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# Development and characterization of a hydroxyl-sulfonamide analog, 5-chloro-N-[2-(4-hydroxysulfamoyl-phenyl)-ethyl]-2-methoxy-benzamide, as a novel NLRP3 inflammasome inhibitor for potential treatment of multiple sclerosis

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**ABSTRACT**: In our efforts to develop novel small molecule inhibitors for the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome as potential disease-modifying agents to treat neurological disorders including multiple sclerosis (MS), a hydroxyl sulfonamide analog **JC-171** has been rationally designed and biologically characterized both *in vitro* and *in vivo*. Our studies established that **JC-171** dose dependently inhibited LPS/ATP induced interleukin-1β (IL-1β) release from J774A.1 macrophages with an $IC_{50}$ of 8.45 ± 1.56 μM. Selective inhibition of the NLRP3 inflammasome induced IL-1β release by this compound was also confirmed using mouse bone marrow derived-macrophages and LPS-challenged mice *in vivo*. Furthermore, immunoprecipitation study revealed that **JC-171** interfered with NLRP3/ASC interaction induced by LPS/ATP stimulation. More importantly, **JC-171** treatment delayed the progression and reduced the severity of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, in both prophylactic and therapeutic settings. This coincided with blocking of IL-1β production and a pathogenic Th17 response. Collectively, these results suggest that **JC-171** is a selective NLRP3 inflammasome inhibitor with biological activity *in vivo*, thus strongly encouraging further development of this lead compound as a potential therapeutic agent for human MS.

**KEYWORDS**: NLRP3 inflammasome, rational design, small molecule inhibitor, experimental autoimmune encephalomyelitis, multiple sclerosis.

## INTRODUCTION

Multiple sclerosis (MS) is considered an immune-mediated neurodegenerative disease of central nervous system (CNS) and is the leading cause of chronic neurological disability among young adults.[1] Epidemiological studies estimated 400,000 cases of MS in the United States with 200 new cases being added every week. Three types of MS exist: Relapsing-remitting MS, primary progressive MS, and secondary progressive MS. Although several classes of drugs have been successfully developed to ameliorate symptoms, speed recovery and slow the progression of the disease, unfortunately, there is currently no cure for MS patients. Furthermore, not all MS patients are responsive to the available treatment agents. Therefore, there is still urgent need to develop new agents with novel mechanism of action for this CNS disease.

Neuroinflammation persists as one of the pathologic hallmarks of MS throughout the course of MS and is closely associated with axonal injury observed in MS. The immunopathology of MS is characterized by the infiltration of myelin-reactive T cells into the CNS and induction of demyelination which disrupts the communication of the nervous system.[2] Although the exact etiology and pathogenesis of MS remain elusive, emerging evidence supports a critical role for caspase-1, IL-1β and IL-18 in the pathogenesis of MS, as demonstrated by studies from both MS patients and experimental autoimmune encephalomyelitis (EAE) mouse model, an animal model of MS.[3-6] Notably, activation of caspase-1 and maturation of cytokine interleukin (IL)-1β with biological activity are closely associated with a multimeric protein platform, termed inflammasome, in response to damage associated molecular pattern molecules or pathogen-associated molecular patterns.[7, 8] Among the known inflammasomes, the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, composed of NLRP3, ASC (apoptosis-associated speck-like protein containing a CARD), caspase-1, is the most extensively

studied and widely implicated regulator of caspase-1 activation, the maturation and production of IL-1β and IL-18.[8-11] There are a number of reports that strongly suggest the involvement of the NLRP3 inflammasome in the development of MS. Intriguingly, MS-like lesions were observed in a Muckle-Wells syndrome (MWS) patient who had a disease-susceptible mutation in the Nlrp3 gene.[12] Expression of caspase-1 is elevated in MS plaques and peripheral blood mononuclear cells of MS patients.[3, 4] A correlation between severity of MS and IL-1β has been well documented.[4, 5] Additionally, increased NLRP3 expression has been observed in the spinal cord of EAE mice, and NLRP3 deficiency substantially delayed onset and reduced severity of EAE symptoms, decreased neuroinflammation, demyelination and oligodendrocyte loss progression,[13-15] thus strongly suggesting the general involvement of NLRP3 inflammasome in the pathogenesis of MS. Importantly, it has been demonstrated that the effectiveness of IFN-β, a drug that has been used for more than 15 years as a first-line treatment for MS, is NLRP3 inflammasome dependent.[16] The presence of an NLRP3 inflammasome-independent subset in EAE,[16] indicates a multifactorial and heterogeneous nature of this disease. However, given the fact that many MS patients fail to respond to currently available MS treatments, including IFN-β and the demonstrated critical role of NLRP3 inflammasome in MS, development of novel small molecule inhibitors that selectively target NLRP3 inflammasome may provide new opportunities to MS intervention for therapeutic benefits in the clinic.

Recently, we started development of small molecule compounds based on the structure of glyburide, a FDA approved anti-diabetic agent and has been shown to inhibit NLRP3 inflammasome,[17] as NLRP3 inflammasome inhibitors. Structural modification of glyburide led to a sulfonamide analog **JC-21** (Figure 1A, referred as 16673-34-0 in previous reports).[18, 19] Biological characterization demonstrated that **JC-21** selectively targets NLRP3 inflammasome,

suppresses the production of IL-1β, and inhibits the activation of caspase-1 *in vitro*. Furthermore, **JC-21** was shown to mitigate the pathology and protect animals from injuries potentially involving NLRP3 inflammasome in acute myocardial infarction (AMI) mouse models.[19, 20] Notably, our results suggested that **JC-21** specifically blocks the assembly of the NLRP3 inflammasome protein complex,[19] thus representing a novel mechanism of action for this chemotype as NLRP3 inflammasome inhibitors. Herein, we report the design and biological characterization of a completely new analog, **JC-171**, for the first time as a potential agent for treatment of MS.

