Pharmacology of a Potent and Novel Inhibitor of the NOD-like receptor pyrin domain-containing protein 3 (NLRP3) Inflammasome that Attenuates Development of NASH and Liver Fibrosis


Jecure Therapeutics, San Diego, CA, USA: DP, ML, CM, GAA, RS, AMS, RFP, JMV, JAS, GB

Department of Pediatrics, University of California San Diego (UCSD), La Jolla, CA, USA: CDJ, MDM, HMH, AEF

*These authors contributed equally to this work
Running Title Page

Running Title: Novel Inhibitor of NLRP3 Active in NASH Models

Corresponding Author:
Davide Povero, PhD
Assistant Professor
Division of Gastroenterology and Hepatology
Mayo Clinic
200 First Street SW
Rochester, MN, USA
Phone: 507-773-9097
povero.davide@mayo.edu

Number text pages: 39
Number of tables: 2
Number of figures: 7
Number of references: 52
Number of words in Abstract: 237
Number of words in Introduction: 705
Number of words in Discussion: 1507

Abbreviations: AMLN, Amylin diet; LPS, Lipopolysaccharide; PBMC, Peripheral Mononuclear Blood Cells; KC, Kupffer cells; NLR, Nucleotide-binding Domain and Leucine-rich Repeat Containing; Nucleotide-binding domain (NOD)-like receptor protein 3; ASC, Apoptosis-Associated Speck-Like Protein Containing CARD; NASH, non-alcoholic steatohepatitis; NAFL(D), non-alcoholic fatty liver (disease); NAS, NASH activity score; CVD, cardiovascular disease; HSC, hepatic stellate cell; TLR, toll-like receptor; MSU, monosodium urate; CHC, cholesterol crystals; PBS, Phosphate buffer saline; DMSO, dimethyl sulfoxide; MPO, Myeloperoxidase; ALT, Alanine aminotransferase; αSMA, Alpha-smooth muscle active;
QD, quaque die; p.o., per os; H&E, Hematoxylin-eosin; DIO, Diet-induced obesity; CDAA, Choline-deficient, l-amino acid-defined; CSAA, Choline-sufficient, l-amino acid-defined; PSR, Picrosirius red; CTGF, Connective tissue growth factor; AST, Aspartate aminotransferase; TG, Triglycerides; TC, Total cholesterol; AUC, Area under the curve; PK, Pharmacokinetics; PD, Pharmacodynamics; mBMDM, mouse bone marrow-derived macrophages; MWS, Muckle-Wells syndrome; CAPS, cryopyrin-associated periodic syndromes; DILI, drug-induced liver injury; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

**Recommended Section:** Drug Discovery and Translational Medicine
Abstract

The NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome is a multiprotein complex and component of the innate immune system that is activated by exogenous and endogenous danger signals to promote activation of caspase-1 and the maturation and release of the pro-inflammatory cytokines IL-1β and IL-18. Inappropriate activation of NLRP3 has been implicated in the pathophysiology of multiple inflammatory and autoimmune diseases, including cardiovascular disease, neurodegenerative diseases and nonalcoholic steatohepatitis (NASH), thus increasing the clinical interest of this target. We describe in this study the preclinical pharmacologic, pharmacokinetic and pharmacodynamic properties of a novel and highly specific NLRP3 inhibitor, JT001 (6,7-dihydro-5H-pyrazolo[5,1-b][1,3]oxazine-3-sulfonylurea). In cell-based assays, JT001 potently and selectively inhibited NLRP3 inflammasome assembly resulting in the inhibition of cytokine release and the prevention of pyroptosis, a form of inflammatory cell death triggered by active caspase-1. Oral administration of JT001 to mice inhibited IL-1β production in peritoneal lavage fluid at plasma concentrations that correlated with mouse in vitro whole blood potency. Orally administered JT001 was effective in reducing hepatic inflammation in three different murine models, including the Nlrp3A350V/+CreT model of Muckle-Wells syndrome (MWS), a DIO NASH model, and a choline-deficient diet-induced NASH model. Significant reductions in hepatic fibrosis and cell damage were also observed in the MWS and choline-deficient models. Our findings demonstrate that blockade of NLRP3 attenuates hepatic inflammation and fibrosis and support the use of JT001 to investigate the role of NLRP3 in other inflammatory disease models.
Significance Statement

Persistent inflammasome activation is the consequence of inherited mutations of NLRP3 and results in the development of cryopyrin-associated periodic syndromes associated with severe systemic inflammation. NLRP3 is also upregulated in NASH, a metabolic chronic liver disease currently missing a cure. Selective and potent inhibitors of NLRP3 hold great promise and have the potential to overcome an urgent unmet need.
Introduction

Cells of the innate immune system, including macrophages and granulocytes, play critical roles in initiating inflammation in response to invading pathogens or signals of cellular stress/damage (Shen et al., 2013; Peiseler and Kubes, 2018). This inflammatory response can be triggered by activation of members of the Nucleotide-binding Domain and Leucine-rich Repeat Containing (NLR) family of receptors that, upon sensing specific stimuli, assemble cytosolic multiprotein complexes termed inflammasomes. Inflammasomes are molecular platforms for the auto-catalytic conversion of procaspase-1 to its active form, which then proteolytically processes pro-IL-1$\beta$ and pro-IL-18 resulting in the release of these potent proinflammatory cytokines (Schroder and Tschopp, 2010; Lamkanfi and Dixit, 2014). Active caspase-1 also cleaves the intracellular protein gasdermin D. Following cleavage, N-terminal fragments of gasdermin D oligomerize and form pores in the plasma membrane, thereby initiating a form of inflammatory cell death termed pyroptosis (He et al., 2015; Man and Kanneganti, 2015; Shi et al., 2015). This membrane pore formation and subsequent cell lysis results in the release of mature IL-1$\beta$ and IL-18 and other soluble mediators, such as IL-1$\alpha$.

NLRP3 is unique in its ability to be activated by diverse stimuli including both pathogen-associated molecules and sterile inflammatory signals. Canonical NLRP3 activation occurs in a two-step process, with the first priming step occurring following toll-like receptor (TLR) activation and signaling. This priming step promotes transcriptional upregulation of NLRP3 and post-translational modifications required for maximal activation (He et al., 2016; Patel et al., 2017; Yang et al., 2019). A second signal leads to activation of NLRP3 and can be provided by pathogen-derived molecules, such as the bacterial pore-forming toxin nigericin; crystalline or...
particulate matter, such as silica or asbestos; or endogenous danger ligands such as extracellular ATP, monosodium urate (MSU) crystals, cholesterol crystals (CHC), β-amyloid aggregates or oxidized mitochondrial DNA (Groslambert and Py, 2018; Yang et al., 2019). Active NLRP3 oligomerizes and in turn recruits and nucleates the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) to form discrete prion-like foci referred to as ASC specks. Through their respective CARD domains, ASC then binds procaspase-1 to form the active inflammasome complex (Yang et al., 2019).

Non-canonical activation of NLRP3 occurs downstream of gasdermin D cleavage through a distinct pathway that involves detection of intracellular LPS by caspase-4, -5 (caspase-11 in mouse) and subsequent cleavage of gasdermin D and pore formation (Baker et al., 2015; Kayagaki et al., 2015; Ruhl and Broz, 2015; Schmid-Burgk et al., 2015). Pore formation results in potassium efflux which promotes non-canonical NLRP3 activation and inflammasome formation (Ruhl and Broz, 2015). More recently, a third mechanism for NLRP3 activation, termed the alternative pathway, has been described. The alternative pathway is activated in human monocytes, but not macrophages or mouse monocytes, by TLR4 stimulation alone and propagated by TRIF-RIPK1-FADD-caspase-8 signaling to activate NLRP3 by an unknown mechanism (Gaidt et al., 2016). Of note, although mature IL-1β is released, activation of the alternative pathway occurs in the absence of pyroptosis and ASC speck formation and is independent of potassium efflux (Gaidt and Hornung, 2017).

The relevance of inappropriately activated NLRP3 to human disease is highlighted in patients with inherited hyperactivating mutations of NLRP3. Patients with NLRP3 gain-of-function mutations develop systemic auto-inflammatory syndromes termed cryopyrin-associated periodic syndromes (CAPS) that are characterized by recurrent fever, rash, and arthralgia.
(Hoffman et al., 2001). More recent studies have implicated persistent NLRP3 activation in the pathophysiology of multiple inflammatory/autoimmune diseases, including cardiovascular disease, inflammatory bowel disease, neurodegenerative diseases and nonalcoholic steatohepatitis (NASH) (Mangan et al., 2018).

NASH is a progressive liver disease caused by the accumulation of fat in the liver and characterized by lobular inflammation, hepatocyte damage/cell death (ballooning hepatocytes) and varying degrees of fibrosis (Friedman et al., 2018). In liver samples from patients with NASH, NLRP3 and other inflammasome components are increased relative to controls or to patients with non-NASH fatty liver and correlate with severity of fibrosis (Wree et al., 2014b). NLRP3 is activated by fatty acid and cholesterol crystals in Kupffer cells, the resident liver macrophages, thus providing a link between NLRP3 and NASH development (Mridha et al., 2017; Pan et al., 2018). Persistent NLRP3 activation has been shown to promote progression to NASH in a choline-deficient diet-induced mouse model, whereas Nlrp3 knockout mice were protected from diet-induced liver injury, hepatic inflammation and fibrosis (Wree et al., 2014b).

MCC950, a small-molecule inhibitor of NLRP3, partially reduced hepatic inflammation and fibrosis in two additional mouse models of NASH (Mridha et al., 2017).

In this study, we describe the preclinical pharmacologic, pharmacokinetic and pharmacodynamic properties of JT001, a novel, potent, and selective inhibitor of NLRP3 inflammasome assembly and activation. JT001 demonstrated prolonged inhibition of IL-1β production after oral administration to mice and robust anti-inflammatory activity in a mouse model of CAPS. When dosed therapeutically, JT001 treatment significantly attenuated hepatic inflammation in two different mouse models of NASH with additional effects in reducing markers of fibrosis.
MATERIALS AND METHODS

Reagents

JT001 was synthesized at Jecure Therapeutics (San Diego, CA, USA). Emricasan (IDN-6556) was purchased from Selleckchem (Houston, TX, USA) and TAK-242 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nigericin, lipopolysaccharide (LPS) E. Coli O26:B6, Poly(dA:dT) and ATP were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flagellin (FLA-BS Ultrapure), MSU, L18-MDP muramyl-dipeptide, and PAM3CSK4 were purchased from Invivogen (San Diego, CA, USA). FuGENE HD and CellTiter-Glo luminescent cell viability assay were purchased from Promega (Madison, WI, USA). The following primary antibodies were used: anti-ASC mouse monoclonal antibody (clone 2EI-7, Millipore-Sigma, St. Louis, MO, USA), anti-MPO rabbit polyclonal antibody (ThermoFisher, Waltham, MA, USA), anti-procaspase-1 + p10 + p12 rabbit monoclonal (clone EPR16883, Abcam, Cambridge, UK), anti-αSMA rabbit polyclonal antibody (clone EPR5368, Abcam, Cambridge, UK), anti-NLRP3 mouse monoclonal (clone Cryo-2, Adipogen, San Diego, CA, USA) and anti-β-actin mouse monoclonal (clone AC-74, Millipore-Sigma, St. Louis, MO, USA). Secondary antibodies included goat anti-mouse IgG Alexa Fluor-488 (Life Technologies, Carlsbad, CA, USA) and horse-radish peroxidase-conjugated secondary antibodies (Azure Biosystems, Dublin, CA, USA).

