai Institute of Materia Medica (Shanghai, China) according to the standard protocol (Nomme et al., 2014). Briefly, SD rats (weighing 200-220 g) were intravenously injected with 10 mg/kg or fed 20 mg/kg SH479 by oral gavage. Approximate values of the area under the curve (AUC), half-life (t1/2), maximum concentration in the plasma (Cmax), and time to reach the maximum concentration (Tmax) were calculated using Boomer/Multi-Forte PK functions from Microsoft Excel.

Induction and treatment of EAE
C57BL/6 female mice (8 to 10-week old) were housed 4-5 per cage and allowed to access to chow diet and water freely. 300 μg/mouse myelin oligodendrocyte glycoprotein (MOG) fragment 35–55 (MOG_{35–55}) (>95% purity, sequence as Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys, from GL Biochem, Shanghai, China) was used for EAE induction. Immunizations were conducted by mixing MOG with Complete Freund’s Adjuvant (Sigma), containing 5 mg/ml heat-killed H37Ra strain of Mycobacterium tuberculosis (Difco Laboratories). Mice were subcutaneously immunized on day 0. Pertussis toxin (List Biological Laboratories) in PBS-50 mM NaCl was injected intraperitoneally (i.p.) immediately after MOG and then again 24 h later. The course and severity of EAE in mice was examined daily in a blinded manner by two independent examiners, using the following standard score system: 0, no obvious changes; 0.5, Limp tail; 1.0, Limp tail and no straight and upright tail function; 1.5 Wobbly gait; 2.0, Hind limb weakness; 2.5, Unilateral hind limb paralysis; 3.0, Bilateral hind limb paralysis; 3.5, Bilateral hind limb paralysis and fore limb weakness; 4.0, Hind limb and fore limb paralysis; 5.0, Death. For treatment of EAE, 20mg/kg SH479 or DMSO as vehicle control was administrated i.p. daily from day 7 to day 35. The prevention protocol differed from the treatment protocol only in the SH479 administration time, which was administered beginning 3 days before induction of EAE in the prevention protocol.

T-cell differentiation in vitro
Naïve CD4+ T cells were isolated from the spleen using Mouse CD4+ CD62L+ T cell isolation kit (MACS) according to manufacturer’s instructions. Cells were cultured in 48-well plates and stimulated with anti-CD3 (eBioscience, 16-0031-86) and anti-CD28 (eBioscience, 16-0281-86) antibodies, supplemented with cytokines and neutralization antibodies for the desired polarization as follows: IL-12 (10 ng/ml) and anti-IL4 (10 μg/ml) for Th1; TGF-β (3 ng/ml), IL-23 (10 ng/ml), IL-6 (30 ng/ml), anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml) for Th-17 cells, TGF-β (5 ng/ml), anti-IL2 (10 μg/ml) and anti-IFN-γ (10 μg/ml) for Treg cells. Cells were cultured for 3 days and analyzed by flow cytometry.

MTS assay

Splenocytes were separated from normal or EAE mice by Ficoll (GE Health Care). 2 × 10^5 cells per well were cultured in 96-well plates in RMPI 1640 medium (Gibco) with 10% FBS added with or without 20 μg/ml MOG and different concentrations of SH479. Cultures were maintained at 37°C in 5% CO₂ for 72 h, then 20 μl MTS solution (CellTiter 96® AQueous Assay, Promega) per well was added. After 2 to 4 h incubation, the absorbance at 490nm was detected.

Flow cytometry

Antibodies used for FACS analysis were rat-anti-mouse IFNγ-APC (BD Biosciences, Cat. No. 554413), rat-anti-mouse IL-17A-PE (BD, Cat. No. 559502), rat-anti-mouse CD4-FITC (BD, Cat. No. 553729), and anti-mouse Foxp3-APC (eBioscience, Cat. No. 119...
17-5773). All the antibodies were used at 0.2 μg per 10⁶ cells in each flow cytometry experiment. Lymphocytes from the brain and spinal cord (CNS) were separated by Percoll (GE Healthcare) and cells from lymph nodes and spleen were separated by Ficoll (GE Healthcare). All cells were stimulated by PMA (5 ng/ml) and ionomycin (75 g/ml) for 5 h. Golgiplug was used at the same time. Cytofix/Cytoperm™ Plus Fixation/Permeablization Kit (BD555028) was used for intracellular staining. Experiments were conducted according to the manufacturer’s instructions.

**Histopathology and immunofluorescent analysis**

Spinal cords were isolated from each mouse and perfused in 4% paraformaldehyde overnight and then washed with running water for 12 h. Alcohol and dimethylbenzene treatment all were done as per conventional protocols and then samples were embedded in paraffin. Sections were cut at 5 μm thickness and stained with H&E or Luxol Fast Blue. Frozen sections of 5 μm thickness were used for CD4⁺ T cell immunohistochemistry. Frozen sections of spinal cord and brain were blocked with 5% BSA for 30 min. at room temperature. Then the sections were incubated with primary antibody Rat anti mouse CD4 (Biolegend, Cat. No. 100401, 1:500 dilution) and second antibody Alexa Fluor 594 goat anti-rat IgG (Abcam, Cat. No. ab150160) for 1 h each at room temperature. Cell nuclei were stained with DAPI for 10 min at room temperature.