## RESULTS AND DISCUSSION

**Design of JC-171**. Although **JC-21** has shown promising activities both *in vitro* and *in vivo* as a NLRP3 inflammasome inhibitor,[18-20] solubility was recognized an issue for further development. Therefore, we designed a new analog **JC-171** with a hydroxamic acid on the sulfonamide moiety with an intention to increase the polarity of this compound (Figure 1A). The LogP value for **JC-21** and **JC-171** is 0.19 and 0.81, respectively, thus reflecting the significant difference in polarity. Furthermore, N-hydroxylation has been indicated as active metabolite of sulfonamide analogs and N-hydroxy sulfonamide analogs have been shown to exhibit anti-inflammatory effects.[21, 22] In addition, another goal of designing this analog was that the results will provide insight into whether structural modifications are tolerated at this specific position, thus facilitating further structure-activity relationship studies to develop more potent analogs. The chemical synthesis of **JC-171** is relatively straightforward as outlined in Scheme 1.

**JC-171 is an active NLRP3 inflammasome inhibitor**. Our previous studies have established that mouse J774A.1 macrophages upon stimulation with lipopolysaccharide (LPS) and adenosine

triphosphate (ATP) represent a reliable model for testing NLRP3 inflammasome activation and IL-1β release.[18, 19] After chemical synthesis, we first examined the cytotoxic effects of **JC-171** on J774A.1 cells. As shown in Figure 1B, **JC-171** did not show any significant cytotoxicity on J774A.1 cells themselves at up to 30 μM concentration. Upon challenge with LPS/ATP, significant cell death was observed for J774A.1 cells and **JC-171** dose dependently protected J774A.1 cells from LPS/ATP induced cell death (Figure 1C). We next tested whether **JC-171** inhibits the release of IL-1β by J774A.1 cells upon stimulation with LPS/ATP. As shown in Figure 1D, **JC-171** reduced the release of IL-1β due to NLRP3 inflammasome activation after LPS and ATP challenge. The inhibition potency of **JC-171** ($IC_{50}$: 8.45 ± 1.56 μM) is about two and half fold less than that of **JC-21** ($IC_{50}$: 3.25 ± 1.35 μM). Analysis of the effects of **JC-171** on caspase-1 activation by Western blotting also confirmed that treatment of J774A.1 cells with **JC-171** resulted in the inhibition of caspase-1 activation as evidenced by the decreased level of cleaved caspase-1 fragments (p20 and p10) (Figure 1E). On the other hand, no significant change was observed for the level of NLRP3, pro-IL-1β, or pro-caspase-1 upon treatment of J774A.1 cells with **JC-171** under the experimental conditions (Figure 1E). Thus the results rule out the possibility that the observed suppression of IL-1β or caspase-1 cleavage by **JC-171** is due to the interference of expression of NLRP3, pro-IL-1β, or pro-caspase-1.

We next employed primary mouse bone marrow-derived macrophages (BMDMs) to confirm the inhibitory effects of **JC-171** on the production of IL-1β and to also test the effect of **JC-171** on the production of other cytokines. As shown in Figure 2A, upon treatment of BMDMs with **JC-171** following the same experimental condition as used in J774A.1 cells, the release of IL-1β upon stimulation with LPS/ATP was significantly blocked in a dose dependent

manner. Notably, the production of TNF-α and IL-6 was not affected by **JC-171** even at high concentration (Figure 2B), indicating its selective effect on the NLRP3 inflammasome.

Our previous studies employing various cellular models including BMDMs carrying constitutively active NLRP3 suggested that this chemical scaffold might function as inhibitor by specifically blocking the assembly of the NLRP3 inflammasome protein complex.[19] To test this, co-immunoprecipitation (IP) study was performed using primary BMDMs after stimulation with LPS and ATP. As shown in Figure 2C, treatment of BMDMs with **JC-171** blocked the recruitment of ASC during NLRP3 activation, thus providing further evidence to the mechanistic hypothesis of this novel chemical scaffold as NLRP3 inflammasome inhibitor.

Given the fact that **JC-171** retained an inhibitory effect on the NLRP3 inflammasome activation, although slightly less potent than **JC-21** *in vitro*, and the fact that it is a potential metabolite of **JC-21**, we decided to evaluate the biological effect of **JC-171** *in vivo*. We pretreated the mice with **JC-171** before intraperitoneal injection of LPS, which has been shown to trigger IL-1β production in a NLRP3-dependent manner.[23] Serum levels of IL-1β was sharply reduced by **JC-171** treatment, whereas the amount of TNF-α was not significantly altered, further indicating the *in vivo* selective activity of **JC-171** on the NLRP3 inflammasome (Figure 2D).

**JC-171 treatment suppresses disease progression of EAE**. Since CNS inflammation represents one of the pathologic hallmarks of MS, and NLRP3 inflammasome and IL-1 signaling have been suggested to play a critical role in MS, [13, 24, 25] we decided to test **JC-171** in mouse EAE model as a proof-of-concept for such inhibitors as potential agents for treatment of human MS. As shown in Figure 3A, prophylactic treatment of EAE mice with **JC-171** (treatment started on the day of immunization) substantially delayed the disease onset and reduced the severity of