Animal Care

All animal procedures were performed in accordance with the national guidelines for the care and use of laboratory animals and approved by the local institutional animal care and use
committee (IACUC). Male 5- to 8-wk-old C57BL/6 mice were obtained from Charles River Laboratories (Hollister, CA, USA), Taconic (Rensselaer, NY, USA) or Janvier Labs (Le Genest Saint Isle, France). Animals were given free access to food and water and were maintained on a 12-hour light/dark schedule.

**Synthesis of sodium (**6,7-dihydro-5H-pyrazolo[5,1-b][1,3]oxazin-3-yl)sulfonyl)((1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl)amide (JT001)**

To a solution of 1,2,3,5,6,7-hexahydro-s-indacen-4-ylamine (15.0 g, 87 mmol) and triethyl amine (13.3 mL, 95.4 mmol) in tetrahydrofuran (300 mL) was added triphosgene (8.5 g, 28.6 mmol) in one portion at 0–5°C and the mixture was stirred at 70°C under N₂ for 1 hour. The reaction mixture was then filtered through diatomite and the filter cake washed with 30 mL petroleum ether (PE). The filtrate was concentrated to dryness and dissolved in 100 mL n-hexane and the mixture filtered through a silica gel pad. The filtrate was concentrated to dryness to give the 4-isocyanato-1,2,3,5,6,7-hexahydro-s-indacene (14.6 g, yield: 84%) as a pink oil. The suspension of 6,7-dihydro-5H-pyrazolo[5,1-b][1,3]oxazine-3-sulfonic acid amide (14.3 g, 70.4 mmol) in methanol (500 mL) was stirred at 80°C until getting a clear solution, then sodium methoxide (3.8 g, 70.4 mmol) was added and the mixture stirred for 5 min. The solution was concentrated to dryness and the residue co-evaporated with acetonitrile (100 mL). The residual solid was suspended in acetonitrile (320 mL) and 4-isocyanato-1,2,3,5,6,7-hexahydro-s-indacene (14.6 g, 73.3 mmol) was added. The mixture was stirred for 16 hours at room temperature and filtered. The filter cake was triturated with ethanol (250 mL), PE/ethyl acetate (5/1, 250 mL) to give the product. The product was dissolved in H₂O (200 mL) and concentrated to dryness to give sodium
((6,7-dihydro-5H-pyrazolo[5,1-b][1,3]oxazin-3-yl)sulfonyl)((1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl)amide (24.5 g, yield: 82%) as a white solid.

Primary Cells

Human primary cryopreserved Kupffer cells (KCs) were provided by Samsara Sciences (San Diego, CA, USA). Mouse bone-marrow derived macrophages (mBMDMs) were isolated and differentiated at iXCells Biotechnologies (San Diego, CA, USA) using bone-marrow from 6- to 8-week-old male C57BL/6 mice and culturing monocytes in medium containing 150 ng/mL Macrophage-Colony Stimulating Factor (M-CSF). Human primary cryopreserved peripheral blood mononuclear cells (PBMCs) were provided by iXCells (San Diego, CA, USA). Cryopreserved mouse, rat, and cynomolgus macaque (non-human primate - nhp) PBMCs were obtained from IQ Biosciences (Berkeley, CA, USA) or BioIVT (Westbury, NY, USA).

NLRP3 Inflammasome Activation Assays

KCs were thawed on the day of the assay and seeded in RPMI 1640 medium with GlutaMAX and supplemented with 10 mM HEPES in poly-D-lysine-coated, black 384-well tissue culture plates at 4x10^4 cells/well. The cells were incubated for 30 minutes at 37°C, 5% CO₂ prior to assaying. PBMCs were thawed the day before the assay and seeded in complete medium (RPMI 1640 medium with GlutaMAX, supplemented with an additional 2.5 g/L D-glucose, 10% Fetal Bovine Serum, 100 mM Sodium Pyruvate, 1% Penicillin/Streptomycin, 10 mM HEPES and 0.05 mM β-mercaptoethanol) in polystyrene V-bottom 96-well plates at 5x10^4 cells per well and incubated overnight at 37°C with 5% CO₂. The next day, PBMC media was replaced with serum-free media prior to assaying. Differentiated mBMDMs were harvested at iXCells and
plated in DMEM/F12 (1:1) with L-glutamine and 100 ng/mL M-CSF at 1x10^4 cells/mL in 384-well tissue culture plates. The cells were incubated overnight prior to delivery to Jecure and used within 3 days. On the day of the assay, the media was replaced with DMEM/F12 (1:1) media with L-glutamine. For screening assays, JT001 or vehicle was added to each well and incubated for 30 minutes at 37°C, 5% CO₂. To activate the canonical NLRP3 inflammasome pathway, cells were primed by adding 100 ng/mL LPS and incubating 4 h, followed by addition of 10 μM Nigericin or 5 mM ATP and incubating an additional 1.5 h or 1 h, respectively. For activation using cholesterol crystals (CHC) or monosodium urate crystals (MSU), mBMDMs were primed for 2-3 hours with 100 ng/mL LPS followed by 1 mg/mL CHC or 300 μg/mL MSU for 6 hours. To activate the noncanonical NLRP3 pathway, mBMDMs were primed with 100 ng/mL PAM3CSK4 for 4 h followed by transfection of 2 μg/mL LPS in OptiMEM (ThermoFisher) containing 0.25% FuGENE HD and incubation overnight. At the end of the incubation period, plates were briefly centrifuged and half the cell culture supernatant removed and stored at -80°C for subsequent cytokine analysis. Cell viability was determined by addition of an equal volume of CellTiter-Glo® 2.0 reagent to the remaining cells/supernatant in each well. Intracellular Caspase-1 activity was measured using the Caspase-Glo® 1 Assay (Promega) according to the manufacturer’s instructions.

**Preparation of Cholesterol Crystals**

Preparation of cholesterol crystals was adapted from Rajamaki et al. (Rajamaki et al., 2010). Briefly, cholesterol was dissolved in 95% ethanol at 12.5 g/L and heated to 60°C. Following filtration through Whatman filter paper, the solution was left to cool at room temperature to allow crystallization. The crystals were collected by filtering and dried under vacuum before
grinding using a mortar and pestle. Crystals were stored at -20°C and were resuspended to 8 mg/mL in assay media prior to use.

**NLRC4, AIM2 and NLRP1 Inflammasomes Activation Assays**

mBMDMs plated as above in 384-well plates were treated with JT001 or vehicle and incubated for 30 minutes prior to priming and stimulation. Cells were primed with 100 ng/mL of LPS for 3 h. For AIM2 activation, cells were stimulated for 3 h with 2 µg/mL poly(dA:dT), prepared in OptiMEM and combined with Lipofectamine 2000 (ThermoFisher). For NLRC4 inflammasome activation, cells were incubated for 3 h with 0.5 µg/mL Flagellin, prepared in OptiMEM and combined with Lipofectamine 2000. For NLRP1 activation, mBMDMs from Nlrp3−/− knockout mice (kindly provided by Prof. Hal Hoffman, UCSD, San Diego, CA, USA) were generated at iXCells as described. After priming Nlrp3−/− mBMDMs with 100 ng/mL of LPS for 3-5 h, cells were exposed to 100 ng/mL L18-MDP and incubated overnight. At the end of each treatment, plates were briefly centrifuged and the cell culture supernatant removed and stored at -80°C for subsequent cytokine analysis.

**Whole Blood Assays**

Human blood, obtained by venipuncture from consenting adult volunteers, was collected into heparin tubes and used within 1 hour of draw. Mouse blood, obtained by cardiac puncture, was collected into heparin tubes, pooled and used within 1 hour of draw. Blood (198 µL) was first added to 96-well polypropylene plates, treated with JT001 or vehicle, and primed with 100 ng/mL LPS at 37°C for 3 h. The NLRP3 canonical pathway was activated by subsequent addition of 1 mM ATP (human blood) or 3 mM ATP (mouse blood) for 1 h. To activate the
NLRP3 alternative pathway, 100 ng/mL of LPS was added to the human blood and incubated at 37°C for 6 h. After incubation, plates were centrifuged and plasma removed and stored at -80°C for subsequent cytokine analysis.

**Cytokine determinations**

Human IL-1β, IL-1α, and TNFα and mouse IL-1β and TNFα, were measured in cell culture supernatants using homogeneous time resolved fluorescence assay kits (Cisbio, Codolet, France). Human IL-18 was measured in cell culture supernatants using an ELISA kit (R&D Systems, Minneapolis, MN, USA). Human and mouse IL-1β were measured in blood plasma using ELISA kits (R&D Systems, Minneapolis, MN, USA).

**Protein Isolation and Western blot**

Mouse BMDMs were treated as described in the NLRP3 inflammasome activation assays. An aliquot of supernatant was reserved and treated with NuPAGE LDS sample buffer for analysis by Western blotting. Cells were solubilized with sample buffer, then frozen at -20°C. Cells were thawed and the plate shaken at 700 rpm for 1 h at room temperature. Clarified cell lysates and supernatants were heated at 95°C for 5 minutes and resolved on a NuPAGE 4-12% Bis-Tris Gel. Proteins were transferred to a PVDF membrane and probed for NLRP3 and pro-caspase-1 and β-actin. Bands were visualized with the Azure Biosystems c300 Imaging System (Azure Biosystems, Dublin, CA, USA) using horse-radish peroxidase conjugated secondary antibodies.

**ASC speck assay in mouse macrophage**
Mouse BMDMs (2.5x10⁴ cells/well) were plated in Collagen-IV coated 96-well plates at iXCells. On the day of the assay, cells were washed with serum-free media then primed with 100 ng/mL LPS for 3 h in DMEM/F12 (1:1) with L-glutamine. JT001 or vehicle was added to each well and incubated at 37°C for 30 minutes prior to stimulation with 10 μM Nigericin for 1.5h. Cells were briefly centrifuged, rinsed with phosphate-buffered saline (PBS), and fixed with 4% formaldehyde in PBS overnight at 4°C. Cells were blocked/permeabilized in PBS with 10% goat serum, 1% fetal bovine serum, 0.5% Triton-X100 for 30 minutes at 37°C and incubated with ASC antibody overnight at 4°C. Cells were incubated with anti-mouse IgG Alexa Fluor-488 for 1 h at 37°C. Nuclei were stained with Hoescht in PBS for 5 minutes and imaged using a CellInsight CX7 High-Content Screening Platform (ThermoFisher) at Phenovista Biosciences (San Diego, CA, USA). The percentage of cells with ASC specks was quantitated.

**Plasma and Liver Pharmacokinetic Analysis**

Normal female C57BL6 mice (n=3/time point), female Sprague-Dawley rats (n=3) and male C57BL6 mice fed AMLN diet for 42 weeks (n=3) were administered a single dose of 10 mg/kg JT001 sodium salt orally (p.o.) in a vehicle of 0.5% Methylcellulose, 0.25% Tween-80, in 50 mM NaH₂PO₄/Na₂HPO₄ at pH 8.0. Blood samples were collected into K₂EDTA vacutainer tubes at various time points post-dosing by cardiac puncture and plasma prepared by centrifugation. Livers were removed and flash frozen prior to homogenization in 75% isopropyl alcohol. Analysis of JT001 plasma and liver concentrations was performed using liquid chromatography with mass spectrometry (LC-MS) as follows. Known concentrations of JT001 (0.5-5000 ng/mL) were added to naïve mouse plasma or liver homogenate to generate a standard curve. A sample of plasma or liver homogenate from the dosed animals (50 μL) was precipitated with 200 μL
acetonitrile containing tolbutamide as an internal standard and centrifuged at 4000g for 10 minutes at 4°C. The supernatant was injected with a Waters Autosampler into a Waters Acquity H-Class system (Waters, Milford, MA, USA) containing a Phenomenex Kinetex C18 column (Phenomenex, Torrance, CA USA) with elution performed at a gradient of 0.6 mL/min using 0.1% formic acid (Mobile Phase A) and 0.1% formic acid in acetonitrile (Mobile Phase B). Mass spectrometry was performed using an AB Sciex API5500 system (SCIEX, Concord, Ontario, Canada) and data were analyzed using the non-compartmental model of WinNonlin Phoenix 64 (Pharsight Inc. Mountain View, CA). Area under the plasma concentration-time curve (AUC) from time zero to the last measured concentration was estimated by the linear trapezoidal rule up to Cmax (maximum observed plasma concentration), followed by the log trapezoidal rule for the remainder of the curve.