**Cytokine analysis**
Splenocytes were separated from vehicle- or SH479-treated EAE mice. The cells were stimulated by MOG (20 μg/ml) for 48 h, and supernatants were collected for cytokine analysis. Serum samples were also collected for analysis. Assays for IFNγ, IL-6 and IL-17 were performed in triplicate using commercial ELISA kits (IFNγ kit and IL-6 kit from BD, IL-17 kit from R&D) according to the manufacturers’ instructions.

**Real-time PCR and RT-PCR**

Total RNA was extracted from splenocytes using TRIzol (Invitrogen) and reverse-transcribed to cDNA using Prime Script RT kit (Takara). Quantitative PCR was performed with the SYBR Green Kit (Takara) according to the manufacturer’s instructions. Quantitative PCR results were calculated through MXPro4.1. (Stratagene, USA). Primers used for quantitative PCR and RT-PCR were synthesized by Shanghai Biosune (Shanghai, China), and sequences are listed in Table1.

**Electrophoretic Mobility Shift (EMSA) Assay**

The EMSA assay was carried out as described with modifications (Li et al., 2011). Briefly, splenocytes were isolated from EAE mice, and then the cells were stimulated with MOG (20 μg/ml) for 24 h and washed with cold PBS. After centrifugation at 14000 rpm for 1 min, the cells were suspended using cold lysis buffer with protease and phosphatase inhibitors, then treated with 10% NP-40 and nuclear extraction solution to obtain nuclear proteins. For electrophoresis, about 2 μg lysate protein was mixed with 2 μl of binding buffer, 2 μl NP-40, and 0.5 μl oligo-probe, then double
distilled water was added up to 20 μl followed by 2 μl orange loading buffer. Then 2 μl of the mixture was loaded onto the gel and run at 80V. Analysis was conducted with the Odyssey infrared imaging system.

**ChIP assay**

ChIP assays were performed as described with modifications (Yoon et al., 2003). Briefly, splenocytes were isolated from EAE mice. Then the cells were incubated with MOG peptide and SH479 (10 μM) for 72 hours. After that, the cells were cross-linked with formaldehyde, and the nuclei were isolated and sonicated. The DNA-protein complexes were immunoprecipitated by anti-STAT3 or anti-STAT5 antibodies (1μl for each reaction) (Cell signaling Technology) using protein-A sepharose beads preblocked with salmon sperm DNA. The DNA was isolated for real-time PCR with IL-17 promoter primers 5′-CACCTCACACGAGGCACAAG-3′, 5′-ATGTTTGCCTCTGATC-3′.

**Statistics**

Student’s t test was used to analyze the differences. For comparison among multiple groups, one-way ANOVA was used. Data were presented as mean ± standard deviation (SD). Differences between values were considered statistically significant when p < 0.05.
Results

The BA derivative SH479 had a greater anti-inflammatory effect than BA

It has been reported that BA has an inhibitory effect on IL-17 and IFN-γ production (Blazevski et al., 2013), suggesting that BA could have an inhibitory effect on Th17 cells. However, whether there are BA derivatives with improved efficacy in modulating the Th17/Treg balance and treating EAE is largely unknown. Here we screened 35 BA derivatives which were described in reference (Xu et al., 2012) using the Th17 differentiation assay (Fig.S1A) and found that a heterocyclic ring-fused BA derivative, SH479 (Fig.1A), was the most potent compound at inhibiting Th17 differentiation. To confirm the inhibitory effect of BA and SH479 on CD4⁺ T cell differentiation, we isolated naïve CD4⁺ T cells from the spleen, and induced differentiation into Th1, Th17 and Treg cells under BA or SH479-treatment conditions in vitro. FACS analysis showed that BA treatment mildly reduced the percentage of differentiated Th17 cells, and had no effect on the Th1 percentage and Treg population. However, under the same experimental conditions, SH479 treatment had a greater inhibitory effect on Th17 and Th1 differentiation, and significantly stimulated Treg differentiation (Fig.1C). These results suggested that SH479 was better at inhibiting pro-inflammatory signals and promoting anti-inflammatory T cells than its parent compound, BA.

SH479 ameliorated the clinical and histological signs of EAE

To examine the effect of SH479 on autoimmune disease, we established an EAE
mouse model by MOG\textsubscript{35-55} immunization. As shown in Fig. 2A and 2B, EAE mice developed a typical course of EAE characterized by disease onset at day 9, rapidly reaching peak severity at day 20, followed by a characteristic recovery phase (day 20 to day 35). In contrast, 20 mg/kg SH479 significantly reduced EAE severity in both prevention protocol and therapeutic protocols (Fig. 2A and 2B). Our data showed that compared to vehicle, the cumulative clinical score of EAE in the prevention protocol was 2.5±0.6 (vehicle) versus 1.0±0.4 (SH479) and in the therapeutic protocol was 2.8±0.4 (vehicle) versus 1.2±0.2 (SH479), which indicated that SH479 was able to prevent as well as treat EAE.

To further evaluate the effect of SH479 treatment on EAE, we isolated spinal cord tissue from vehicle- or SH479-treated EAE mice and performed histopathological analysis. Compared with the vehicle-treated group, SH479-treated mice showed significantly decreased leukocyte infiltration by H&E staining and less neural demyelination by Luxol fast blue staining (Fig. 2C). Considering the crucial role of CD\textsuperscript{4}\textsuperscript{+} T-cells in EAE development, we also investigated CD\textsuperscript{4}\textsuperscript{+} T cells by immunofluorescent staining in spinal cord tissue. As expected, the number of CD\textsuperscript{4}\textsuperscript{+} T cells in the spinal cord was obviously decreased in the SH479 treated group (Fig. 2D), which suggested that SH479 treatment may function through modulating CD\textsuperscript{4}\textsuperscript{+} T cells.