EAE. We next examined the effect of **JC-171** treatment on a pathogenic T-helper 17 (Th17) response that is known to be involved in the pathogenesis of EAE, [26, 27] and plays a causative role in autoimmune damage to the CNS in MS.[28, 29] Intracellular cytokine staining and flow cytometry analysis of lymph node cells showed significantly reduced frequencies of IL-17A$^+$CD4$^+$ Th17 cells in **JC-171**-treated mice as compared to the vehicle-treated mice (Figure 3B). Consistent with the attenuated Th17 response, as shown in Figure 3C, the transcription of *rora* and *rorc*, two transcription factors that are critical for Th17 cell differentiation, was also substantially decreased upon treatment with **JC-171**. These data also further support a critical role of NLRP3 and IL-1β in the induction of IL-17-producing T cells that mediate EAE.[13, 24] Notably, treatment with **JC-171** also reduced serum levels of IL-1β in EAE mice, but not the levels of TGF-β and IL-6, indicating the *in vivo* selectivity of **JC-171** on the NLRP3 inflammasome (Figure 3D). Given a well-documented role of IL-1β signaling in promoting the differentiation of Th17 cells,[30] we demonstrate that inhibition of IL-1β production via blocking activation of the NLRP3 inflammasome by **JC-171**, can significantly reduce a pathogenic Th17 response and alleviate the disease severity. While these data have clearly established the activity of **JC-171** in suppressing the activation of NLRP3 inflammasome as well as its downstream effects (e.g., Th17 response) in the context of EAE, future studies involving genetic manipulation of NLRP3 inflammasome are necessary for precisely delineating its contribution.

We next evaluated the therapeutic potency of **JC-171** by administration of the compound starting when the clinical scores of individual mice have reached 1 (flaccid tail). Despite the use of a low dose of **JC-171** (10 mg/kg), treatment of mice with **JC-171** efficiently suppressed EAE progression compared with vehicle treatment (Figure 4A). Notably, the therapeutic effect of **JC-171** treatment was quite durable and lasted throughout the chronic phase of EAE. **JC-171**

exhibited an *in vivo* therapeutic activity comparable with MCC950, a small molecular inhibitor for NLRP3 inflammasome recently reported to block EAE development.[31] Histologic analysis showed that, consistent with the reduced clinical score, myelin injury was reduced in the white matter of spinal cords from **JC-171**-treated EAE mice, indicated by Luxol fast blue staining (Figure 4B). Treatment with **JC-171** also resulted in a substantial decrease in the frequency of $MOG_{35–55}$-specific Th17 cells in the spleens and spinal cords of EAE mice (Figure 4C).

In summary, we rationally designed a hydroxyl-sulfonamide analog, **JC-171**, from parent compound **JC-21**. *In vitro* biological characterization using mouse J774A.1 macrophage and primary BMDMs demonstrated that **JC-171** is a selective inhibitor of the NLRP3 inflammasome, which is further supported by *in vivo* data from LPS-challenged mice. The inhibition potency of **JC-171** on IL-1β release by LPS/ATP stimulation in J774A.1 cells is comparable to the parent compound **JC-21**, thus indicating that structural modifications at the sulfonamide moiety can be tolerated. Furthermore, inhibition of the NLRP3 inflammasome by **JC-171** administration effectively blocks disease progression and ameliorates the clinical symptoms in EAE mice, which is associated with significantly reduced levels of IL-1β. Collectively, the results of **JC-171** together with that of **JC-21** suggest that this chemical scaffold represents a promising template to develop novel small molecule inhibitors for the NLRP3 inflammasome. These data also wanrrant further development of **JC-171** and analogs as potential agents for treatment of human MS as well as other inflammatory diseases involving NLRP3 inflamamsome.

## MATERIALS AND METHODS

### Chemical synthesis

Reagents and solvents were obtained from commercial suppliers and used as received unless otherwise indicated. Reactions were monitored by thin-layer chromatography (TLC) (precoated silica gel 60F254 plates, EMD Chemicals) and visualized with UV light or by treatment with phosphomolybdic acid (PMA). Flash chromatography was performed on silica gel (200-300 mesh, Fisher Scientific) using solvents as indicated. $^{1}$HNMR and $^{13}$CNMR spectra were routinely recorded on a Bruker ARX 400 spectrometer. The NMR solvent used was $CDCl_3$ or DMSO-*d*6 as indicated. Tetramethylsilane (TMS) was used as the internal standard.

5-chloro-2-methoxy-*N*-phenethylbenzamide (**3**). 5-Chloro-2-methoxybenzoic acid 1 (1 mmol) and trimethylamine ($Et_3N$, 2 mmol) were dissolved in dimethyl formaldehyde (DMF, 25 mL) and cooled to 0 ˚C. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 1.5 mmol) was added and after 30 min, hydroxybenzotriazole (HOBt, 1.5 mmol) was added. After 1 h, 2-phenylethanamine 2 (1 mmol) was added to the mixture and the reaction was allowed to warm to room temperature for overnight. The solution was concentrated under reduced pressure and $H_2O$ was added. The solution was extracted with ethyl acetate (EtOAc) and combined organic phase was concentrated and purified by column chromatography (EtOAc/Hexane 20/80) to give compound 3 as a viscous oil (yield: 82%). $^{1}$H NMR (400 MHz, DMSO-*d*6) δ 8.22 (br. s., 1H), 7.66 (br. s., 1H), 7.50 (dd, J = 3.00, 8.80 Hz, 1H), 7.32 (d, J = 7.22 Hz, 2H), 7.28 (d, J = 8.03 Hz, 2H), 7.20 - 7.24 (m, 1H), 7.16 (d, J = 9.46 Hz, 1H), 3.81 (s, 3H), 3.54 (q, J = 7.06 Hz, 2H), 2.85 (t, J = 6.20 Hz, 2H); $^{13}$C NMR (100 MHz, DMSO-*d*6) δ 163.4, 155.7, 139.4, 131.5, 129.6, 128.7, 128.3, 126.1, 124.7, 124.3, 114.1, 56.2, 40.7, 34.9.