**In vivo Pharmacodynamic Evaluation**

For the dose response study, groups of female C57BL/6, 8-wk-old mice (Taconic Biosciences) were dosed once orally with PBS (n=8) or 0.5-5 mg/kg JT001 sodium salt (n=8/dose group) in PBS one hour prior to the intraperitoneal (i.p.) administration of 1 µg of LPS in 0.5 mL of PBS. For the time course study, groups of mice were administered either single doses of JT001 at 30 mg/kg at 1, 6, 12 or 22 hours prior to i.p. injection of LPS or vehicle (0.5% methylcellulose) at 1 hour prior to injection of LPS. For both the dose-response and time-course studies, two hours after the administration of LPS, the mice were injected i.p. with 0.5 mL of 80 mM ATP disodium salt, pH adjusted to 7.2. At 30 minutes post the ATP injection, the mice were euthanized via CO2 inhalation and blood collected into K$_2$EDTA tubes by retro-orbital bleeding and plasma prepared for determination of JT001 concentrations as described above. Immediately after blood
collection, the peritoneal cavity was lavaged with 3 mL ice-cold PBS containing 25 U/mL heparin sodium salt, Complete ULTRA protease inhibitor tablet and 10% fetal bovine serum. Approximately 1 mL of the lavage fluid was centrifuged to remove cells and debris and stored at -80°C for subsequent determination of IL-1β concentrations using the Becton Dickinson CBA analysis kit according to the manufacturer’s protocol. The differences in mean IL-1β production were evaluated using one-way analysis of variance (ANOVA) followed by Holm-Sidak’s multiple comparisons test and were considered significant at a calculated probability (p) ≤ 0.05.

*Nlrcp3*A350V/+CreT knock-in model of MWS

*Nlrcp3*A350V/+ CreT knock-in mice were provided by Hal M. Hoffman, University of California, San Diego, USA. These mice carry a transgenic tamoxifen-inducible Cre and an intronic floxed neomycin-resistance gene (NeoR) that prevents the expression of *Nlrcp3*A350V/+ in the absence of Cre expression (McGeough et al., 2012). The study was performed using two randomized groups of male and female *Nlrcp3*A350V/+ CreT mice (n=3-4/group) ranging from 6- to 8-wks of age at the start of the study. Vehicle (n=3) or JT001 sodium salt (n=4) at 30 mg/kg in phosphate-buffered saline was administered orally once daily for 30 days starting on Day 1. Tamoxifen (free base) (MP Biomedicals, Solon, OH, USA) was prepared in 90% sunflower seed oil and 10% ethanol and administered at 50 mg/kg i.p. daily for 4 consecutive days, starting on Day 2, followed by a booster injection on Day 17. Mice were weighed every day. One mouse in the vehicle treatment group was found dead on Day 21 of the study. All animals in the JT001 treatment group survived until study termination. On Day 29, a submandibular cheek bleed was performed to obtain whole blood for total blood cell counting. Mice were euthanized on Day 30 and blood and livers were harvested and stored appropriately. Tissue specimens were fixed in 10% formalin and
subsequently embedded in paraffin for histopathological assessments, or snap frozen in liquid N₂ and stored in low temperature freezers. Complete blood counts and differentials were conducted on a HemaVet Auto Blood Analyzer (Drew Scientific, Miami Lakes, FL, USA). In vitro quantitative determination of alanine aminotransferase (ALT) in serum was performed using the Infinity™ ALT (GPT) Liquid Stable Reagent (cat. n. TR71121, Fisher Diagnostics, Middletown, VA, USA). Immunohistochemistry for hepatic mouse myeloperoxidase (MPO) and alpha-smooth muscle actin (α-SMA), TUNEL staining and PSR staining was performed as described previously (Wree et al., 2014b).

**Diet-Induced Obesity (DIO) Model of NASH**

Mice for the diet-induced obesity (DIO) murine model of NASH were fed a diet high in trans-fat (40%), fructose (20%) and cholesterol (2%) referred to as the Amylin (AMLN) diet. The study was conducted at Gubra Aps (Hørsholm, Denmark) using male C57BL/6J 5-wk-old mice (Janvier Labs, Le Genest-Saint-Isle, France) fed the AMLN diet *ad libitum* for 40 weeks. At week 37, all animals underwent baseline liver biopsies as previously described and nonalcoholic fatty liver disease activity score (NAS) and fibrosis stage scores were determined from the biopsies by histological analyses following hematoxylin and eosin (H&E) staining and Picrosirius red staining (PSR), respectively (Kristiansen et al., 2016). Only mice with fibrosis stage ≥ 1 and steatosis score ≥ 2 were included in the treatment phase of the study. Compound treatments were initiated at the beginning of week 41, with one group of mice (n=12/group) receiving 10 mg/kg JT001 sodium salt, a second group receiving 30 mg/kg JT001 sodium salt and the third group receiving vehicle (0.5% methylcellulose). Mice were dosed p.o. once daily for 10 weeks while maintaining the DIO NASH diet feeding. Mice were weighed every day...
during treatment period and food intake was monitored. At study termination, blood was drawn by cardiac puncture under isoflurane anesthesia and plasma prepared for analysis of alanine aminotransferase (ALT), triglycerides (TG) and total cholesterol (TC) as described previously (Kristiansen et al., 2016). Livers were harvested and fixed in 10% formalin and subsequently embedded in paraffin for histopathological assessments and immunohistochemistry for MPO as described previously (Wree et al., 2014a).

**CDAA-Induced Murine Model of NASH**

C57BL/6 7- to 8-wk-old male mice (Jackson Labs, Bar Harbor, ME, USA) were fed a choline-deficient L-amino acid-defined diet (CDAA) diet (cat. no. 518753, Dyets, Inc., Bethlehem, PA, USA) or the corresponding control choline-supplemented L-amino acid-defined diet (CSAA, cat. n. 518754, Dyets, Inc., Bethlehem, PA, USA) ad libitum for 15 weeks. After 15 weeks the animals on each diet were weighed and randomized into two groups (n = 10/group). Either vehicle or JT001 sodium salt (30 mg/kg) was administered by oral gavage in 0.5% methylcellulose once daily to groups on both diets for the last 5 weeks of the 20-week study. At study termination, mice were euthanized after 4 h of fasting with a ketamine/xylazine cocktail, and blood was collected into serum separator tubes by cardiac puncture. The tubes were allowed to sit at room temperature for 30 minutes before centrifuging at 2,000 x g for 10 minutes at 4°C. The serum was stored at -80°C prior to analysis of liver enzymes. Serum values of alanine aminotransferase (ALT) were measured at the end of the study according to the manufacturer’s instruction (Infinity™ ALT, ThermoFisher). After blood collection, livers were harvested, weighed and different lobes either fixed in 10% neutral-buffered formalin overnight, placed in RNALater® solution (ThermoFisher), or snap frozen in liquid N₂ for analysis of hepatic
hydroxyproline content. The differences in response between the disease control group (CDAA/Veh) and the non-disease control group (CSAA/Veh) and between the disease control group and disease treatment group (CDAA/JT001) was analyzed using Welch’s unequal variances t-test and were considered significant at a calculated probability (p)≤0.05. For each bar graph, the CDAA/Veh group mean was considered the maximum signal and the CSAA/Veh group mean considered the baseline signal. The mean value from the CDAA/JT001 group was normalized to the control groups to determine % inhibition.

**Histological Analyses**

For the CDAA efficacy study, liver tissue was fixed in 10% neutral-buffered formalin for 24 h and paraffin embedded for routine processing. Liver sections cut from paraffin blocks were stained with H&E for blinded scoring of macrovesicular and microvesicular steatosis, inflammation and hepatocyte ballooning as previously described (Kleiner et al., 2005). Blinded scoring was performed by an experienced pathologist at UCSD (San Diego, CA, USA). Immunohistochemistry for MPO and alpha-smooth muscle actin (α-SMA), TUNEL staining, and PSR staining were performed as described previously (Wree et al., 2014b). Stained liver sections were quantitated using bright field images captured with NanoZoomer S210 Digital Slide Scanner (Hamamatsu, Iwata City, Japan). For the DIO model of NASH, paraformaldehyde-fixed liver pre- and post-biopsies were paraffin-embedded, sectioned and stained with hematoxylin-eosin (Dako, Glostrup, Denmark), Picrosirius red (Sigma-Aldrich, Broendby, Denmark), anti-type I Collagen (cat. no. 1310-01, SouthernBiotech, Birmingham, AL, USA), and anti-MPO (cat. no. RB-373-A1, ThermoFisher) as described previously (Tolbol et al., 2018).
RNA Isolation and qPCR

Total RNA was isolated from liver tissue (~50 mg) via syringe homogenization in TRIzol™ and cDNA synthesized using the iScript™ cDNA Synthesis kit (Biorad, Hercules, CA, USA). Real-time PCR was performed using TaqMan Fast Advanced Master Mix (ThermoFisher) and samples were amplified using QuantStudio-7 (ThermoFisher) in accordance with manufacturer instructions. The following TaqMan Gene Expression Assays (cat. no. 4331182, Applied Biosystems, Foster City, CA) were used: α-SMA (Acta2, Mm00725412_s1), CollagenIα1 (Col1a1, Mm00801666_g1), Connective tissue growth factor (Ctgf, Mm01192933_g1), caspase-1 (Casp1, Mm00438023), ASC (Pycard, Mm00445747), IL-1β (Il1b, Mm00434228). For the diet-induced DIO-NASH study, liver tissue RNA isolation and gene expression were performed as previously described (Tolbol et al., 2018).

Hepatic Hydroxyproline Content

Liver tissue (~100 mg) was homogenized in 6N HCl and heated overnight at 110°C. Samples cooled to room temperature (RT) and centrifuged at 12,000 x g for 2 min. The hydrolysate was filtered through a 100 μm nylon cell strainer. Samples were measured in triplicate along with a hydroxyproline standard curve. Citrate-acetate buffer (pH 6) was added to each well followed by Chloramine T solution, and incubated at room temperature for 20 min. Ehrlich’s reagent was added, and incubated at 65°C for 15 min. The plate was cooled to RT and the absorbance measured at 558 nm.

Fibroblast Activation Studies
JT001 was profiled in a BioMAP fibrosis panel using four different primary human fibroblast systems at Eurofins DiscoverX at concentrations ranging from 10 nM-10 µM. The human fibroblast systems used were lung fibroblasts, dermal fibroblasts, and dermal fibroblasts co-cultured with either human bronchial epithelial cells or keratinocytes. Readouts included collagen I, III and IV, α-SMA, TIMP-2 and PAI-1. A close analogue of JT001 was profiled against primary normal human hepatic stellate cells and primary NASH human hepatic stellate cells at iXCells Biotechnologies (San Diego, CA) according to their standard protocol with readouts of α-SMA and collagen I immunostaining.