To examine whether SH479 affected other immune cells, we tested the effect of SH479 on macrophage cells. Our results showed that SH479 had little effect on inflammatory factor (\textit{Il}-6 and \textit{Tgf}-\textit{β}) expression induced by LPS, suggesting that SH479 specifically affected T cells but not macrophages. (Fig. S1B).
SH479 specifically inhibited EAE mouse splenocyte cell viability without affecting normal splenocyte viability

Next, we examined the toxicity of SH479. Our data showed that SH479 had no adverse effects on body weight at the treatment dose and time frame in EAE mice and control mice (Fig. 3A). SH479 also had little cytotoxic effect on splenocytes isolated from normal mice (Fig. 3B). However, when splenocytes were harvested from EAE mice and stimulated with MOG \textit{in vitro}, SH479 inhibited cell viability in a dose-dependent manner (Fig. 3C), suggesting that SH479 specifically inhibited MOG-induced splenocyte cell viability but not normal splenocyte viability.

SH479 exerted an anti-inflammatory effect by regulating the Th17/Treg balance

MS and EAE have been considered a T cell (especially CD4$^+$ T cell) mediated autoimmune disease (Duffy et al., 2014; O'Brien et al., 2010). To confirm the effect of SH479 on T cell regulation \textit{in vivo}, we isolated CD4$^+$ T cells of the CNS, spleen and lymph nodes from vehicle- or SH479-treated EAE mice on day 18 postimmunization and analyzed the percentage of Th1 (IFN$\gamma$ positive), Th17 (IL-17 positive) and Treg (Foxp3 positive) cells by flow cytometry. In these tissues, SH479 significantly decreased the percentage of pro-inflammatory T cells - Th1 and Th17, and increased the percentage of anti-inflammatory Treg cells compared with the vehicle-treated group in the CNS, spleen and lymph nodes (Fig. 4A and B). Furthermore, compared to the vehicle-treated EAE group, SH479 treatment significantly decreased serum
levels of the pro-inflammatory cytokines IL-6, INF-γ and IL-17 (Fig. 4C), while the anti-inflammatory cytokine IL-10 was significantly increased in the SH479-treated EAE group (Fig. 4D).

To further confirm the effect of SH479 on inflammation, we isolated splenocytes from vehicle- or SH479-treated EAE mice on day 18 postimmunization, incubated them in the presence of MOG35-55 peptide (20μg/ml) for 48 hours, and then analyzed supernatant cytokines by ELISA. Compared to vehicle-treated EAE mice, SH479 remarkably reduced IL-6, IFN-γ and IL-17 levels and increased the IL-10 level (Fig. 5A and 5B). Consistent with the ELISA results, SH479 also significantly increased the mRNA level of Treg-associated cytokines and transcription factors (including IL-4, IL-13, IL-10 and Foxp3), and significantly decreased Th17-associated cytokine and transcription factor mRNA expression (including IL-17A, IL-17F, IL-22, and RORγt) (Fig. 5C) while it had little effect on the expression of RORα. Compared to ROR-γt, RORα deficiency reduced IL-17A and IL-23R, but not IL-17F or IL-22 expression (Jetten, 2009). The reduction in IL-17F or IL-22 expression in the SH479 treatment group further suggested that SH479 inhibited Th17 differentiation by ROR-γt rather than RORα. Furthermore, we examined whether SH479 inhibited the transcription level of cytokines in a dose-dependent manner. As expected, SH479 dose-dependently reduced Th17- and Th1-associated cytokine expression (including IL-17A, IL-17F and IFN-γ) and increased Treg associated transcription factor Foxp3 expression (Fig. 5D). In conclusion, our data indicated that SH479 regulated the Th17/Treg balance by inhibiting Th17 associated cytokines while promoting Treg associated cytokines.
SH479 inhibited Th17 differentiation by regulation of STAT3 and STAT5 signaling pathways

STAT family transcription factors play important roles in the differentiation of Treg and Th17 cells. Stat3 is pivotal for Th17 differentiation and Treg inhibition. Conversely, STAT5A/B is essential for the differentiation and maintenance of Treg cells, while downregulating Th17 differentiation. To determine the signaling pathways mediating the effect of SH479 on T cell differentiation, we employed four separate approaches to examine whether SH479 regulated the STAT3 and STAT5 signaling pathways. First, we isolated CD4+ T cells from vehicle- or SH479-treated EAE mouse splenic lymphocytes and stimulated them with or without MOG. Our data showed that though the total STAT3 and STAT5 protein level did not change, SH479 treatment significantly reduced STAT3 phosphorylation, and increased STAT5A/B phosphorylation (Fig. 6A). Phosphorylation of JAK1 and JAK2, the upstream kinases of STAT3, was significantly reduced by SH479 treatment. ROR-γt, the master transcription factor of Th17 differentiation, was also reduced by SH479, while another master transcription factor ROR-α had little change (Fig. 6A), which is consistent with our real-time PCR analysis results (Fig. 5C). Second, we examined the inhibitory effect of SH479 on STAT3 phosphorylation in spinal cord tissue from vehicle- and SH479-treated EAE mice by immunohistochemistry. Our data showed that STAT3 phosphorylation was much less in the SH479-treated group (Fig. 6B), suggesting that SH479 could inhibit the STAT3 signaling pathway in vivo. Third, we examined...
whether SH479 inhibited the DNA-binding activity of STAT3 by EMSA using STAT3 specific probes and CD4+ T cell nuclear extracts. As shown in Fig. 6C, the DNA-binding activity of STAT3 was markedly decreased in SH479-treated EAE mice, suggesting that SH479 could inhibit the DNA-binding activity of STAT3. Finally, we examined whether SH479 regulated the IL-17 promoter by modulating STAT3 and STAT5 activity. Our results showed that MOG peptide treatment notably induced STAT3 recruitment to the IL-17A promoter in CD4+ cells from the EAE mice, while SH479 dramatically abrogated STAT3 recruitment (Fig. 6D). The recruitment of STAT3 to the IL-17A promoter was also inhibited in SH479-treated EAE mice compared to the vehicle control EAE mice (Fig. 6D). As expected, the recruitment of STAT5 to the IL-17A promoter was dose-dependently enhanced by BA or SH479 treatment as determined using the CHIP assay, and the enhancement effect was more potent in the SH479 treatment group (Fig. 6E). Taken together, all of our data demonstrated that SH479 inhibited Th17 differentiation by regulation of the STAT3 and STAT5 signaling pathways.

