The NLRP3 inhibitor MCC950 inhibits IL-1β production in PBMC from 19 patients with Cryopyrin-Associated Periodic Syndrome and in 2 patients with Schnitzler’s Syndrome

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Abstract

Background: The cryopyrin-associated periodic syndromes (CAPS) are a group of inherited disorders associated with systemic auto-inflammation. CAPS result from gain-of-function mutations in NLRP3, which result in formation of an intracellular protein complex known as the NLRP3 inflammasome. This leads to overproduction of IL-1β and other pro-inflammatory signals, resulting in inflammatory symptoms. Treatments for NLRP3-related diseases are biologic agents that directly target IL-1β. We sought to determine if the orally available small molecule NLRP3 inhibitor MCC950 could inhibit IL-1β ex vivo in a cohort of patients with autoinflammatory disease.

Methods: Patients were recruited to donate blood, from which PBMCs were isolated and assayed in the presence of MCC950 to determine inhibitory efficacy.

Results: We found that apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and mature IL-1β was higher in ex vivo PBMCs from CAPS patients than healthy donors. MCC950 inhibited production of mature IL-1β in PBMC from CAPS patients with a range of mutations and blocked NLRP3 activity in an in vitro mutation reconstitution assay. Similar results were observed with
PBMC from two patients with Schnitzler’s Syndrome, another auto-inflammatory disease.

**Conclusions:** The NLRP3 inflammasome inhibitor MCC950 blocked constitutive activation of NLRP3 observed in the PBMCs of CAPS patients. This study highlights the potential utility of NLRP3 inhibition by a small molecule for rare autoinflammatory diseases that are driven by NLRP3.

**Keywords**
Inflammasome, NLRP3, CAPS, MCC950, Schnitzer’s Syndrome, ASC

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Introduction

Families of highly conserved pattern recognition receptors (PRRs) have evolved to occupy cellular membranes and cytosolic compartments of certain immune cells such as macrophages and dendritic cells. Upon activation, a subset of intracellular receptors denoted Nod-like receptors (NLRs) assemble and oligomerize to form multiprotein intracellular complexes called inflammasomes which can initiate a caspase-1 dependent cascade leading to the production of pro-inflammatory cytokines IL-1β and IL-18. The NLRP3 inflammasome has recently become the subject of intense clinical and scientific interest. NLRP3 drives the release of the pro-inflammatory cytokines IL-1β/IL-18 and a type of cell death called pyroptosis (lytic cell death). NLRP3-driven inflammation contributes to chronic inflammation in humans and has been implicated in the development of many diseases, ranging from autoimmune conditions to indications such as atherosclerosis, heart failure, stroke, obesity and type 2 diabetes. Specific pharmacological inhibition of NLRP3 may have therapeutic potential in these conditions and represents a promising avenue for clinical investigation.

MCC950 has been identified as a potent and specific NLRP3 inhibitor. The precise mechanism of action of MCC950 was described by two recent complementary studies. MCC950 binds to both active and inactive NLRP3, in a high-affinity non-covalent interaction at or adjacent to the Walker B motif, thereby blocking the ability of NLRP3 to hydrolyse ATP for NLRP3 inflammasome function. Tapia-Abellán et al. report that NLRP3 undergoes structural rearrangements to an open conformation during activation, and that MCC950 counters this via interaction with the NLRP3 Walker B site, maintaining NLRP3 in a closed, inactive conformation. As the open conformation is required for NLRP3 activity, binding of MCC950 to NLRP3 inhibits its oligomerization and inflammasome function.

Cryopyrin-associated periodic syndromes (CAPS) are a rare family of heterogeneous autoinflammatory diseases characterized by IL-1β-mediated systemic inflammation and clinical symptoms involving skin, joints, central nervous system, and eyes. CAPS is associated with an activating mutation in NLRP3, of which approximately 200 mutations have been defined. While some mutations have been classed as benign and present with no deleterious clinical phenotype, approximately 100 of the reported mutations result in a chronic pro-inflammatory presentation. CAPS is categorized into three clinical subgroups of increasing severity: familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease (NOMID), also called chronic infantile neurologic cutaneous and articular syndrome or CINCA. Mice that harbour the mutant forms of NLRP3 that occur in CAPS die in the neonatal period and have increased concentrations of circulating IL-1β and IL-18. Coll et al. previously demonstrated that MCC950 inhibited NLRP3 activation in a mouse model of MWS. Peripheral blood mononuclear cells (PBMC) from patients with low penetrance NLRP3 variants (Q703K and V198M) have been shown to display enhanced IL-1β levels following inflammasome activation compared to healthy controls. Furthermore, release of IL-1β has been shown to be NLRP3-dependent as it was blocked by MCC950. The IL-1 receptor antagonist anakinra is conventionally used to control the symptoms of the syndrome, but patients relapse when treatment is withdrawn.