**Statistical Analysis**

All data were graphed and analyzed using GraphPad Prism 8.0.1 software (GraphPad, La Jolla, CA, USA). All results, except JT001 plasma concentrations, are shown as mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Holm-Sidak’s multiple comparisons test (in vivo pharmacodynamic data), Welch's unequal variances t-test (quantitative data from CDAA model), ANOVA followed by Dunnett’s multiple comparison test (MPO data from the DIO model), Fisher’s exact test (histology scores from the DIO model), Student’s t-test (quantitative data from A350V knock-in model) or Mann-Whitney U-test (ordinal data from A350V knock-in model). Data were considered significant when P ≤ 0.05.
Results

In Vitro Potency, Selectivity and Mechanism of Action of JT001

The potency of JT001 (Figure 1A) in inhibiting NLRP3-dependent IL-1β and IL-18 production was evaluated in human Kupffer cells primed with LPS and stimulated by nigericin. Treatment of human Kupffer cells with LPS for 4 hours followed by nigericin treatment for an additional 1 hour resulted in robust production and release of IL-1β and IL-18 (Supplementary Figure 1A). JT001 displayed potent, concentration-dependent inhibition of IL-1β and IL-18 production and release from human Kupffer cells with average IC₅₀ values of 62 and 74 nM, respectively (Figure 1B and Table 1). In addition to the observed increases in IL-1β and IL-18 production, treatment with LPS plus nigericin increases the production and release of the pro-inflammatory cytokine TNF-α in a NLRP3-independent manner via the activation of TLR4. JT001 treatment had no effect on TNF-α secretion from human Kupffer cells at concentrations up to 10 µM (Supplementary Figure 1A, Figure 1B and Table1).

LPS plus nigericin activation of NLRP3 in human Kupffer cells also induced pyroptosis, as evidenced by the substantial decrease in cellular ATP content followed by the release of IL-1α (Supplementary Figure 1B). JT001 showed concentration-dependent inhibition of pyroptosis as measured by a reduction in IL-1α release and an increase in cell viability (ATP content) with average IC₅₀ values of 94 and 43 nM, respectively (Figure 1C, Table 1).

The recruitment of inflammatory monocytes to the liver is implicated in the progression of NASH and the development of fibrosis (Tacke, 2017). Therefore, JT001 was tested for its ability to inhibit NLRP3-mediated IL-1β production from monocytes using peripheral blood mononuclear cells (PBMCs). Treatment of human PBMCs with LPS and nigericin resulted in robust production and release of IL-1β at concentrations typically higher than those observed in
the human Kupffer cells (Supplementary Figure 2). JT001 displayed concentration-dependent inhibition of IL-1β release from human PBMCs with an average IC₅₀ value of 66 nM, similar to the IC₅₀ value obtained in Kupffer cells (Figure 1D, Table 1). Comparison experiments were also performed with PBMCs isolated from mouse, rat and non-human primate to evaluate cross-species potencies. Although we observed clear induction of IL-1β with LPS plus nigericin stimulation of mouse, rat and non-human primate PBMCs, the levels of IL-1β reached were significantly lower than those reached using human PBMCs (Supplementary Figure 2). Despite the differences in total IL-1β production, JT001 showed similar potency in inhibiting IL-1β production from human, mouse and non-human primate PBMCs. However, the IC₅₀ value showed a reduction in potency of approximately 10-14-fold using rat PBMCs (Figure 1D, Table 1).

Lipotoxic compounds such as palmitic acid and cholesterol crystals (CHC) accumulate in the liver due to steatosis and can act as NLRP3-activating signals (Rajamaki et al., 2010; Mridha et al., 2017; Pan et al., 2018). Gouty arthritis is characterized by the precipitation of monosodium urate (MSU) crystals in the joints, also leading to NLRP3 inflammasome activation (Jhang et al., 2018). We evaluated the potential of JT001 to inhibit IL-1β production using crystals of cholesterol or MSU as pathophysiologically relevant activators of NLRP3. Treatment of mouse bone-marrow-derived macrophages (mBMDMs) with LPS plus CHC or MSU crystals resulted in increased IL-1β production that was inhibited by JT001 in a concentration-dependent manner, with mean IC₅₀ values of 9 and 20 nM, respectively (Supplementary Figure 3A, Figure 1E, Table 1).

Small molecule inhibitors such as JT001 may substantially bind albumin or other blood proteins, effectively lowering the free fraction of drug available for binding the target protein. As
such, measurement of compound potency in whole blood is often regarded as a better predictor of in vivo potency. NLRP3-dependent IL-1β production can be induced in circulating cells in the context of whole blood by treatment with LPS and ATP (Grinstein et al., 2018). LPS plus ATP treatment of isolated human and mouse PBMCs induced IL-1β production to a similar extent as LPS plus nigericin (Supplementary Figure 3), and JT001 showed concentration-dependent inhibition of LPS plus ATP stimulated IL-1β production in human and mouse PBMCs with mean IC₅₀ values of 30 and 25 nM, respectively (Figure 2A and Table 1). The potencies of JT001 for inhibiting LPS plus ATP stimulated IL-1β production in human and mouse whole blood were reduced as compared to the serum-free PBMC assays, with mean IC₅₀ values of 463 and 1,112 nM, respectively (Figure 2B and Table 1). The loss of potency between the serum-free cell-based and whole blood assays was not due to compound instability as JT001 was stable in human and mouse whole blood for at least 4 hours at 37°C (Supplementary Figure 4A). Of note, the extent of LPS plus ATP stimulated IL-1β production in human blood varied considerably across different blood donors, with IL-1β concentrations ranging from 1,953 to 21,970 pg/mL across the five different healthy donors used (Supplementary Figure 4B). Despite the differences in magnitude of IL-1β production between donors, we observed little variability in IL-1β production within the same human donor between experiments run on different days, and there was no correlation of compound potency with total IL-1β production in the human blood studies (Supplementary Figure 4B and C). We observed considerably less variability in IL-1β production across the mouse blood studies, with concentrations ranging from 1,206 to 3,865 pg/mL (Supplementary Figure 4B). LPS alone can stimulate IL-1β production from monocytes in human blood through activation of the alternative NLRP3 inflammasome pathway, so the potency of JT001 on the alternative pathway was also examined. IL-1β production from 6-hour
LPS-treated human blood varied from 10,692 to 26,192 pg/mL, and JT001 concentration-dependently inhibited LPS-induced IL-1β production with a mean IC₅₀ value of 109 nM (Supplementary Figure 4D, Figure 2C and Table 1).

Studies were undertaken to investigate the molecular mechanism of action of JT001. First, cell lysates from LPS plus nigericin stimulated human PBMCs treated with varying concentrations of JT001 were evaluated for caspase-1 activity. As shown in Figure 3A, JT001 showed concentration-dependent inhibition of caspase-1 activity with a mean IC₅₀ value of 146 nM. The observed decrease in caspase-1 activity was not a result of direct inhibition of caspase-1 protease activity, as JT001 showed no inhibition of the protease activity across twelve different human caspase enzymes, including caspase-1 (Supplementary Figure 5).

The ability of JT001 to inhibit the autocatalytic cleavage of procaspase-1 was evaluated in mBMDMs by pre-incubation with 10 µM test compound for 30 minutes prior to stimulation with LPS plus nigericin. Following stimulation, levels of the procaspase-1 zymogen (p45) and cleaved caspase-1 p10 subunit were evaluated in cell culture supernatants and lysates by Western blotting. Treatment of mBMDMs with LPS alone (DMSO + LPS) resulted in increased NLRP3 protein levels, consistent with the NF-κB-mediated transcriptional upregulation of Nlrp3 downstream of LPS/TLR4 signaling (Figure 3B, Supplementary Figure 6). Treatment with both LPS plus nigericin promoted the autocatalytic cleavage of the procaspase-1 zymogen (p45) and the accumulation of active caspase-1 (p10 subunit) in cell lysates. LPS plus nigericin stimulation also promoted the appearance of caspase-1 p10 in the cell supernatants, presumably due to increased pyroptosis and release from the dying cells. JT001 inhibited autocatalytic cleavage of the procaspase-1 zymogen (p45) and accumulation of active caspase-1 (p10 component) in cell lysates and supernatants. In contrast, neither the pan-caspase inhibitor emricasan, nor the TLR4
inhibitor TAK-242 inhibited autocatalytic cleavage of the procaspase-1 p45 zymogen. However, emricasan, but not TAK242, resulted in reduced levels of the p10 subunit in the cell supernatants, presumably by attenuating caspase-1 mediated pyroptosis. These data suggest little to no effect of TAK242 on blocking pyroptosis.

Based on the above results, we reasoned that JT001 acts upstream in the pathway to block the cleavage of procasapse-1 into its active form. To better understand the mechanism of action of JT001, we evaluated intracellular ASC speck formation by immunofluorescence in LPS plus nigericin stimulated mBMDMs. Untreated mBMDMs displayed diffuse cytosolic staining for ASC, while in LPS plus nigericin treated cells ASC aggregated into a single speck per cell, consistent with assembly of the NLRP3 inflammasome (Figure 3C). Preincubation of mBMDMs with either 0.5 or 2 μM JT001 completely prevented ASC speck formation in mBMDMs with just 0.09% and 0.06% of the JT001-treated cells exhibiting ASC specks, respectively, compared to 10.3% for the vehicle-treated cells (Figure 3C and D). In concentration-response experiments, JT001 inhibited ASC speck formation in mBMDMs with a mean IC₅₀ value of 43 nM (Figure 3E), a value that was similar to its IC₅₀ value for inhibiting IL-1β production in serum-free cell-based assays.

To evaluate inhibition of the non-canonical NLRP3 pathway, mBMDMs were stimulated with the TLR2 agonist, Pam3CSK4, followed by transfection of LPS and compared to canonical NLRP3 activation in mBMDMs using LPS plus nigericin. Both non-canonical and canonical NLRP3 pathway activation led to robust IL-1β production from mBMDMs, with typical concentrations ranging from 5,000 to 12,000 pg/mL (Supplementary Figure 7A and B). JT001 showed concentration-dependent inhibition of IL-1β production in mBMDMs after non-
canonical and canonical pathway activation with mean IC$_{50}$ values of 198 and 225 nM, respectively (Figure 3F, Table 1). These data, combined with whole blood assay results, showed that JT001 potently inhibited the NLRP3 inflammasome independent of whether inflammasome activation was by the canonical, non-canonical, or alternative pathway.

To determine the selectivity of JT001 for the NLRP3 inflammasome, relative to other inflammasome family members, we examined the effects of JT001 treatment following activation of the NLRC4 and AIM2 inflammasomes in mBMDMs treated with LPS plus flagellin or LPS plus poly(dA:dT), respectively. Although we observed substantial production of IL-1$\beta$ after activation of either NLRC4 or AIM2, JT001 showed no significant inhibition of these inflammasomes at concentrations up to 10 µM (Supplementary Figure 7C and D and Figure 3G and H). As expected, the pan-caspase inhibitor emricasan fully inhibited IL-1$\beta$ production downstream of NLRC4 and AIM2 activation at concentrations $\geq$300 nM (Figure 3G, H).

Muramyl dipeptide (MDP), the minimal bioactive peptidoglycan motif common to bacteria, can activate both the NLRP1 and NLRP3 inflammasomes (Martinon et al., 2004; Faustin et al., 2007). Therefore, to evaluate inhibition of NLRP1, we stimulated mBMDMs generated from Nlrp3 knockout mice with LPS plus L18-MDP, a synthetic and more potent derivative of muramyl dipeptide. Treatment of Nlrp3 knockout-derived mBMDMs with L18-MDP stimulated NLRP1-dependent IL-1$\beta$ production and JT001 showed no significant inhibition at concentrations up to 10 µM (Supplementary Figure 7E and F). Collectively, these results demonstrated that JT001 was a selective inhibitor of NLRP3 inflammasome activation and not an inhibitor of the NLRC4, AIM2, and NLRP1 inflammasomes.