**SH479 inhibited NF-κB signaling in CD4+ T cells**

The NF-κB activation pathway is also involved in Th17 differentiation. To investigate whether SH479 affected Th17 differentiation through the NF-κB pathway, we examined the NF-κB signaling pathway in CD4+ T cells from vehicle- or SH479-treated EAE mice. As shown in Figure 7A, the phosphorylation of p65 and IκBα significantly decreased, and the degradation of IκBα decreased in SH479-treated
EAE mice with MOG peptide re-stimulation when compared with the vehicle control EAE mice (Fig. 7A). Interestingly, without MOG peptide re-stimulation, the phosphorylation of p65 and IκBα was already decreased, and the degradation of IκBα was decreased in SH479-treated EAE mice compared to the vehicle control EAE mice (Fig. 7A). These data suggested that SH479 could inhibit the NF-κB signaling pathway in CD4+ T cells. To further confirm our observation, the DNA binding activity of NF-κB (p65) was investigated in splenic CD4+ T cells. Our data showed that the DNA binding activity of p65 was significantly lower in SH479-treated EAE mice with MOG peptide re-stimulation (Fig. 7B). Together, all of the results indicated that SH479 could inhibit the NF-κB signaling pathway in T cells.

**The stability and pharmacokinetic properties of SH479**

To evaluate the stability and pharmacokinetic properties of SH479, we examined its stability when exposed to human liver microsomes. The human liver microsomal stability assay data showed that when incubated with human liver microsomes, at least 46.5% of SH479 was unchanged after 1 hour incubation at 1 μM (Supplemental Table 1). To further explore the metabolic stability of this compound, we determined its *in vivo* pharmacokinetics. The plasma concentrations of SH479 after oral gavage administration and intravenous injected were measured. The results showed that SH479 possessed a plasma half-life of ($t_{1/2}$) 1.63 hours upon intravenous injection and $t_{1/2} = 2.45$ hours upon oral gavage administration (Supplemental Table 2 and Table 3). The plasma concentration of SH479 reached a value of 212 ng/mL ($C_{max}$) in
0.44 hours (Supplemental Table 3).
Discussion

Betulinic acid (BA), a natural pentacyclic triterpenoid, is found in many species of plants. In recent years, a variety of biological activities including anti-inflammation have been reported for BA (Mukherjee et al., 1997). BA can inhibit pro-inflammatory cytokine IL-17 and IFN-γ production (Blazevski et al., 2013). To find derivatives of BA with greater anti-inflammatory efficacy, we screened more than 30 BA derivatives using the Th17 differentiation assay. We found that SH479, a heterocyclic ring-fused BA derivative, was the most potent compound to inhibit Th17 differentiation. Further investigation found that SH479 inhibited Th1 and Th17 while promoting Treg differentiation. These results indicated that SH479 may be a potential drug candidate in MS prevention and treatment.

CD4+ T cell subsets Th17 and Treg have opposite biological activities in the inflammatory response but the same cytokines and transcription factors (such as TGF-β, STAT3 and STAT5A/B) operate in their respective differentiation processes. STAT3 can be activated by Th17-induced IL-6, IL-23 and IL-21, and then induces ROR-γt gene expression. In the absence of IL-6, Foxp3 is induced by TGF-β, and then interacts with ROR-γt protein to prevent ROR-γt from binding DNA to regulate Th17-associated gene transcription. In contrast, when IL-6 is present with low concentrations of TGF-β, STAT3 will be activated, overcoming Foxp3 inhibition of ROR-γt transcription (Zhou et al., 2008). STAT5 can directly bind the Foxp3 gene to promote development and maintenance of Treg cells (Burchill et al., 2007; Yao et al., 2007; Zorn et al., 2006). However, STAT5 also directly binds the Il17 gene to repress
Th17 related gene transcription (Stockinger, 2007). NF-κB signaling also was reported to play a critical role in EAE development and Th17 cell differentiation (McGuire et al., 2013; Ruan et al., 2011). In our results, SH479 inhibited Th17 and promoted Treg differentiation by inhibiting STAT3 and the NF-κB pathway and promoting the STAT5 pathway. These results indicated that through STAT3, STAT5 and NF-κB pathway regulation, SH479 could shift the Th17/Treg balance towards an anti-inflammatory response.