4-(2-[(5-chloro-2-methoxyphenyl)formamido]ethyl)benzene-1-sulfonyl chloride (**4**). Compound **3** (1.73 mmol) was dissolved in methylene chloride (2 mL). To this, excess chlorosulfonic acid (1 mL) was added, and the solution stirred at 70 °C for 2 h. The reaction was cooled to room

temperature, and then poured over crushed ice. The product was extracted into methylene chloride, and then concentrated under reduced pressure. The product was purified by column chromatography (EtOAc/Hexanes: 20/80 to 50/50) yielding compound 4 as a white solid (yield: 65%). $^{1}$H NMR (400 MHz, DMSO-*d6*) δ 8.20 (br. s., 1H), 7.66 (s, 1H), 7.56 (d, J = 8.28 Hz, 2H), 7.50 (dd, J = 2.76, 8.78 Hz, 1H), 7.22 (d, J = 8.53 Hz, 2H), 7.16 (d, J = 9.04 Hz, 1H), 3.80 (s, 3H), 3.51 (q, J = 7.30 Hz, 2H), 2.84 (t, J = 6.80 Hz, 2H); $^{13}$C NMR (100 MHz, DMSO-d6) δ 163.5, 155.7, 146.1, 139.8, 131.5, 129.5, 128.0, 125.6, 124.7, 124.3, 114.2, 56.2, 40.6, 34.6.

5-chloro-N-(2-[4-(hydroxysulfamoyl)phenyl]ethyl)-2-methoxybenzamide (**JC-171**). Hydroxylamine (6.44 mmol) and N-methylmorpholine (0.2 mL) were dissolved in MeOH (2 mL). To this, compound 4 (1.29 mmol) was added, and the solution was stirred for 4 h. $H_2O$ was added, and the product was extracted into methylene chloride and concentrated. The product was purified by column chromatography (dichloromethane/methanol: 94/6) to give compound **JC-171** as a white solid (yield: 56%). $^{1}$H NMR (400 MHz, DMSO-*d6*) δ 9.56 (d, J = 3.26 Hz, 1H), 9.52 (d, J = 3.26 Hz, 1H), 8.27 (t, J = 5.40 Hz, 1H), 7.78 (d, J = 8.28 Hz, 2H), 7.64 (d, J = 2.76 Hz, 1H), 7.48 - 7.53 (m, 3H), 7.15 (d, J = 8.78 Hz, 1H), 3.81 (s, 3H), 3.55 (q, J = 6.70 Hz, 2H), 2.94 (t, J = 6.90 Hz, 2H); $^{13}$C NMR (100 MHz, DMSO-d6) δ 163.6, 155.7, 145.2, 135.3, 131.5, 129.5, 129.2, 128.2, 124.8, 124.3, 114.2, 56.2, 40.2, 34.7.

**Cell culture**

J774A.1 murine macrophage cells were purchased from Americal Type Cell Culture (ATCC), Manassas, VA and were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. Bone marrow cells from C57BL/6 mice was differentiated for 8 days in DMEM supplemented with 10% FBS, 1%

penicillin/streptomycin and 30% L929 mouse fibroblast conditioned media that contains macrophage colony-stimulating factor.

**Mice**

C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD). All experiments and procedures involving mice were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Antibodies and reagents**

Fluorochrome-conjugated mouse monoclonal antibodies (mAbs) for flow cytometry analysis of immune cells, including FITC–CD4 (GK1.5), Percp/Cy5.5–IL-17A (TC11-18H10.1), CD16/CD32 (2.4G2), isotype control rat IgG2b (RTK4530) and IgG1 (RTK2071) were purchased from BioLegend (San Diego, CA). Mouse TNF-α, IL-6 and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend. Mouse IL-1β ELISA kits was from eBioscience (San Diego, CA). Monoclonal antibodies for ASC (clone 2EI-7, 04-147, EMD Millipore, Billerica, MA), and NLRP3 (clone D4D8T, Cell Signaling Biotechnology) were purchased from commercial resources as indicated. Anti-NLRP3 (abcam, USA), anti-IL-1β (EMD Millipore, CA, USA), and anti-caspase-1 (p20 and p10) (Santa Cruz Biotechnology, Santa Cruz, USA) for Western blotting analysis were purchased from commercial resources as indicated and used per manufacturer's instruction.

**Cell viability assays**

J774A.1 cells were plated into a 96-well plate ($1 \times 10^5$ cells/well) for 24 h in growth medium. Cells were then treated with different concentrations of JC-171 for 0.5 h. After treatment, 3-(4.5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5% solution) was added. After incubation for 4 h, DMSO (100 μL) was added for 5 min. Absorbance at 570 nm was then immediately recorded using a FlexStation 3 plate reader.

**Cell death rescue assays**

J774A.1 cells were plated into a 96-well plate ($1 \times 10^5$ cells/well) for 24 h in growth medium. Cells were primed with *Escherichia coli* 0111:B4 LPS (Sigma-Aldrich) (final concentration: 1 μg/mL) for 4.5 h. Next, **JC-171** was added for 30 min. ATP (5 mM) was added at the same time when JC-171 was added to induce NLRP3 inflammasome formation, and cells were incubated another 30 min. Cell viability was measured by MTT assay as described above.

***In vitro* NLRP3 activation assays**

J774A.1 cells were primed and activated with LPS/ATP as described above. **JC-171** was added at the time when ATP was added for 30 min. The supernatants were collected and levels of IL-1β were measured with a mouse IL-1β Enzyme-linked Immunosorbent Assay (ELISA) kit following the manufacturer's instructions. BMDMs were similarly treated as described with J774.A1 cell line followed by ELISA analysis of IL-β production. To assess effect of **JC-171** on induction of cytokine TNF-α or IL-6, BMDMs were pretreated with **JC-171** (400 μM) for 2 h and then stimulated LPS (100 ng/mL) for 5.5 h, followed by ATP (5 mM) treatment for 30 min.