Other auto-inflammatory diseases also feature elevated IL-1β, such as Schnitzler’s syndrome. Schnitzler’s syndrome is a rare systemic auto-inflammatory disease. Its main clinical features include fever, a chronic urticarial skin rash, arthralgia and enlarged lymph nodes. Canakinumab has been reported to be an effective treatment.

The hypothetical clinical utility of NLRP3 inhibition in CAPS patients is clear. As detailed above, a number of small ex vivo studies have indicated that MCC950 is efficacious inhibiting pathological IL-1β production in these patients when tested on a small number of ex vivo samples. However, CAPS represent a heterogenous cohort of patients with numerous causative mutations, displaying a wide spectrum of disease severity. Furthermore, recent research has called into question whether all causative CAPS mutations can be inhibited by MCC950. A limitation of these studies is their reliance on artificial in vitro systems to model the CAPS autoinflammatory phenotype. This study aims to both broaden the coverage of CAPS mutations and utilize CAPS patient samples in combination with an artificial in vitro system involving doxycycline-induced expression of mutant forms of NLRP3. This study provides evidence of the efficacy of MCC950 in the inhibition of aberrant IL-1β production with a broad range of ex vivo samples from the largest cohort of CAPS patients reported to date, and for the first time investigates the ex vivo efficacy of an NLRP3 inhibitor in other auto-inflammatory diseases that are characterised by elevated IL-1β levels.

Results

19 CAPS patients were recruited from Irish and UK centres, comprising of 6 FCAS patients, 12 MWS patients and 1 NOMID patient. 4 patients with other auto inflammatory conditions were also recruited (Table 1). We first confirmed that MCC950 could inhibit NLRP3 in human PBMC and also in human whole blood. NLRP3 requires two signals for activation in healthy donor PBMCs, a priming signal to upregulate the expression of NLRP3 inflammasome machinery and the pro form of IL-1β, and a secondary signal to stimulate processing of IL-1β to its mature form. Upon stimulation with LPS and LPS plus nigericin, increased IL-1β was detected in the supernatant. As shown in Figure 1a, the IC50 of MCC950 in human whole blood found to be 627nM. This value is higher than the IC50 reported for isolated bone marrow derived macrophages (BMDM) and human monocyte-derived macrophages (HMDM) by previous studies, presumably due to the binding of MCC950 to protein components in whole blood lowering the effective free drug concentration.
Table 1. Patient demographic information and clinical diagnoses.

<table>
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<tr>
<th>Patient number</th>
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<th>NLRP3 mutation</th>
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<tr>
<td>21</td>
<td>Schnitzler’s Syndrome</td>
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<tr>
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<td></td>
<td>n/a</td>
<td>Adult</td>
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We tested the plasma from CAPS patients for relevant NLRP3-linked markers. Elevated plasma free ASC (Figure 1b) and IL-1β (Figure 1c) were detected in patient samples relative to healthy donor donations.

As observed by others, when compared to healthy donors, CAPS patient PBMCs and whole blood samples required only a priming LPS signal to induce mature IL-1β production into the supernatant, whereas healthy donor samples required both a priming LPS signal and a subsequent nigericin stimulation to initiate IL-1β production to similar levels (Figure 1d).

MCC950 inhibited the production of IL-1β from CAPS patient PBMCs in response to LPS. Significant impairment of IL-1β production was detected in 12 MWS patients harbouring a variety of mutations in NLRP3: V198M, R260W, E311K, A349V, GS64D and E567K (Figure 2a). MCC950 also reduced production of IL-1β in PBMCs from 6 FCAS patients of three defined mutations, Y563N, Y563I and E525K (Figure 2b). While only one NOMID patient with a confirmed D303N mutation was recruited as part of this work, promising inhibition by MCC950 in this patient sample was identified (Figure 2c). Data from extended dose responses in 4 MWS patients were combined to calculate an IC₅₀ for MCC950 of 70.4 nM, which is comparable to that of healthy donor PBMC at 41.3 nM (Figure 2d).

To provide a second line of evidence for inflammasome inhibition, western blots were also performed in PBMC cell lysates and supernatants from CAPS patients to quantify IL-1β and caspase-1 in these samples. MCC950 treatment reduced IL-1β p17 and caspase-1 p20 levels in the cell supernatant and boosted pro-caspase-1 levels in the lysate (Figure 1e).

An in vitro reconstitution model of a mutation found in CAPS patients was also used. IL-1β production following the introduction of NLRP3 mutation D303N into a NLRP3 deficient iBMDC cell line was inhibited by MCC950 (Figure 2f).