Th17-specific transcription factors, ROR-γt and RORα, are orphan nuclear receptors. Both ROR-γt and RORα have a degree of functional redundancy in positive regulation of Th17 differentiation. Th17 differentiation was completely impaired in both ROR-γ and RORα deficient mice (Yang et al., 2008b; Yang et al., 2008c). However, ROR-γt plays a major role in Th17 differentiation because loss of ROR-γt has a more pronounced effect on Th17 cytokine expression than loss of RORα (Jetten, 2009). Compared to ROR-γt, RORα deficiency reduced IL-17A and IL-23R, but not IL-17F or IL-22 expression (Jetten, 2009). RORα negatively interferes with the NF-κB signaling pathway by reducing p65 translocation (Delerive et al., 2001), while ROR-γt expression inhibition was mediated by the suppression of NF-κB (Lee et al., 2015), suggesting that ROR-γt should be the downstream of NF-κB and RORα. In our data, we found that SH479 repressed the transcription level of ROR-γt but not RORα, suggesting that SH479 has effects on the transcriptional regulation of ROR-γt rather than RORα during Th17 differentiation. Though the expression of both ROR-γt and RORα was STAT3-dependent (Harris et al., 2007; Nurieva et al., 2007; Yang et al.,
2007), the difference between ROR-γt and RORα in Th17 differentiation implies that there may be different mechanisms in up- and down-stream transcription regulation. The detailed mechanisms of ROR-γt-specific regulation need to be further investigated.

Many small molecule compounds have been used to suppress Th17 responses including Th17-mediated autoimmune disease. Given the crucial role of RORα in circadian rhythm and metabolism regulation, an agonist for both ROR-γt and RORα may cause undesirable side effects. Consequently, developing a ROR-γt-selective agonist is desirable. Many ROR-γt specific agonists were synthesized and used to suppress Th17 and stimulate Treg differentiation (Solt et al., 2012; Xiao et al., 2014). However, these ROR-γt specific agonists cannot affect Th1 cells (Xiao et al., 2014). In our data, we found that SH479 can suppress both Th1 and Th17 differentiation, and stimulate Treg differentiation by repressing transcription of ROR-γt but not RORα, suggesting SH479 may have a stronger effect on autoimmune diseases including EAE.

Moreover, we have previously reported that SH479 can act as an inhibitor of osteoclast differentiation and bone resorption (Xu et al., 2012). Rheumatoid arthritis (RA), as a chronic, systemic inflammatory disorder, exhibits many symptoms including inflammation, osteoporosis and nervous system problems. The dual functions of SH479 in reducing both inflammation and osteoporosis suggest that SH479 may be a potentially excellent drug candidate for RA. Thus the activity and mechanism of SH479 on RA need to be examined.
This study for the first time identified a BA derivative, SH479, which exhibited good anti-inflammatory activity as a regulator of the Th17/Treg balance in an EAE mouse model. We believe that SH479 will be of great value to further understand the anti-inflammatory mechanism of BA derivatives, and has the potential to be developed into therapeutics against a variety of autoimmune diseases.
Authorship contributions

Participated in research design: Jing Li, Ji Jing, Yang Bai, Zhen Li, Mingyao Liu, Huaqing Chen and Jian Luo

Conducted experiments: Jing Li, Ji Jing, Yang Bai, Zhen Li, Roumei Xing, Binhe Tan, Contributed new reagents or analytic tools: Wenwei Qiu, Fan Yang and Jie Tang

Performed data analysis: Jing Li, Ji Jing, Yang Bai, Zhen Li, Stefan Siwko, Xueyun Ma, Changsheng Du, Bing Du, Stefan Siwko, Mingyao Liu, Huaqing Chen and Jian Luo

Wrote or contributed to the writing of the manuscript: Jing Li, Stefan Siwko, Mingyao Liu, Huaqing Chen and Jian Luo
References


Footnotes

The authors declare no conflict of interest. This work is supported by grants from the National Key Research and Development Program of China [2016YFC0902102], National Basic Research Program of China [2012CB910402], the National Natural Science Foundation of China [81472048, 81272911, 81330049, 31200678, 31271468], Innovation Program of Shanghai Municipal Education Commission [14ZZ051], General Program of Shanghai Municipal Commission of Health and Family Planning [201540090], the Science and Technology Commission of Shanghai Municipality [15140903600].

Jing Li, Ji Jing and Yang Bai contributed equally to this work.
Figure Legends

Figure 1. SH479 had a greater anti-inflammatory effect than Betulinic Acid (BA).

(A) Chemical structure of Betulinic Acid (BA) and its derivative SH479.

(B and C) Naïve CD4+ T cells were isolated from normal spleen, cultured in Th1, Th17 and Treg polarizing conditions in vitro, together with 10 μM BA (B) or 10 μM SH479 (C). Cells were then subjected to flow cytometric analysis for percentages of Th1, Th17 and Treg cells in CD4+ subsets (n = 6). The data represents the mean ± SD of three independent experiments. *P <0.05, **P <0.01 , ***P<0.001. N.S. means no significant difference.

Figure 2. Amelioration of EAE by SH479 treatment.

(A and B) SH479 significantly reduced the severity and cumulative clinical score of EAE. Clinical scores of EAE mice treated with SH479 (20 mg/kg) or vehicle control once daily via i.p. injection, beginning day 3 before immunization (n=8) (A) or day 7 post-immunization (n=10) (B) (Day 0 indicates MOG injection; red arrow indicates the treatment initiation time point).