**Western blotting assays**

J774A.1 cells were seeded on 6-well plates ($2 \times 10^6$ cells/well) in RPMI 1640 medium for 24 h. Cells were then primed and activated with LPS/ATP and treated with JC-171 as described above. After treatment, cells were collected and wash with ice-cold PBS twice. Cells pellets were lysed

by sonication in a radioimmunoprecipitation (RIPA) buffer solution. Protein samples were quantified by the Bradford method. Equal amounts of protein (20.0 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Proteins were probed with primary antibodies overnight at 4 °C: anti-NLRP3 (1:800, abcam, USA), anti-IL1β (1:500, EMD Millipore, CA, USA), anti-caspase-1 (P20) (1:1000, Cell Signaling Technology, MA, USA), anti-caspase-1 (P10) (1:200, Santa Cruz Biotechnology, Santa Cruz, USA) and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated rabbit or mouse secondary antibodies (Cell Signaling Technology, USA). After washing three times in TBS-Tween 20 for 10 min, the proteins were visualized by employing chemiluminescent reagent (Thermo Fischer Scientific, Waltham, MA). The blots were also probed with antibodies against α-tubulin to ensure equal loading of proteins.

**Immunoprecipitation and immunoblotting**

BMDM cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in modified lysis buffer (50 mM Tris-Cl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml each of aprotinin and leupeptin, and 1 mM $Na_3VO_4$). 1 mg protein of cell extracts was incubated with 2 μg of anti-NLRP3 antibodies for 2 h at 4 °C, followed by incubation with 40 μl of Protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology, Inc.) overnight at 4 °C. The beads were washed with lysis buffer, and immune complexes were eluted by boiling in 2 × sodium dodecyl sulfate (SDS) Laemmli loading buffer for 5 min prior to immunoblotting analyses with indicated antibodies. For immunoblotting, the immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were immunoblotted with antibodies for

NLRP3 or ASC, followed by HRP-conjugated secondary antibodies. Specific bands were visualized using Pierce™ ECL Western Blotting Substrate from ThermoFisher Scientific (Waltham, MA). Band intensities of immunoprecipitated ASC or NLRP3 panel were quantified using Image Studio LITE (LI-COR biotechnology) software. The ratios of ASC/NLRP3 association were calculated with PBS control group set as 1.

**LPS challenge *in vivo* and JC-171 treatment**

C57BL/6 mice were injected intraperitoneally (i.p.) with 20 mg/kg LPS (Sigma-Aldrich) or PBS one hour after **JC-171** (100 mg/kg) or vehicle treatment. 2.5 h after LPS injection, serum levels of IL-1β and TNF-α were measured by ELISA.

**EAE induction and JC-171 treatment**

EAE was induced as we previously described. [32] Briefly, mice were immunized subcutaneously with 200 μg Myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (ProSpec, East Brunswick, NJ) emulsified in Complete Freund's Adjuvant (CFA) on day 0 followed by injection of 200 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO) i.p. on days 0 and 2. After the first immunization, the severity of EAE was monitored and graded daily in a blinded fashion on a scale of 0–5 as we previously described. For prophylactic treatment, **JC-171** was administered to mice (100 mg/kg, i.p.) on days 0, 1 and 2; and every other days thereafter. Therapeutic treatment with **JC-171** (10 mg/kg, i.p.) or MCC950 (Sigma-Aldrich, 10 mg/kg, i.p.) was initiated when the clinical scores of individual mice have reached 1 (flaccid tail), and given every other days. Upon termination of the experiment, mice were transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde. Spinal cords with vertebrae were removed. Paraffin-embedded sagittal sections of cervicothoracic spinal cord were stained with Luxol fast blue/periodic acid–Schiff to determine demyelination.

**Analyses of immune response in EAE mice**

Mononuclear cells (MNCs) were isolated from spinal cords of EAE mice using percoll gradient centrifugation after perfusion with PBS as we previously described. [32] MNCs were stimulated for 5 h in the presence of phorbol 12-myristate 13-acetate (10 nM; Sigma-Aldrich), ionomycin (1 μM; EMD Biosciences) with the addition of brefeldin A (5 μg/ml, Biolegend) during the last 3 hours of culture. After surface staining with anti-CD4 mAbs for 30 min at 4°C, cells were fixed, permeabilized, and stained with anti–IL-17A mAbs for 30 min at 4°C. For analysis of Th17 cells in lymphoid organs, cells were stimulated with $MOG_{35–55}$ (1 μg/ml) overnight at 37°C, 5% CO2 first and stained with the mAbs as decribed above. Data were acquired using BD FACSCalibur and analyzed using FlowJo software (Tree Star, Ashland, OR). Cytokine levels in the serum were assayed using the ELISA kits according to the manufacturer's instructions.

## AUTHOR INFORMATION

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Author contributions: Chemical synthesis was completed by J.F. and J.C.; Cellular assays with J774A.1 cells were completed by J.F.; Western blotting studies with J774A.1 cells were completed by Y.J.; Studies employing mouse BMDMs and immunoprecipitation study were completed by X.L.; Animal studies were completed by C.G.; Experiment design, data analysis, writing, and editing were completed by C.G.; J.F.; J.W.; X.Y.W.; and S.Z.