Pharmacokinetic and Pharmacodynamic Profile
The PK of JT001 was assessed in female CD-1 mice and female Sprague-Dawley rats after intravenous (i.v.) and oral (p.o.) administration. After intravenous administration of 1 mg/kg, JT001 displayed low systemic clearance (Cl_p) with values of 1.1 mL/min/kg and 1.7 mL/min/kg in mouse and rat, respectively (Table 2). JT001 displayed a short elimination half-life (t_1/2) of 1.4-1.5 hours in both species and a low volume of distribution at steady state (V_ss) with values of 0.1-0.2 L/kg. The sodium salt form of JT001 was readily absorbed after oral administration, showing good bioavailability (62-85%), and reaching maximum plasma concentrations of 26.9 µg/mL (67 µM) in mouse and 17.8 µg/mL (44 µM) in rats at thirty minutes following oral dosing. Dose-adjusted exposures (dose-adjusted AUC) from oral administration were comparable between mouse and rat (9.4 versus 8.3), consistent with the comparable bioavailability and clearance values observed in both species (Table 2).

The relationship between JT001 pharmacokinetics and pharmacodynamics (PD) was evaluated in vivo using a murine peritonitis model of NLRP3-dependent IL-1β production (Xiang et al., 2013). In a dose-response study, single oral doses of 0.5-5 mg/kg JT001 or vehicle were administered 1 hour prior to the i.p. delivery of LPS. Two hours post-LPS injection, ATP was injected i.p. and 30 minutes later blood and peritoneal lavage fluid collected for determination of JT001 and IL-1β concentrations, respectively. As shown in Figure 4A, NLRP3-dependent IL-1β production was significantly decreased with increasing JT001 dose, with doses of ≥1.5 mg/kg resulting in 99% inhibition and the 0.5 mg/kg dose yielding 85% inhibition of IL-1β production. Plasma concentrations of JT001 increased in a dose-dependent manner over the range tested, with 99% mean IL-1β inhibition observed at mean plasma concentrations ≥3.8 µM and 85% mean IL-1β inhibition observed at a mean plasma concentration of 1.3 µM (Figure 4B, C).
In a time-course study, groups of test mice were administered a single dose of 30 mg/kg JT001 p.o. at 1, 6, 12 or 22 hours prior to the i.p. injection of LPS. Two hours post-LPS injection, ATP was injected i.p. and 30 minutes later (at 3.5, 8.5, 14.5 or 24.5 hours post-JT001 dose) blood and peritoneal lavage fluid collected for determination of JT001 and IL-1β concentrations, respectively. As shown in Figure 4D, production of peritoneal IL-1β was significantly decreased by JT001 treatment over the time interval tested, as compared to vehicle treated mice, with peritoneal concentrations of IL-1β remaining at <1% of the vehicle control level from 3.5h to 14.5h post dosing before rising to 55% of the control value by 24.5h post-JT001 dosing. Plasma concentrations of JT001 decreased with time after dosing, but sustained 99% mean inhibition of IL-1β production at mean plasma concentrations ≥11 µM and 45% mean inhibition at a mean plasma concentration of 0.5 µM (Figure 4E, F). The 45% inhibition of IL-1β observed in vivo at a mean plasma concentration of 0.5 µM is in close alignment with the in vitro mouse blood IC_{50} value of 1.1 µM, underscoring the tight correlation between in vivo NLRP3 inhibition and in vitro inhibition in whole blood.

**In Vivo Efficacy of JT001 in a Nlrp3 Knock-in Murine Model of Muckle-Wells Syndrome.**

Dominantly inherited activating mutations in the NLRP3 gene are responsible for the excessive cytokine production, autoinflammation and clinical presentation characteristic of CAPS (Hoffman et al., 2001). In humans, a mutation in NLRP3 that replaces alanine 352 with valine (A352V) is strongly associated with a type of CAPS called Muckle Wells syndrome (MWS) (Hoffman et al., 2001). JT001 was evaluated in a mouse Nlrp3^{A350V/+}CreT mutant knock-in model of MWS in which expression of the A350V mutant allele (analogous to the human
A352V mutation) occurs only in mice expressing tamoxifen-inducible Cre recombinase (Brydges et al., 2009; McGeough et al., 2012).

Tamoxifen-induced expression of A350V-mutant NLRP3 resulted in pronounced weight loss, with vehicle-treated animals losing an average 32% of their body weight by day 30 (Figure 5A). There were three animals in the vehicle group at study initiation, however, one animal died on Day 21 of the 30-day study. There were no deaths in the JT001 treatment group, and animals treated once daily by oral gavage with 30 mg/kg JT001 were significantly protected from the body weight loss, losing an average of only 11% of their body weight by day 30 (Figure 5A). Tamoxifen treated Nlrp3A350V/+CreT mice developed splenomegaly as a result of marked inflammation and displayed increased systemic inflammation, with white blood cell, neutrophil and eosinophil counts 5-7-fold higher than normal (Figure 5B and C). Treatment with JT001 significantly reduced spleen weight and restored the numbers of circulating inflammatory cells to normal or near normal levels (Figure 5B and C).

Persistent expression of mutant A350V and D301N Nlrp3 in knock-in mice was previously shown to promote extensive liver inflammation and fibrosis. Observed effects included increased numbers of macrophages, neutrophil infiltration, increased hepatic stellate cell activation, marked hepatocyte pyroptotic cell death and increased hepatic collagen deposition (Wree et al., 2014a; Wree et al., 2014b). Therefore, treatment with JT001 was evaluated for effects on these liver-related endpoints in the Nlrp3A350V/+CreT mice. As shown in Figure 5E, treatment with JT001 decreased hepatic mRNA levels of the inflammasome-related genes, casp1, Il1b and Pycard. JT001 also attenuated hepatic inflammation as assessed by H&E staining and immunohistochemistry for MPO, a neutrophil marker, although with the limited number of animals, the data did not reach statistical significance (Figure 5F). The degree of liver
fibrosis was evaluated by picrosirius red (PSR) staining of liver tissue and immunohistochemical analysis of $\alpha$-smooth muscle actin ($\alpha$-SMA), a marker of hepatic stellate cell (HSC) activation. Additionally, mRNA quantitation of the profibrotic mediator Ctgf was assessed. JT001 treatment reduced both the % PSR positive area and $\alpha$-SMA staining and also inhibited hepatic Ctgf expression, consistent with preventing fibrosis progression (Figure 5F and G). Finally, JT001 treatment prevented hepatocyte cell death as evidenced by the significant reduction in serum ALT and TUNEL positive cells in the liver (Figure 5D and F).

**Efficacy of JT001 in a Murine Diet-Induced Obesity (DIO) Model of NASH**

The effect of JT001 on hepatic inflammation, nonalcoholic fatty liver disease activity scores (NAS) and fibrosis was next evaluated in a murine diet-induced obesity (DIO) model of NASH (Kristiansen et al., 2016). Prior to study initiation, plasma and liver drug levels in mice fed the AMLN diet for 42 weeks were investigated to confirm appropriate exposure and target coverage. Compared to naïve C57BL6 mice, mice fed the AMLN diet for 42 weeks showed 7-8-fold lower plasma drug concentrations, but higher liver drug concentrations (Supplementary Figure 8). Importantly, liver drug levels at 8 hours post-dose in the AMLN fed mice reached concentrations similar to the mouse whole blood $IC_{90}$ value of 9 $\mu$M, suggesting good target coverage through 8 hours post-dosing with a 10 mg/kg dose. For the efficacy study, mice were fed the AMLN diet for 39 weeks at which time a baseline liver biopsy was performed; only animals with a steatosis score $\geq 2$ from H&E stained liver sections and fibrosis score $\geq 1$ based on PSR stained sections were included in the study (Kristiansen et al., 2016). Animals were randomized into groups based on body weight and liver biopsy collagen I$\alpha$1
immunohistochemistry, and treated with either 10 or 30 mg/kg JT001 or vehicle once daily by oral gavage for an additional 8 weeks while maintaining the AMLN diet.

Chronic treatment with 10 or 30 mg/kg JT001 was well tolerated and exerted no adverse effect on body weight or food consumption of the AMLN fed animals when compared to the vehicle control group (Supplementary Figure 9A). At study termination, a blinded histological assessment of steatosis, ballooning degeneration and inflammation was performed and the scores compared to the baseline biopsy scores for determination of the number of animals in each treatment group showing a worsening (higher score), improvement (lower score) or no change (same score) in each parameter. As shown in Figure 6A, there was no progression of steatosis during the treatment period as all animals in the vehicle group displayed the maximum steatosis score of 3 both pre- and post-treatment. JT001 administration did not promote steatosis resolution or reduce liver triglycerides or liver total cholesterol (Figure 6A and Supplementary Figure 9B). This NASH model exhibited limited ballooning degeneration with only 2, 7, and 5 out of 12 animals showing perceptible pre-treatment ballooning in the vehicle control, 10 mg/kg JT001, and 30 mg/kg JT001 groups, respectively. Despite the limited ballooning degeneration present in the study animals, there was improvement in the JT001 treatment groups, with animals from only these groups showing lowered ballooning degeneration scores over the 8-week treatment period (Figure 6A).

In the vehicle-treated group, 7 of the 12 animals showed a worsening of lobular inflammation during the treatment period, compared to only 1 animal in the 10 mg/kg JT001 group and no animals in the 30 mg/kg JT001 group (Figure 6A). Additionally, 4 of the 12 animals in the 10 mg/kg JT001 group and 1 animal in the 30 mg/kg group showed an improvement in inflammation score over the treatment period, demonstrating a pronounced
effect for JT001 treatment on lobular inflammation. The effect on inflammation was further confirmed by immunohistochemistry for the neutrophil marker MPO, which was significantly decreased in mice treated with 10 or 30 mg/kg JT001 (Supplementary Figure 9C). Overall, administration of JT001 resulted in a significant therapeutic effect on NAS, a composite of the histological scores for steatosis, ballooning degeneration, and inflammation, as 6 and 5 out of 12 animals in the 10 mg/kg and 30 mg/kg JT001 dose groups, respectively, showed an improvement in NAS while 7 out of 12 animals in the vehicle control group showed a worsening of NAS (Figure 6B). The effect on NAS was primarily due to a decrease in lobular inflammation and to a lesser extent ballooning degeneration.

The effect of JT001 on hepatic collagen deposition in the DIO NASH model was assessed by scoring fibrosis from PSR stained liver sections. There was limited fibrosis progression during the treatment period with only three out of 12 vehicle treated animals displaying an increase in fibrosis score during the treatment period (Figure 6C). In comparison, 1 out of 12 animals in the lower dose JT001 group and 0 out of 12 animals in the higher dose group showed a worsening of fibrosis scores over the treatment period. While no animals in the vehicle group showed an improvement in fibrosis, 1 animal in the lower dose group and 3 animals in the higher dose group showed lower fibrosis scores between the pre- and post-treatment analyses (Figure 6C). Therefore, while the data did not reach statistical significance, JT001 treatment showed a trend for reducing fibrosis in this DIO model of NASH.

**Efficacy of JT001 in the CDAA Diet-Induced Murine Model of NASH Fibrosis**

The therapeutic effect of JT001 treatment on hepatic steatosis, inflammation, and fibrosis was evaluated in a second murine NASH model, the choline-deficient L-amino acid-defined diet
(CDAA) model (De Minicis et al., 2014). Animals were fed a CDAA diet or the control CSAA diet (choline-supplemented L-amino acid-defined) for 20 weeks with once daily oral dosing of 30 mg/kg JT001 during the last 5 weeks of the CDAA/CSAA feeding. After 15 weeks of diet feeding, the CDAA-fed animals displayed a slightly reduced body weight as compared to the control CSAA fed animals, however, JT001 treatment was well tolerated by both diets fed groups and showed no adverse effects on body weight as compared to the vehicle-treated animals (Supplementary Figure 10A and B). At study termination, mice fed a CDAA diet showed increased liver weight to body weight ratio and serum ALT relative to the CSAA control diet animals, and these endpoints were not affected by JT001 treatment (Supplementary Figure 10C and D).