(C) Histopathology of spinal cord tissue from vehicle and SH479-treated EAE mice. H&E staining and Luxol fast blue staining of paraffin sections of spinal cords from vehicle- or SH479- (20 mg/kg, starting from day 7) treated EAE mice on day 18 after immunization (left). The number of infiltrating leukocytes and the extent of demyelination were quantified (right). The data represents the mean ± SD of three independent experiments. *P <0.05.
(D) Immunofluorescent staining of CD4$^+$ T cells in frozen sections of spinal cords isolated from vehicle-, or SH479 (20 mg/kg, starting from day 7) treated EAE mice on day 18 post-immunization. The staining cells were quantified in the right panel. The data represents the mean ± SD of three independent experiments. *P <0.05.

Figure 3. SH479 specifically inhibited MOG-induced splenic lymphocyte cell viability in EAE mice.

(A) 20 mg/kg SH479 had little effect on mouse body weight in EAE mice and control mice. Body weights of the vehicle-treated and SH479-treated (20 mg/kg) EAE mice and control mice were recorded every day (n=10).

(B) SH479 had little effect on normal splenic lymphocyte viability. Splenic lymphocytes isolated from normal mice were cultured in the presence of indicated concentrations of SH479 and the cell viability was measured by MTS assay. The data represents the mean ± SD of three independent experiments. (n=4).

(C) SH479 inhibited MOG-induced splenic lymphocyte cell viability in EAE mice. Splenic lymphocytes from EAE mice were cultured with the MOG peptide (20 μg/ml) and various concentrations of SH479, and then the cell viability was measured by MTS assay. The data represents the mean ± SD of three independent experiments. (n=4).

Figure 4. SH479 induces an anti-inflammatory effect by regulating the Th17/Treg balance in vivo.
(A and B) Treatment of SH479 reduced pro-inflammatory T cells and promoted anti-inflammatory T cells in the CNS and peripheral lymphoid tissues. Total lymphocytes from CNS, spleen or lymph nodes were isolated from vehicle- or SH479-treated mice (20 mg/kg) on day 18 post-immunization and subjected to flow cytometry analysis. The population was gated on CD4+ T cells, and then analyzed for different subpopulations. Representative FACS images of Th1 (IFNγ positive, IL-17 negative), Th17 (IL-17 positive, IFNγ negative) and Treg (Foxp3 positive, IL-17 negative) cells are shown (A). The statistics of flow cytometry data were shown (B). The data represents the mean ± SD of three independent experiments. *P <0.05, **P <0.01.

(C and D) SH479 treatment reduced pro-inflammatory cytokines (IL-6, IFN-γ, IL-17) (C) and increased anti-inflammatory cytokines (IL-10) (D) in vivo. Mouse serum was isolated from the vehicle-treated and SH479-treated (20 mg/kg) EAE mice and control mice on day 18 post-immunization and analyzed by ELISA analysis. The data represents the mean ± SD of three independent experiments. **P <0.01, n=10.

Figure 5. SH479 mediates an anti-inflammatory effect by regulating the Th17/Treg balance ex vivo

(A and B) SH479 treatment reduced expression of pro-inflammatory cytokines (A) and increased anti-inflammatory cytokines (B) in splenic lymphocytes. Splenic lymphocytes isolated from vehicle- or SH479-treated mice on day 18 post-immunization were incubated in the presence of MOG (20 μg/ml) for 48 h. The
supernatants were collected and subjected to ELISA analysis with the indicated antibodies. The data represents the mean ± SD of three independent experiments. *P <0.05,

(C) Splenic lymphocyte cells were isolated from vehicle- and SH479-treated mice on day 18 post-immunization and incubated in the presence of MOG (20 μg/ml) for 24 h. The total RNA were collected and analyzed for the expression of different cytokines (IL-4, IL-5, IL-13, IL-10, IL-17a, IL-17f, and IL-22) and transcription factors (Foxp3, Rorgt, and Rora) by real-time PCR. The data represents the mean ± SD of three independent experiments. *P <0.05,

(D) SH479 reduced pro-inflammatory cytokines and increased anti-inflammatory transcription factors in splenic lymphocyte in a dose-dependent manner. Splenic lymphocyte cells from EAE mice were cultured with the MOG peptide (20 μg/ml) and indicated concentrations of SH479. Transcription levels of IL-17a, IL-17f, IFN-γ and Foxp3 were determined by real time-PCR. The data represents the mean ± SD of three independent experiments.

Figure 6. SH479 inhibited Th17 differentiation by the STAT3 and STAT5 pathways.

(A) SH479 regulated STAT3 and STAT5 signaling pathways in CD4+ T cells. CD4+ T cells were isolated from vehicle- or SH479-treated mice at day 18 after immunization in the presence or absence of MOG (20 μg/ml), and then cell extracts were collected and subjected to Western blot analysis with the indicated antibodies. β-actin was used
as a loading control. The relative protein levels were quantified in the graph (right panel). The data represents the mean ± SD of three independent experiments. *P < 0.05,

(B) SH479 inhibited the phosphorylation of STAT3 in vivo. Immunohistochemistry staining (p-STAT3) of spinal cord tissue from vehicle- or SH479- treated EAE mice on day 18 post-immunization. The stained cells were quantified in the right panel. The data represents the mean ± SD of three independent experiments. *P < 0.05.