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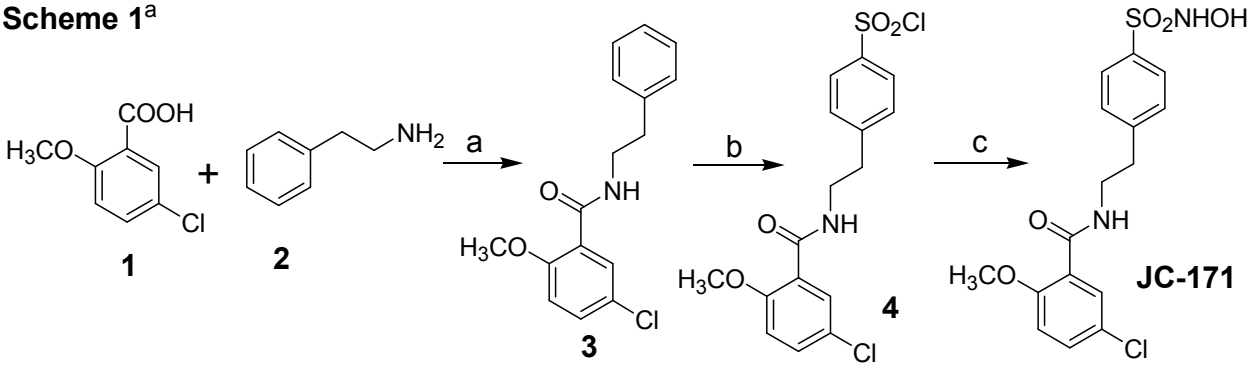
## ABBREVIATIONS

Acute myocardial infarction (AMI), apoptosis-associated speck-like protein containing a CARD (ASC), adenosine triphosphate (ATP), central nervous system (CNS), Dulbecco's Modified Eagle Medium (DMEM), dimethyl formaldehyde (DMF), experimental autoimmune encephalomyelitis (EAE), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), ethylenediaminetetraacetic acid (EDTA), Enzyme-linked Immunosorbent Assay (ELISA), ethyl acetate (EtOAc), fetal bovine serum (FBS), hydroxybenzotriazole (HOBt), immunoprecipitation (IP), interleukin-1β (IL-1β), intraperitoneally (i.p.), lipopolysaccharide (LPS), monoclonal antibodies (mAbs), mononuclear cells (MNCs), multiple sclerosis (MS), myelin oligodendrocyte glycoprotein (MOG), NOD-like receptor family pyrin domain containing 3 (NLRP3), phosphate-buffered saline (PBS), phosphomolybdic acid (PMA), phenylmethylsulfonyl fluoride (PMSF), polyvinylidene fluoride (PVDF), radioimmunoprecipitation (RIPA), sodium dodecyl sulfate (SDS), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), T-helper 17 (Th17), trimethylamine ($Et_3N$).

**Scheme 1**[a]




[a]Reagents and conditions: a) EDC, HOBt, $Et_3N$, methylene chloride; b) Chlorosulonic acid, methylene chloride; c) $NH_2OH$, N-methylmorpholine, MeOH.

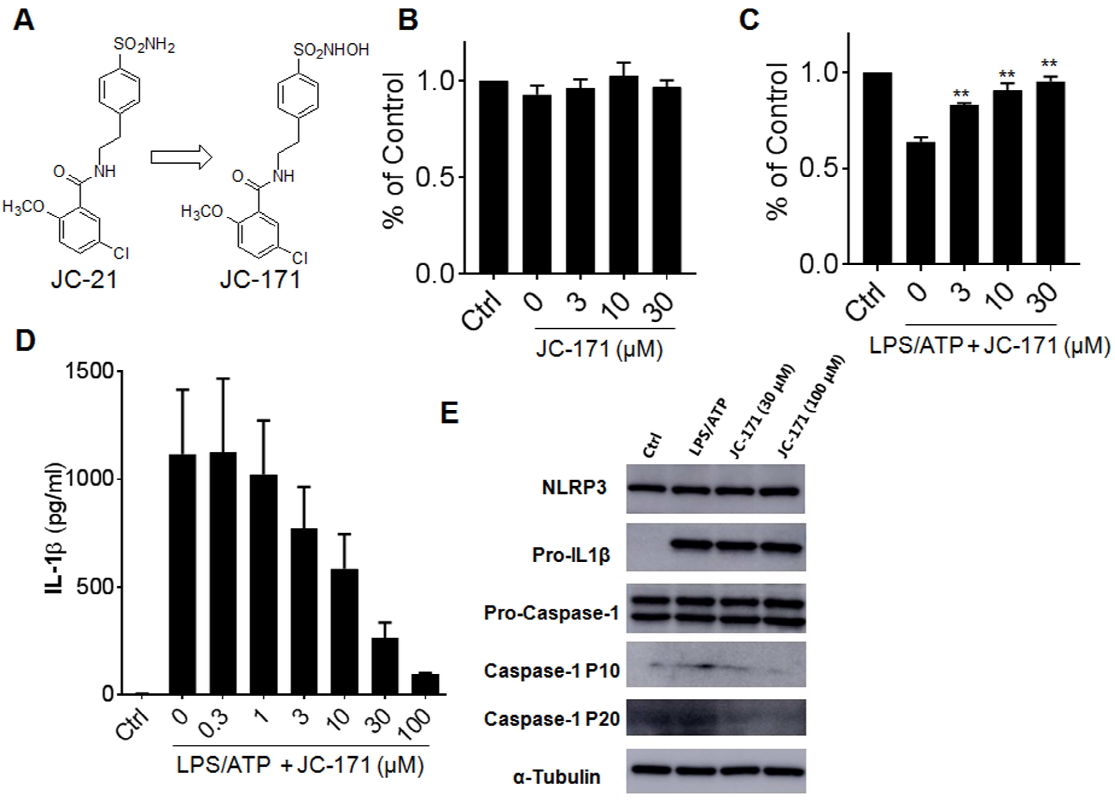



**Figure 1. JC-171 inhibits the release of IL-1β in J774A.1 cells upon stimulation with LPS/ATP.** Rational design of JC-171 (A). J774A.1 cells were treated with JC-171 at indicated concentrations in the absence (B) or presence (C) of LPS priming (1 μg/mL, 4.5 h) and ATP (5 mM, 30 min). JC-171 was added at the time of ATP addition. Cell viability was then measured

by MTT assay. J774A.1 cells were activated with LPS/ATP and treated with JC-171 as described above. Supernatant were collected and level of IL-1β was assayed with mouse ELISA kit per manufacture's instruction (D). J774A.1 cells were primed with LPS/ATP and treated with JC-171 as described above. Cells were collected and lysed and the lysates were analyzed by Western blotting with corresponding antibodies (The image represents the results from one of three independent experiments). Error bar represents SEM. **, $p < 0.01$