Liver tissue was H&E stained and scored for macrovesicular and microvesicular steatosis and inflammation using non-alcoholic fatty liver disease (NAFLD) activity scoring (NAS) criteria (Kleiner et al., 2005). Histopathological assessment of liver tissue revealed no significant difference in median hepatic inflammation or steatosis scores between the CDAA/Veh group and the CSAA/Veh control group (p>0.05) (Supplementary Figure 10E and F). Despite the lack of significant difference in inflammation scores, there was a trend for an increase in inflammation in the CDAA/Veh group as compared to the CSAA/Veh group and also a strong trend for reduction in inflammation score with JT001 treatment (CDAA/JT001), as only 20% of the animals in the JT001 treatment group displayed an inflammation score of 2 as compared to 60% in the vehicle group (CDAA/Veh) (Supplementary Figure 10E). In contrast, there were no differences in steatosis scores between the CDAA/Veh and CDAA/JT001 animals (Supplementary Figure 10F). The effect of JT001 on hepatic inflammation was further assessed by immunostaining liver tissue with antibodies to MPO, a marker of neutrophils. CDAA diet
feeding resulted in a significant increase in hepatic neutrophil infiltration (p <0.0001) that was suppressed by 80% with JT001 treatment (p <0.0001) (Figure 7A, B).

The extent of liver fibrosis was determined histologically by PSR staining for the presence of collagen I and III fiber deposition and biochemically by analysis of hydroxyproline content. As shown in Figures 7C and D, quantification of PSR staining revealed a significant 2.8-fold increase in collagen I and III deposition in the CDAA/Veh group relative to the CSAA/Veh control group (p = 0.01) and JT001 treatment of CDAA-fed animals resulted in a substantial but non-significant 45% normalization of PSR positive area. The observed JT001-induced reduction in fibrosis was further confirmed by hydroxyproline analysis of hepatic collagen content. Relative to the CSAA control group, the CDAA-fed animals exhibited a significant 1.9-fold increase in hydroxyproline concentration (p = 0.003), which was substantially, but not significantly, decreased by 41% upon treatment with JT001 (Figure 7E). The effect of JT001 on hepatic fibrosis was further assessed by evaluating hepatic gene expression of three fibrosis-related genes: α-Smooth Muscle Actin (Acta2), Collagen Type I Alpha 1 Chain (Col1a1) and Connective Tissue Growth Factor (Ctgf). Quantitative polymerase chain reaction (qPCR) analyses revealed significant increases in mean expression of all three genes in the CDAA/Veh group relative to the CSAA/Veh control group: 3.1-fold for Acta2 (p = 0.0005), 2.7-fold for Col1a1 (p = 0.03), and 1.6-fold for Ctgf (p = 0.03) (Figure 7F). JT001 treatment in CDAA-fed animals resulted in a significant 80% normalization of Acta2 expression (p = 0.003) and substantial, but non-significant, inhibitions in mean expression of Col1a1 and Ctgf of 65%, and 66%, respectively (Figure 7F). Plasma and liver drug levels were determined at the end of the study to estimate target engagement. Plasma drug concentrations in the CDAA HFD mice were higher than the mouse whole blood IC₉₀ value of 9 µM for >8 hours post-dosing and were similar.
to the mouse blood IC$_{50}$ value at 24 hours post-dose. Liver concentrations, determined 24 hours after the last dose, were between the mouse blood IC$_{50}$ and IC$_{90}$ value, suggesting robust, but not complete target coverage over the dosing period.

**Effect of JT001 on Hepatic Stellate Cell Activation**

Since activated hepatic stellate cells (HSCs) are key drivers of liver fibrosis, we evaluated the effect of NLRP3 inhibition on hepatic stellate cell activation and markers of fibrosis. Human normal and NASH primary hepatic stellate cells were serum-starved in vitro, then incubated with a close analog of JT001 or the TGF$_\beta$-RI inhibitor LY2157299 as a positive control. NLRP3 inhibition appeared to reduce $\alpha$-SMA expression but had no effect on collagen I production (Supplementary Figure 11). Effects of JT001 on fibroblast activation were also tested in TGF-$\beta$ activated human primary dermal, lung fibroblasts, and dermal fibroblasts co-cultured with human keratinocytes or bronchial epithelial cells. We saw no effect on any of the fibrotic endpoints measured, including expression of collagen I, III, or IV, or $\alpha$-SMA, TIMP-2 or PAI-1.
Discussion

Inflammation in the absence of infection, so-called sterile inflammation, is required for normal physiological wound healing in response to tissue injury. However, chronic persistent sterile inflammation contributes to the development of acute and chronic inflammatory diseases, such as ischemia-reperfusion injury, atherosclerosis, Type II diabetes, neurodegenerative diseases and non-alcoholic steatohepatitis (Peiseler and Kubes, 2018). Inflammasome complexes in cells of the innate immune system act as key regulators of sterile inflammation through the sensing of intracellular or extracellular triggers such as nuclear proteins, oxidized mitochondrial DNA, cholesterol crystals, monosodium urate crystals and β-amyloid aggregates, leading to the subsequent activation of caspase-1 and processing of the pro-inflammatory cytokines IL-1β and IL-18 (Schroder and Tschopp, 2010; Lamkanfi and Dixit, 2014). NLRP3 is unique among the NOD-like family of receptors in its ability to sense and be activated by a diverse set of sterile inflammatory stimuli, thus underscoring the clinical potential of NLRP3 inhibitors to treat chronic inflammatory diseases driven by aberrant sterile inflammation. Here we describe the preclinical pharmacologic, pharmacodynamic and pharmacokinetic properties of JT001, which was observed to be a novel, potent and selective inhibitor of the NLRP3 inflammasome that blocked production and secretion of IL-1β and IL-18 from multiple cell types and prevented pyroptosis. JT001 treatment exhibited a robust PK/PD relationship, demonstrating 99% inhibition of IL-1β production in vivo at 14.5 hours after a single oral dose of 30 mg/kg. In preclinical efficacy models, therapeutic administration of JT001 reduced systemic and hepatic inflammation and attenuated fibrosis progression.

During the last several years, a number of putative NLRP3 inhibitors have been described (Baldwin et al., 2017; He et al., 2018; Huang et al., 2018; Mangan et al., 2018). Among the best
characterized of these molecules is MCC950 (CRID3, CP-456,773), which demonstrated specific inhibition of NLRP3 inflammasome assembly, with in vivo PD activity and efficacy in mouse models (Coll et al., 2015; Primiano et al., 2016). MCC950 was advanced into Phase 1 of clinical development, however its development was halted when elevated liver transaminases were observed at a 1200 mg total dose (Shah et al., 2015). Although the exact cause of the MCC950 drug-induced liver injury (DILI) is unknown or has not been reported, JT001 was developed to address possible causes, for example by removing the furan moiety present in MCC950. While furans are present in approved drugs, the furan moiety has also been identified as a possible toxicophore due to its potential to undergo bioactivation resulting in the generation of toxic reactive metabolites that have the potential to trigger toxicities. Additionally, JT001 has a reduced clogP relative to MCC950 and higher lipophilicity, two additional characteristics proposed to be contributors to the risk of DILI (Chen et al., 2013).

Data generated over the last several years has supported a role for NLRP3 as a modulator of hepatic inflammation and NASH-induced liver fibrosis. In human livers, the upregulated expression of NLRP3 and inflammasome related genes in tissue from NASH patients as compared to non-NASH patients with fatty liver disease suggests a role for NLRP3 in the transition from benign steatosis to NASH (Csak et al., 2011; Ganz et al., 2014; Wree et al., 2014b). NLRP3 is activated in liver Kupffer cells directly by lipotoxic compounds that accumulate in the fatty liver, such as palmitic acid and cholesterol crystals (Mridha et al., 2017; Pan et al., 2018). Additionally, therapeutic dosing of MCC950 demonstrated anti-inflammatory and anti-fibrotic efficacy in two models of experimental steatohepatitis: mice fed a methionine-choline deficient diet and foz/foz mice fed an atherogenic diet (Mridha et al., 2017). Our in vivo data with JT001 extend the previous analyses by demonstrating therapeutic anti-inflammatory
and anti-fibrotic efficacy in two additional murine models of NASH: a DIO model and a CDAA model, and provide further support for NLRP3 as a modulator of progression from NAFL to NASH. We also observed potent inhibition of NLRP3 activity by JT001 in human Kupffer cells, the resident liver macrophages, as well as in mBMDMs following activation by cholesterol crystals.

NLRP3 knock-in mice develop severe liver inflammation and fibrosis which is accompanied by hepatic stellate cell activation (HSC) with collagen deposition and hepatocyte pyroptosis (Wree et al., 2014a). These data raise the possibility that NLRP3 activation in these non-myeloid cell types may contribute to NASH pathogenesis. However, we observed no significant effect of JT001 on hepatic stellate activation and fibrosis-related endpoints in vitro and no significant effect in preventing cell death in primary cultured hepatocytes in vitro (unpublished data). Our data is in agreement with a recent publication which showed that myeloid-specific knockout of NLRP3, but not hepatocyte- or hepatic stellate cell-specific knockout, reduced HSC activation and liver fibrosis in a model of NASH. Additionally, using monocyte and HSC co-cultures, these authors demonstrated that NLRP3 activation in monocytes promotes the activation of HSCs and increases the production of a number of pro-fibrotic mediators (Kaufmann et al., 2022).

We have characterized the mechanism of action of JT001 using a number of cell-based assays and shown that JT001 inhibits NLRP3 activity downstream of the canonical, non-canonical and alternative pathways with similar potency and regardless of the secondary trigger, but does not inhibit IL-1β production downstream of the NLRP1, NLRC4 or AIM2 inflammasomes. JT001 blocks ASC speck formation upon canonical pathway activation, thus demonstrating the ability to block inflammasome assembly, however alternative NLRP3
pathway activation occurs in the absence of speck formation and JT001 showed equivalent potency in inhibiting the alternative pathway. Taken together, these data suggest that JT001 binds NLRP3 directly, and this is further supported by a recent publication that used cryo-EM to show direct binding of a structurally similar compound to an allosteric pocket in the NACHT domain of NLRP3 (McBride et al., 2022).

We used an in vivo murine peritonitis model to evaluate the in vivo potency of JT001 and found that a single dose of 30 mg/kg JT001 led to complete inhibition of IL-1β production through 14.5 hours post-dosing and doses of 10-30 mg/kg demonstrated anti-inflammatory and anti-fibrotic efficacy in preclinical efficacy models. These data suggest the ability to attain sustained NLRP3 inhibition at therapeutically reasonable doses. In the DIO and CDAA models, therapeutic dosing of JT001 at either 10 and/or 30 mg/kg showed clear anti-inflammatory effects, due, at least in part, to reductions in hepatic neutrophil infiltration. JT001 treatment had no effect on hepatic steatosis, liver triglycerides or total cholesterol in the DIO model, which is consistent with the anti-inflammatory mechanism of NLRP3 and supports the proposed role of NLRP3 in mediating the transition from NAFL to NASH (Mridha et al., 2017).