(C) SH479 inhibited the DNA binding activity of STAT3 in CD4+ T cells. CD4+ T cells isolated from vehicle- or SH479-treated EAE mice were re-stimulated with MOG (20 μg/ml) for 24 h. The nuclear extracts were collected and subjected to EMSA assay using fluorescent-labeled double-stranded STAT3 binding oligonucleotide as a probe.

(D and E) SH479 modulated the recruitment of STAT3 and STAT5 to the Il-17a promoter. CD4+ T cells isolated from vehicle-treated EAE mice (EAE) or SH479-treated EAE mice (EAE+SH479) were re-stimulated with or without MOG (20 μg/ml) for 24 h. After treated with indicated dose of BA or SH479, the cells were lysed and immunoprecipitated with an anti-STAT3 antibody (D) or anti-STAT5 antibody (E) or control IgG, and then chromatin immunoprecipitate was subjected to real-time PCR. The data represents the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7. SH479 directly inhibited the NF-κB signaling pathway in CD4+ T cells.
(A) SH479 regulated the NF-κB signaling pathway in CD4+ T cells. CD4+ T cells isolated from vehicle- or SH479-treated mice were re-stimulated with MOG (20 μg/ml) for 24 h, and then the cell lysates were subjected to Western blot analysis with indicated antibodies. β-actin was loaded as a control. The relative protein levels were quantified in the right panel. The data represents the mean ± SD of three independent experiments. *P <0.05,

(B) SH479 inhibited the DNA binding activity of NF-κB (p65) in CD4+ T cells. CD4+ T cells isolated from vehicle- or SH479-treated mice were re-stimulated with MOG (20 μg/ml) for 24 h. The nuclear extracts were collected and subjected to the EMSA assay using a fluorescent-labeled double-stranded NF-κB binding oligonucleotide as a probe.
Table 1: Primers used in PCR analysis

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<td>Reverse</td>
<td>GTCACGCACGATTTCCTCT</td>
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<tr>
<td>Ifn-γ</td>
<td>Forward</td>
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<td>Reverse</td>
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<td>Reverse</td>
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<td>TTGCAGAAAGCTGGGAACT</td>
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<td>Foxp3</td>
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<td></td>
<td>Reverse</td>
<td>CAGGGAGGAGTTCAGTAGAG</td>
</tr>
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</table>
Figure 1

A

Betulinic Acid

SH479

B

Cell percentage (%)

Th1  Th17  Treg

Ctrl  BA

C

Cell percentage (%)

Th1  Th17  Treg

Ctrl  SH479

N. S.  **  N. S.

**  ***  **
Figure 2

A

Clinical score

- EAE (n=8)
- EAE+SH479 (n=8)

Prevention

B

- EAE (n=10)
- EAE+SH479 (n=10)

Treatment

C

H&E

Luxol fast blue

EAE

EAE+SH479

D

CD4+

DAPI

Merge

EAE

EAE+SH479

Demyelination (%)

Infiltrating cells (numbers/slide)

EAE

EAE+SH479

Numbers of CD4+ Cell

EAE

EAE+SH479
**Figure 3**

A

![Graph showing weight over time for different groups.](image)

- **Legend:**
  - Ctrl (n=10)
  - Ctrl+SH479 (n=10)
  - EAE (n=10)
  - EAE+SH479 (n=10)

B

**Normal mice**

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<th>5</th>
<th>10</th>
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<th>20</th>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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</table>

C

**EAE mice**

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<tr>
<th>MOG</th>
<th>SH479 (μM)</th>
<th>-</th>
<th>-</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
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<tr>
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<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
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</table>

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Figure 4

A. Flow cytometry analysis of IFN-γ and IL-17 expression in different tissues (CNS, Spleen, lymph node) in EAE and EAE+SH479 groups.

B. Graph showing the percentage of CD4+IFN-γ+ cells in different tissues (CNS, Spleen, lymph node) in EAE and EAE+SH479 groups.

C. Graph showing the concentration of cytokines (IL-6, IFN-γ, IL-17) in serum from normal, EAE, and EAE+SH479 groups.

D. Graph showing the concentration of IL-10 in serum from EAE and EAE+SH479 groups.
Figure 5

A

Supernatant

IL-6 (pg/ml)

EAE  EAE  EAE  EAE

+SH479  +SH479  +SH479  +SH479

0  80  160  240  320

IFN-γ (pg/ml)

EAE  EAE  EAE  EAE

+SH479  +SH479  +SH479  +SH479

0  80  160  240  320

IL-17 (pg/ml)

EAE  EAE  EAE  EAE

+SH479  +SH479  +SH479  +SH479

0  20  40  60  80

IL-10 (pg/ml)