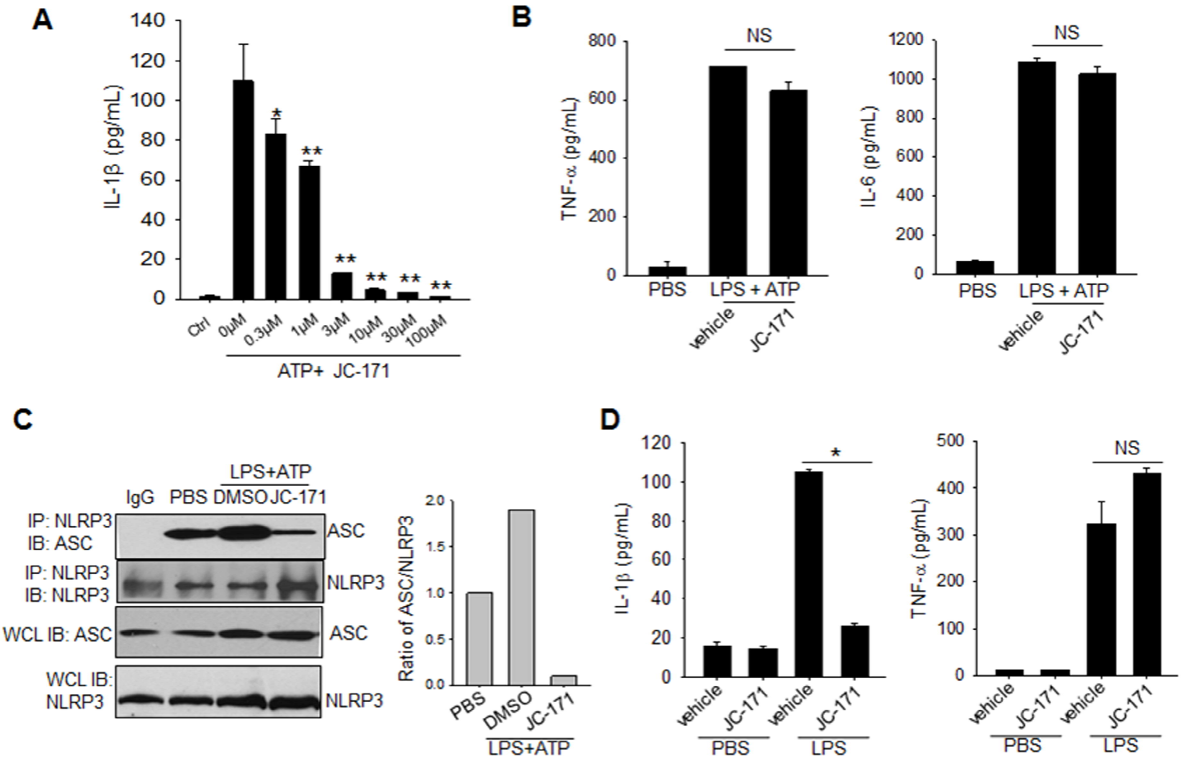



**Figure 2. JC-171 blocks NLRP3 inflammasome activation and IL-1β production in primary macrophages and LPS-challenged mice.** BMDMs were primed with LPS (1 μg/mL) for 4.5 h and then treated with indicated doses of JC-171 when adding ATP (5 mM) stimulation for 30 min. IL-β in the culture media was assayed by ELISA (A). JC-171 treatment has no effect on the production of TNF-α and IL-6. BMDMs were pre-treated with JC-171 (400 μM) for 2 h followed by stimulation with LPS and ATP. Cytokines in the culture media was assayed by ELISA (B). JC-171 interferes with association of NLRP3 and ASC during activation of NLRP3 inflammasome. Cells were also collected after LPS/ATP stimulation for IP assay using anti-

NLRP3 antibodies (C, left). Pull-downed immunocomplexes were subjected to immunoblotting with anti-ASC antibodies to detect association of ASC with NLRP3. Whole cell lysates were also used as input controls. The ratios of ASC and NLRP3 band intensities in the complex were quantified and calculated, with PBS control group set as 1 (C, right). Serum levels of IL-1β and TNF-α from C57BL/6 mice pretreated with JC-171 (100 mg/kg) or vehicle control were measured by ELISA 2.5 h after i.p. injection of LPS (20 mg/kg) (D). *, $p < 0.05$; **, $p < 0.01$; NS, not significant.