The DIO model exhibits limited ballooning degeneration and fibrosis with little to no progression of either pathology over the course of the treatment period making it difficult to discern effects on these endpoints. However, consistent with the previously demonstrated effects of MCC950 on liver injury and fibrosis, only animals in the JT001 DIO groups showed improvements in fibrosis and ballooning scores over the treatment period, and it is possible that longer treatment times would result in improved effects on these endpoints. Despite the lack of effect on steatosis and marginal effect on ballooning, JT001 treatment in the DIO model resulted in a robust and statistically significant reduction in NAS, due primarily to the anti-inflammatory
effect. Anti-fibrotic activity of JT001 was more evident in the CDAA model with therapeutic dosing achieving 40-80% reductions in multiple fibrosis-related endpoints, including PSR positive area, hydroxyproline content and hepatic gene expression of \textit{Acta2}, \textit{Col1a1} and \textit{Ctgf}. Many of the therapies currently under development for NASH that are showing clinical efficacy target metabolic processes. However, ultimately NASH is likely going to be treated with combination therapies that target the metabolic, inflammatory and/or fibrotic drivers of the disease (Friedman et al., 2018). Our data confirm the anti-inflammatory and anti-fibrotic effects of NLRP3 inhibition and highlight the rationale for combination therapy with metabolic targets, such as the thyroid hormone receptor agonist and FGF21 mimics.

Canakinumab is an anti-inflammatory IL-1\textbeta targeting strategy that lowered the rate of cardiovascular events in high-risk patients without lipid-lowering effects in the CANTOS trial (Canakinumab Anti-Inflammatory Thrombosis Outcome Study)(Baylis et al., 2017; Weber and von Hundelshausen, 2017). However, the FDA declined to approve canakinumab for treatment of cardiovascular disease, possibly due to concerns around the increased incidence of fatal infections. Unlike canakinumab, which inhibits all IL-1\textbeta, JT001 selectively inhibits IL-1\textbeta production downstream of the NLRP3 inflammasome, leaving the AIM2, NLRC4 and NLRP1 inflammasomes intact. Therefore, it is interesting to speculate that selective targeting of NLRP3 may provide clinical benefit similar to the anti-IL-1\textbeta therapies, via targeting of sterile inflammation, with lower risk for increased infections due to the ability of other inflammasome family members to maintain host defense. Additionally, a small molecule based therapeutic approach allows for more effective temporal inhibition of IL-1\textbeta production. JT001 demonstrated preclinical efficacy at doses of 10 and 30 mg/kg which do not provide complete inhibition of IL-1\textbeta over the 24-hour dosing period. These results suggest that clinical efficacy may be achieved
at doses that do not fully inhibit NLRP3, allowing for an optimized dosing regimen that maximizes efficacy without increased risk of infection. Finally, another potential benefit of targeting NLRP3 inflammasome activity, versus IL-1β directly, is the ability to also modulate pyroptosis. Recent reports highlight the relevance of aberrant pyroptosis as a disease driver due to the ability to confer clonal proliferative advantages (Basiorka et al., 2016; Sallman et al., 2016). We have now reported here the identification of JT001, a potent and selective NLRP3 inhibitor with good PK/PD properties and efficacy in animal models of CAPS and NASH. Preclinical profiling of this compound in additional disease models will help inform on the utility of NLRP3 inhibitors to treat a broad range of inflammatory diseases.
Acknowledgements

The work discussed in this manuscript was performed in collaboration with Jecure Therapeutics, Inc. prior to the acquisition of Jecure by Genentech, Inc. The authors would like to thank Dr. Craig Stivala for assistance with navigation of internal publication review at Genentech. We would like to thank Hooke Laboratories (Lawrence, MA) for conducting the murine peritonitis studies and Dr. Kristoffer Voldum-Clausen and Dr. Michael Feigh of Gubra ApS (Hørsholm Kongevej 11B, 2970 Hørsholm, Denmark) for coordinating, conducting and interpreting the diet-induced DIO-NASH study. We would like to thank Dr. James G. Evans of Phenovista Biosciences (San Diego, CA, USA) for the technical support associated with the cell imaging. We would like to acknowledge Eileen Stone of iXCells (San Diego, CA, USA), Dr. Sharon Presnell and Dr. Ken Dorko of Samsara Sciences (San Diego, CA, USA) for providing human primary cells used for the studies described herein. We would like to thank Dr. Suresh Chintalapati, Dr. Prasad Manchem and Dr. Sanjay Pandey of Triangulum Biopharma (San Diego, CA, USA) for their preclinical pharmacology services.

Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.
Authorship Contributions:

Participated in research design: Povero, Lazic, McBride, Ambrus-Aikelin, Stansfield, Veal, Hoffman, Feldstein, Stafford, Bain

Conducted experiments: Povero, Lazic, Ambrus-Aikelin, Stansfield, Johnson, Santini, Pranadinata, McGeough

Contributed new reagents or analytic tools: McBride

Performed data analysis: Povero, Lazic, Ambrus-Aikelin, Stansfield, Johnson, Santini, Pranadinata, McGeough

Wrote or contributed to the writing of the manuscript: Povero, Veal, Stafford, Feldstein, Bain
References


Footnotes

This work received no external funding.

No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Figure 1. In vitro potency of JT001 in inhibiting cytokine production and pyroptosis in human Kupffer cells, mBMDMs and PBMCs. (A) Chemical structure of JT001. (B) Concentration-response curves of JT001 for inhibition of IL-1β, IL-18 and TNF-α production in human primary Kupffer cells treated with LPS + nigericin. (C) Concentration-response curves of JT001 for inhibition of LPS plus nigericin-induced pyroptosis as measured by cellular ATP content and IL-1α secretion in human Kupffer cells. (D) Comparison of concentration-response curves of JT001 for inhibition of LPS + nigericin stimulated IL-1β production in human, rat, mouse and non-human primate peripheral blood mononuclear cells (PBMCs). All graphs shown represent the average curves from three to four independent experiments. (E) Average JT001 concentration-response curves for inhibition of LPS + CHC or LPS + MSU-induced IL-1β in mBMDMs. The graphs represent data from two independent experiments.

Figure 2. Effects of JT001 on in vitro and ex vivo NLRP3-mediated IL-1β production downstream of canonical and alternative pathway activation. Concentration-response curves of JT001 for inhibition of LPS + ATP (canonical pathway activation) stimulated IL-1β production in (A) human (h) and mouse (m) PBMCs and (B) human and mouse whole blood. (C) Concentration-response curves of JT001 for inhibition of IL-1β production in human whole blood stimulated with LPS only (alternative pathway activation). All graphs shown represent the average curves from three to eight independent experiments.

Figure 3. Mechanism of action and selectivity of JT001. (A) Average concentration-response curve from two independent experiments showing inhibition of intracellular caspase-1 activity
by JT001 in human PBMCs treated with LPS + Nigericin. (B) Western blot analysis of full-length caspase-1 (p45), cleaved mature caspase-1 (p10 subunit), NLRP3 and β-actin in cell lysates and cell supernatants from mouse bone marrow-derived macrophages (mBMDMs) treated with LPS + nigericin with or without 10 μM JT001 or reference compounds (TAK-242 and emricasan). Blot has been cropped to remove data from other test compounds that were run in wells between JT001 and TAK-242. The full, uncropped blot is shown in Supplementary Figure 6. (C) Representative images and (D) relative quantification of ASC speck formation in mBMDMs stimulated with LPS + nigericin. (E) JT001 concentration-response curve for inhibition of ASC speck formation in mBMDMs treated with LPS + nigericin. (F) Average concentration-response curve of JT001 for inhibition of IL-1β production in mBMDMs after activation of the canonical (LPS + nigericin) and non-canonical (Pam3CSK4 + transfected LPS) NLRP3 pathways. Data shown is from at least two independent experiments. Concentration-response curves of JT001 and the pan-caspase inhibitor Emricasan for IL-1β production downstream of (G) NLRC4 inflammasome activation (LPS + flagellin) or (H) AIM2 inflammasome activation (LPS + poly(dA:dT)) in mBMDMs. Data shown is from a single experiment.

Figure 4. PK/PD profile of JT001 in a murine peritonitis model. (A) Mean ± SEM peritoneal IL-1β concentrations (B) mean ± SD plasma JT001 concentrations from a subset (n=3) of the animals shown in (A) and (C) correlation of plasma JT001 concentrations with mean IL-1β inhibition measured after a single oral dose of JT001 (0.5-5 mg/kg) or vehicle (n=8/group) administered 1 hour prior to the i.p. injection of LPS plus ATP. Dotted lines in (C) represent the mean IC₅₀ and IC₉₀ values of JT001 for the in vitro inhibition of IL-1β production in mouse
whole blood. **(D)** Mean ± SEM peritoneal IL-1β concentrations **(E)** mean ± SD plasma JT001 concentrations and **(F)** correlation of plasma JT001 concentrations with mean IL-1β inhibition measured after a single oral dose of 30 mg/kg JT001 or vehicle (n=8/group) administered at various times (1, 6, 12 or 22 hours) prior to the i.p. injection of LPS plus ATP. Dotted lines in **(F)** represent the mean IC₅₀ and IC₉₀ values of JT001 for the in vitro inhibition of IL-1β production in mouse whole blood. Differences in IL-1β production between JT001 and vehicle treated animals were evaluated using one-way analysis of variance (ANOVA) followed by the Holm-Sidak’s multiple comparisons test and were considered significant at a calculated probability (p) ≤ 0.05.

**Figure 5. Efficacy of JT001 in the Nlrp3 A350V genetic knock-in model of Muckle-Wells syndrome.** Nlrp3A350V/+/CreT mice were treated with 30 mg/kg JT001 (n=4) or vehicle (n=2) for 30 days. Data (except body weight) were plotted as scattergrams showing individual data points and median values ± range for ordinal data and mean values ± SEM for quantitative data. **(A)** body weight change, **(B)** terminal spleen weight as a % of body weight, and **(C)** blood counts for total white blood cells, neutrophils and eosinophils in Nlrp3A350V/+/CreT mice treated with JT001 or vehicle. Dotted lines in cell count graphs indicate representative normal values in naïve C57BL/6 mice. **(D)** Serum ALT concentrations at study termination. **(E)** Hepatic gene expression analyses of Casp1 (caspase-1), Il1b (IL-1β) and Pycard (ASC). **(F)** Representative images and quantitation from histopathological assessment of hepatic inflammation (H&E), neutrophil infiltration (MPO IHC), collagen I/III fiber deposition (PSR), activated hepatic stellate cells (αSMA IHC), and cell death (TUNEL) (Images shown at 40X magnification). **(G)** Hepatic gene expression analyses of Ctgf. Differences in response between treated and control
animals were evaluated using the Mann–Whitney U test for ordinal data and a Student's t-test for quantitative data and were considered significant at a calculated probability (p) ≤ 0.05.

Figure 6. Effect of JT001 treatment on hepatic steatosis, ballooning degeneration and lobular inflammation in a diet-induced AMLN model of NASH. Mice were fed the AMLN diet for 51 weeks and dosed with 10 or 30 mg/kg JT001 for the last 10 weeks of the AMLN diet feeding. Blinded qualitative scoring of H&E stained liver sections for (A) hepatic steatosis, ballooning degeneration, and lobular inflammation. Data are graphed as the number of animals in each group that showed an improvement (lower score), worsening (higher score) or no change (same score) in qualitative score between the pre-treatment biopsy and post-treatment histology. (B) NAFLD activity score (NAS) and (C) qualitative fibrosis score graphed as shown for the data in (A). Differences in scores between treated and control animals were evaluated using the Freeman-Halton extension of the Fisher exact probability test for a 2 by 3 contingency table and were considered significant at a calculated probability (p) ≤ 0.05/2 corrected for multiple comparisons.