EAE  EAE  EAE  EAE

+SH479  +SH479  +SH479  +SH479

0  50  100  150

B

Supernatant

IL-4  IL-5  IL-13  IL-10  Foxp3

EAE  EAE  EAE  EAE

+SH479  +SH479  +SH479  +SH479

0  2  4  6

IL-17a  IL-17f  Rorγt  Rora

EAE  EAE  EAE  EAE

+SH479  +SH479  +SH479  +SH479

0  2  4  6

C

Relative mRNA Expression

EAE  EAE +SH479

II-4  II-5  II-13  II-10  Foxp3

0  2  4  6  8  10

II-17a  II-17f  II-22  Rorγt  Rora

0  2  4  6  8  10

D

Relative mRNA Expression

MOG  SH479  MOG  SH479  MOG  SH479

- - -  5  10

II-17a  II-17f  Ifn-γ  Foxp3

0  1  2  3  4  5  6

0  20  40  60  80

0  1  2  3
Figure 6

A. Comparison of protein expression levels in EAE, EAE+SH479, EAE+SH479, and MOG conditions.

B. Immunohistochemical staining for p-STAT3 in EAE and EAE+SH479 conditions.

C. Staining for STAT3 in MOG conditions.

D. qRT-PCR analysis of Il-17a promoter activity in EAE and EAE+SH479 conditions with SH479 and MOG treatments.

E. qRT-PCR analysis of Il-17a promoter activity in EAE and EAE+SH479 conditions with BA and SH479 treatments.

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Figure 7

A

\[ \alpha-p-p65 \]
\[ \alpha-p65 \]
\[ \alpha-p-\kappa B\alpha \]
\[ \alpha-\kappa B\alpha \]
\[ \alpha-\beta-actin \]

B

Relative amount

<table>
<thead>
<tr>
<th>EAE</th>
<th>EAE+SH479</th>
<th>EAE+MOG</th>
<th>EAE+SH479+MOG</th>
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</table>
| α-p-p65 | * | | *
| α-p65 | | * | *
| α-p-κBα | | | *
| α-κBα | | | *

MOG

p65

Free probe

ex vivo

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SH479, a Betulinic Acid Derivative, Ameliorates Experimental Autoimmune Encephalomyelitis by Regulating the Th17/Treg Balance

Jing Li, Ji Jing, Yang Bai, Zhen Li, Roumei Xing, Binhe Tan, Xueyun Ma, Wenwei Qiu, Changsheng Du, Bing Du, Fan Yang, Jie Tang, Stefan Siwko, Mingyao Liu, Huaqing Chen and Jian Luo
**Figure S1.**

(A) Th17 differentiation assay for BA derivative screening. Naïve CD4⁺ T cells were differentiated into Th17 cells and incubated with BA and BA derivatives. *Il-17* mRNA expression was determined by real-time PCR. The data represents the mean ± SD of three independent experiments. *P <0.05.

(B) Macrophages were isolated from mice and treated with LPS (100 ng/mL) and SH479 (5 μM and 10 μM) as shown. Total RNA was collected and analyzed for the expression of different cytokines (*Il-6*, and *TGF-β*) by real-time PCR. The data represents the mean ± SD of three independent experiments.

**Table S1: human Liver microsomal stability assay.**

**Table S2: The pharmacokinetic parameters of SH479 after intravenous injection of 10 mg/kg SH479 into Rat.**

**Table S3: The pharmacokinetic parameters of SH479 after rat were fed 20 mg/kg SH479 by oral gavage.**
Figure S1

A

Il-17a Relative mRNA Expression

B

Il-6 Relative mRNA Expression

Tgf-β Relative mRNA Expression

Molecular Pharmacology
Table S1: human Liver microsomal stability assay.

<table>
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<th>Sample Name</th>
<th>$R^2$</th>
<th>T$_{1/2}$ (min)</th>
<th>$CL_{\text{int(mic)}}$ (μL/min/mg)</th>
<th>$CL_{\text{int(liver)}}$ (mL/min/kg)</th>
<th>Remaining (T=60min)</th>
<th>Remaining (*NCF=60min)</th>
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<td>113.9%</td>
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<td>Testosterone</td>
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<td>71.3</td>
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<td>Diclofenac</td>
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<td>130.6</td>
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<td>Propafenone</td>
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<td>219.6</td>
<td>197.7</td>
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<td>95.7%</td>
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*NCF: the abbreviation of no co-factor. No NADPH regenerating system is added into NCF sample (replaced by buffer) during the 60 min-incubation, if the NCF remaining is less than 60%, then Non-NADPH dependent system occurs.

$R^2$ is the correlation coefficient of the linear regression for the determination of kinetic constant (see raw data worksheet).

T$_{1/2}$ is half life and $CL_{\text{int(mic)}}$ is the intrinsic clearance.

$CL_{\text{int(mic)}}$ = 0.693/half life/mg microsome protein per mL

$CL_{\text{int(liver)}}$ = $CL_{\text{int(mic)}}$ * mg microsomal protein/g liver weight * g liver weight/kg body weight

Liver weight: 88 g/kg, 40g/kg, 32 g/kg, 30 g/kg and 20 g/kg for mouse, rat, dog, monkey and human.
Table S2: The pharmacokinetic parameters of SH479 after intravenous injection of 10 mg/kg SH479 into rats.

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<th>AUC₀-∞ (ng·h/mL)</th>
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<th>t₁/₂ (h)</th>
<th>CLz (L/h/kg)</th>
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Table S3: The pharmacokinetic parameter of SH479 after administration of 20 mg/kg SH479 into rats by oral gavage.

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<th>$C_{\text{max}}$</th>
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<th>$\text{AUC}_{0-\infty}$</th>
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<th>$t_{1/2}$</th>
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<td>0.13</td>
<td>58</td>
<td>108</td>
<td>117</td>
<td>0.34</td>
<td>0.30</td>
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</tr>
<tr>
<td>CV (%)</td>
<td>28.6</td>
<td>27.3</td>
<td>19.1</td>
<td>18.6</td>
<td>9.3</td>
<td>12.3</td>
<td></td>
</tr>
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