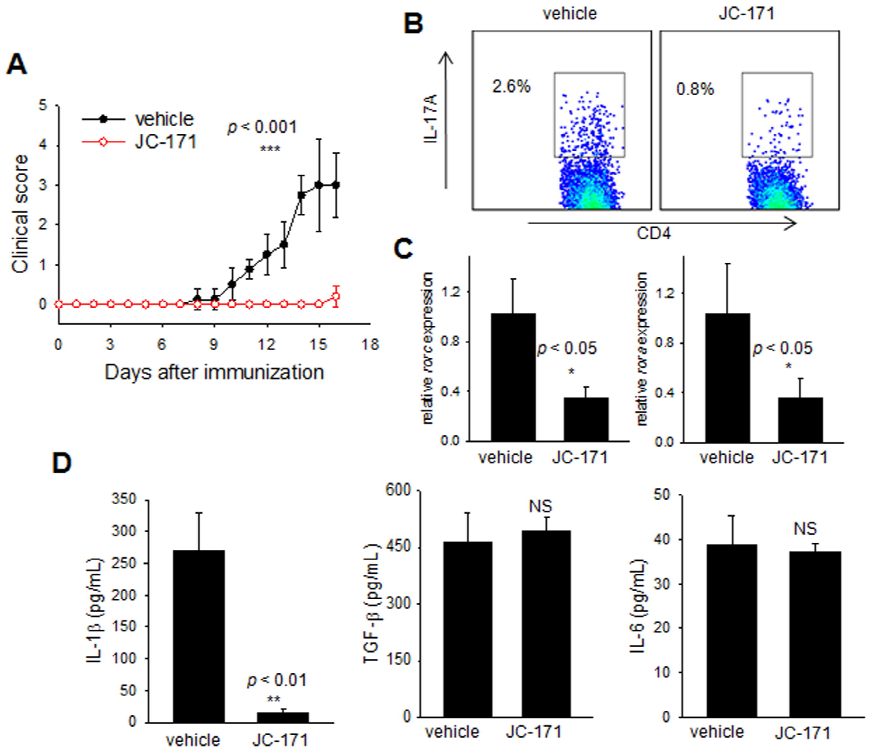



**Figure 3. Administration of JC-171 delays disease progression in EAE mice by blocking IL-1β production.** C57BL/6 mice (n = 5) were immunized with MOG peptide (200 μg) emulsified in CFA on day 0. JC-171 treatment (100 mg/kg) was started on day 0 for three consecutive doses followed by treatment on every other day. EAE development in mice was followed, and clinical scores were recorded (A). Lymph nodes were collected after the 10th treatment, the frequency of IL-17A$^+$CD4$^+$ Th17 cells was assayed by flow cytometry after stimulation with MOG peptide for 3 days (B). Transcription of rorc and rora genes in the spleen was determined by real-time PCR (C). IL-1β, TGF-β and IL-6 levels in the serum were assayed by ELISA on day 17 after immunization (D). NS, not significant

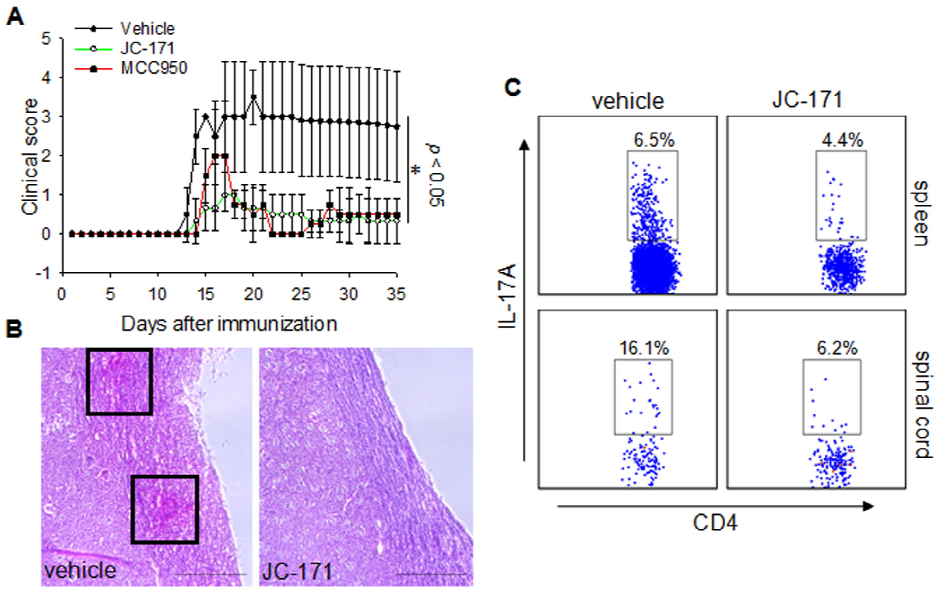



**Figure 4. Therapeutic JC-171 treatment reduces EAE severity and a Th17 response in EAE mice.** C57BL/6 mice (n = 5) were immunized with MOG peptide (200 μg) emulsified in CFA on day 0. JC-171 or MCC950 treatment (10 mg/kg) was started when the clinical scores of individual mice have reached 1 (flaccid tail) and continued every other day. EAE development in mice was followed, and clinical scores were recorded (A). Twenty-eight days after immunization, spinal cords were dissected from mice and subjected to Luxol fast blue/periodic acid–Schiff staining for analysis of demyelination (B). The frequency of IL-17A$^+$CD4$^+$ Th17 cells in the spleen and spinal cord was determined by flow cytometry (C). Bar=50 μm

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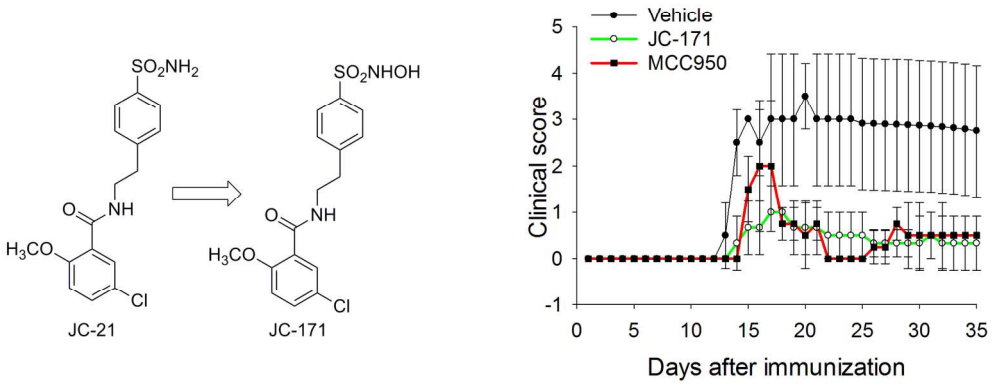


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