Figure 7. JT001 treatment decreases liver fibrosis and neutrophil infiltration in a CDAA-induced model of NASH. Mice were fed a CDAA or control CSAA diet for 20 weeks and dosed with 30 mg/kg JT001 for the last 5 weeks of the diet feeding. (A) Representative images and (B) quantitation of hepatic neutrophil infiltration as assessed by immunohistochemistry for MPO. (C) Representative PSR images of liver tissue and (D) morphometric quantitation of PSR staining from the CDAA-fed mice treated with JT001 or vehicle. (All images shown at 80X magnification) (E) Quantitative analysis of hepatic hydroxyproline content. (F) Hepatic gene expression analyses of Acta2 (αSMA), Col1α1 (Collagen Iα1) and Ctgf. All graphed data is
represented as mean ± SEM. For each graph, the CDAA/Veh group mean was considered the maximum signal (100%) and the CSAA/Veh group mean was considered the baseline signal (0%). The CDAA/JT001 group mean was normalized to these values to determine % inhibition/normalization by JT001 treatment. Statistical significance was assessed using Welch’s unequal variances t-test and considered significant at a calculated probability (p) ≤ 0.05.
## TABLE 1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>NLRP3 activator</th>
<th>Endpoint measured</th>
<th>Ave IC$_{50}$ (nM)$^a$</th>
<th>N number$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hKupffer cells</td>
<td>LPS + nigericin</td>
<td>IL-1β</td>
<td>62 x/± 1.8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-1β</td>
<td>74 x/± 1.1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>&gt;10,000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-1α</td>
<td>94 x/± 1.0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATP</td>
<td>43 x/± 4.8</td>
<td>3</td>
</tr>
<tr>
<td>Human PBMC$^c$</td>
<td>IL-1β</td>
<td>IL-1β</td>
<td>66 x/± 1.4</td>
<td>13</td>
</tr>
<tr>
<td>Mouse PBMC</td>
<td>IL-1β</td>
<td>IL-1β</td>
<td>90 x/± 2.1</td>
<td>4</td>
</tr>
<tr>
<td>Rat PBMC</td>
<td>IL-1β</td>
<td>IL-1β</td>
<td>945 x/± 1.1</td>
<td>2</td>
</tr>
<tr>
<td>NHP$^d$ PBMC</td>
<td>IL-1β</td>
<td>IL-1β</td>
<td>70 x/± 2.1</td>
<td>3</td>
</tr>
<tr>
<td>Human PBMC</td>
<td>LPS + ATP</td>
<td>IL-1β</td>
<td>30 x/± 2.0</td>
<td>3</td>
</tr>
<tr>
<td>Mouse PBMC</td>
<td>IL-1β</td>
<td>IL-1β</td>
<td>25 x/± 1.8</td>
<td>3</td>
</tr>
<tr>
<td>Human Blood</td>
<td>IL-1β</td>
<td>IL-1β</td>
<td>463 x/± 2.1</td>
<td>9</td>
</tr>
<tr>
<td>Mouse Blood</td>
<td>IL-1β</td>
<td>IL-1β</td>
<td>1,112 x/± 1.6</td>
<td>8</td>
</tr>
<tr>
<td>Human Blood</td>
<td>LPS alone</td>
<td>IL-1β</td>
<td>109 x/± 2.4</td>
<td>4</td>
</tr>
<tr>
<td>Mouse BMDM$^e$</td>
<td>LPS + CHC$^f$</td>
<td>IL-1β</td>
<td>9 x/± 1.9</td>
<td>2</td>
</tr>
<tr>
<td>Mouse BMDM$^e$</td>
<td>LPS + MSU$^g$</td>
<td>IL-1β</td>
<td>20 x/± 2.8</td>
<td>2</td>
</tr>
<tr>
<td>Mouse BMDM$^e$</td>
<td>LPS + nigericin</td>
<td>IL-1β</td>
<td>225 x/± 1.6</td>
<td>2</td>
</tr>
<tr>
<td>Mouse BMDM$^e$</td>
<td>Pam3CSK4 + LPS</td>
<td>IL-1β</td>
<td>198 x/± 1.7</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$All IC$_{50}$ values shown are the geometric mean (nM) x/± geometric standard deviation; $^b$LPS = lipopolysaccharide; $^c$PBMC = peripheral blood mononuclear cells; $^d$NHP = non-human primate; $^e$BMDM = bone-marrow derived macrophages; $^f$CHC = cholesterol crystals; $^g$MSU = monosodium urate; $^h$N = number of tests
**TABLE 2**
Pharmacokinetic parameters for JT001 in mouse and rat after intravenous (i.v.) and oral (p.o.) administration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mouse</th>
<th></th>
<th>Rat</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.v.</td>
<td>p.o.</td>
<td>i.v.</td>
<td>p.o.</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)$^a$</td>
<td>-</td>
<td></td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/mL)$^b$</td>
<td>9.3</td>
<td>26.9</td>
<td>5.7</td>
<td>17.8</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)$^c$</td>
<td>1.4</td>
<td>1.4</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>AUC (h*µg/mL)$^d$</td>
<td>15.0</td>
<td>93.6</td>
<td>9.8</td>
<td>41.6</td>
</tr>
<tr>
<td>Dose-adjusted AUC</td>
<td>15</td>
<td>9.4</td>
<td>9.8</td>
<td>8.3</td>
</tr>
<tr>
<td>$C_{\text{pl}}$ (mL/min/kg)$^e$</td>
<td>1.1</td>
<td></td>
<td>1.7</td>
<td>-</td>
</tr>
<tr>
<td>$V_{\text{ss}}$ (L/kg)$^f$</td>
<td>0.1</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>$%F$</td>
<td>-</td>
<td>62.4</td>
<td>-</td>
<td>85.2</td>
</tr>
</tbody>
</table>

$^aT_{\text{max}}$ = time of maximum concentration; $^bC_{\text{max}}$ = maximum concentration; $^c t_{1/2}$ = half-life; $^d$AUC = area under the curve; $^e$ $C_{\text{pl}}$ = plasma clearance; $^f$ $V_{\text{ss}}$ = volume of distribution at steady state; $^g$ $\%F$ = oral bioavailability
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

Panel A: Images showing CDAA/Veh versus CDAA/JT001.

Panel B: Graph showing MPO IHC with 80% normalization.

Panel C: Images showing CDAA/Veh versus CDAA/JT001.

Panel D: Graph showing PSR with 45% normalization.

Panel E: Graph showing hydroxyproline with 41% normalization.

Panel F: Graphs showing relative Acta2, Coll1a1, and Ctgf mRNA (fold-change) with respective normalization percentages.
“Pharmacology of a Potent and Novel Inhibitor of the NOD-like receptor pyrin domain-containing protein 3 (NLRP3) Inflammasome that Attenuates Development of NASH and Liver Fibrosis”

Journal of Pharmacology and Experimental Therapeutics
Manuscript #JPET-AR-2023-001639

Supplementary Figure 1

Supplementary Figure 1. Graphs showing mean ± SEM cytokine concentration in cell supernatants from untreated vs. LPS + nigericin treated human Kupffer cells. Data shown is from one representative experiment.
Supplementary Figure 2. Graphs showing mean ± SEM IL-1β concentrations in cell supernatants from untreated and LPS ± nigericin treated peripheral blood mononuclear cells (PBMCs) from various species. Data shown is from one representative experiment.
Supplementary Figure 3. Graphs showing mean ± SEM IL-1β concentrations in cell supernatants from (A) mBMDMs treated with LPS + cholesterol crystals (CHC) or LPS + monosodium urate crystals (MSU) or (B) human or mouse PBMCs treated with LPS + ATP. Data shown is from one representative experiment.
Supplementary Figure 4. (A) Stability of JT001 as measured by LC-MS/MS after incubation at 37°C in human or mouse whole blood for up to 4 hours. (B) Mean ± SD IL-1β concentrations in plasma from human and mouse whole blood stimulated with LPS + ATP (canonical NLRP3 pathway). Individual points represent the average IL-1β production from 4-6 LPS + ATP treated wells from 7 (human) or 8 (mouse) independent experiments. For the human studies, blood from 5 different donors was used for the 7 independent experiments and each donor is identified by a different color. (C) Plot of IL-1β production versus JT001 IC₅₀ value from the LPS + ATP human blood studies shown in B. (D) Mean ± SD IL-1β concentrations in plasma from human whole blood stimulated with LPS alone (alternative NLRP3 pathway). Individual points represent the average IL-1β production from 4-6 LPS treated wells from 4 independent experiments using blood from 2 different donors. Each donor is identified by a different color.
**Supplementary Figure 5.** Evaluation of caspase protease activity. Screening results of JT001 (upper half) and positive controls (lower half) against the protease activity of 12 different caspase enzymes. Data shown is the % activity relative to the vehicle control. IETD-CHO was used as the positive control for caspases-1, -2, -4, -5, -8, -9, 10, and -11; DEVD-CHO was used as the positive control for caspases-3, -6 and -7 and WEHD-CHO was used as the positive control for caspase-14.
Supplementary Figure 6. Full Western Blot of mBMDM cell lysates and cell supernatants generated from cells stimulated with LPS +/- nigericin in the presence or absence of 10 μM test compound. Lanes for two additional Jecure test compounds, Cmpd B and Cmpd C, were cropped out of the blot shown in Figure 3B.
Supplementary Figure 7. Graphs showing mean ± SEM IL-1β production from wild-type mouse BMDMs treated with (A) LPS + nigericin, (B) Pam3CSK4 + transfected LPS, (C) LPS + transfected flagellin and (D) LPS + transfected poly(dA:dT). (E) Graph showing mean ± SEM IL-1β production from NLRP3 knockout mouse BMDMs treated with L18-MDP. (F) IL-1β production from JT001 treated and LPS + L18-MDP stimulated NLRP3 KO mouse BMDMs as a percent of vehicle control. Data shown for each panel is from one representative experiment.
Supplementary Figure 8

<table>
<thead>
<tr>
<th>Time post-dose</th>
<th>WT Mice*</th>
<th>AMLN NASH Mice*</th>
<th>CDAA HFD Mice**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma (μM)</td>
<td>Liver (μM)</td>
<td>Plasma (μM)</td>
</tr>
<tr>
<td>1h</td>
<td>51.9</td>
<td>24.2</td>
<td>7.3</td>
</tr>
<tr>
<td>2h</td>
<td>6.7</td>
<td></td>
<td>57.9</td>
</tr>
<tr>
<td>4h</td>
<td>29.0</td>
<td>15.7</td>
<td>3.5</td>
</tr>
<tr>
<td>8h</td>
<td>1.6</td>
<td></td>
<td>8.4</td>
</tr>
<tr>
<td>12h</td>
<td>7.6</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>0.04</td>
<td>0.04</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Supplementary Figure 8.** Plasma and Liver Drug Levels in Normal and Diseased Mice. *Drug levels determined in a PK cohort after a single dose of JT001; **Drug levels determined using efficacy study animals at the end of the study.
Supplementary Figure 9. Mouse AMLN DIO Model of NASH. (A) Mean ± SEM body weight during the 10-week drug treatment period. (B) Mean ± SEM liver triglycerides and total cholesterol at study termination. (C) Hepatic MPO expression as a percent of fractional area. Statistical significance assessed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test at p≤0.05.
Supplementary Figure 10. Mouse CDAA model of NASH. Mean ± SEM body weight (A) for the first four weeks of the treatment period and (B) at study termination. Mean ± SEM (C) liver weight as a percent of body weight and (D) serum ALT at study termination after 5 weeks of treatment. Individual, median and range of (E) hepatic inflammation scores (F) and steatosis scores at study termination.
Supplementary Figure 11. Human normal and NASH hepatic stellate cells were serum-starved for 12h then incubated for 24h with 1 μM of NLRP3i (JT001 analogue) or the TGFβR inhibitor LY2157299. Images from control and NLRP3 inhibitor treated cells stained with DAPI and antibodies to α-SMA and collagen I are shown (upper panels) with quantitation of the staining and cell confluency (bar graphs).