As NLRP3 can drive lytic cell death, LDHA was quantified in supernatant from LPS-treated CAPS PBMCs to determine if MCC950 could impact LDHA levels, but no significant change from baseline was determined (Figure 3a). IL-6 and TNFα were also measured in the supernatant of LPS-stimulated CAPS patient PBMCs. MCC950 had no effect on the production of TNFα and IL-6 (Figure 3b and c).

As IL-1β production in response to LPS is highly variable between individual CAPS patients, the use of normalised values for inhibition calculations was used, however raw data are also provided. Significant inhibition was seen at 500 nM MCC950 in the MWS cohort (Figure 3d). An inhibitory trend was measured in FCAS patient PBMC (Figure 3e).

Canakinumab has been documented to form a complex with free IL-1β in the serum of patients and serves to extend the half-life of IL-1β in serum, allowing for its detection. However, we saw no correlation between increased IL-1β plasma levels in patients known to be receiving Canakinumab over those taking Anakinra (Figure 3f).

Patients with other auto-inflammatory conditions were recruited as part of this study. PBMCs were isolated from 3 patients with Schnitzler’s Syndrome. While this conditions are not known to be specifically driven by NLRP3, IL-1β production by LPS-treated PBMCs was showed a trend towards inhibition with MCC950 pre-treatment (Figure 2g).

Discussion

The current therapeutic approach for CAPS targets IL-1β and its cognate receptor. This is achieved via the use of biologics such as canakinumab (a human monoclonal targeting IL-1β) and anakinra (a recombinant version of the human IL-1 receptor antagonist). However, the use of IL-1β blockade presents problems. Firstly, it is currently only achieved with biologics, which can cause injection site reactions, which in the case
Figure 1. MCC950 is a potent and specific inhibitor of NLRP3 inflammasome activation. Cryopyrin-associated periodic syndrome (CAPS) patients have increased plasma biomarkers NLRP3 activation relative to healthy donors. IL-1β inhibition by MCC950 in (A) human whole blood (n=186). (B) Plasma quantification of free ASC in CAPS patients. Healthy donors n=6, Muckle-Wells syndrome (MWS) n=13, Familial cold autoinflammatory syndrome (FCAS) n=5, neonatal-onset multisystem inflammatory disease (NOMID) n=1 (C) Plasma quantification of IL-1β in CAPS patients. Healthy donors n=7, MWS n=13, FCAS n=6, neonatal-onset multisystem inflammatory disease (NOMID) n=1 (D) IL-1β concentrations in the supernatant of healthy donor, CAPS and other autoinflammatory patient peripheral blood mononuclear cells (PBMC) left unstimulated, LPS primed (1 hr) or stimulated with LPS plus nigericin for 20 min. P values calculated using one-way ANOVA for multiple comparisons. Data represent mean ± SEM. Blots are representative of three independent experiments.
Figure 2. MCC950 inhibits IL-1β production from cryopyrin-associated periodic syndrome (CAPS) patient peripheral blood mononuclear cells (PBMC). (A) Normalised IL-1β quantification in the supernatant of LPS-primed (1 hr) Muckle-Wells syndrome (MWS) n=12, (B) Familial cold autoinflammatory syndrome (FCAS) n=6 and (C) NOMID patient n=1 PBMC in the presence or absence of MCC950 for 3 hr (D) IC50 for MCC950 in MWS patient PBMC (E) Western blot for IL-1β, pro-IL-1β, Caspase-1 P20, pro-Caspase-1 and Beta Actin from CAPS patient PBMC left unstimulated or LPS primed (1 hr) in the presence or absence of MCC950 for 3 hr. (F) NLRP3 mutant D303N reconstitution assay in the presence of MCC950 (G) IL-1β quantification in PBMC supernatants from Schnitzler Syndrome patients (n=3). Cells were LPS-primed (1 hr) in the presence or absence of MCC950 for 3 hr, P values calculated using one-way ANOVA for multiple comparisons or two-tailed Student’s t-test for paired comparisons. Blots are representative of three independent experiments. Each n represents an individual donor.

of anakinra can require daily administration. Secondly there is a risk of infection, since IL-1β driven by other inflammasomes which sense pathogens (e.g. the inflammasome NLRC4 is activated by bacterial flagellin) is also blocked. Furthermore, some biologic agents have long half-lives, which means that they cannot be quickly withdrawn should the patient...
Figure 3. MCC950 has no effect on LDHA release or TNF and IL-6 production by LPS-treated PBMC from CAPS patients. IL-1β production by LPS-treated PBMC from CAPS patients is highly variable and IL-1β blockade does not impact plasma IL-1β quantification. (A) LDHA release by LPS-primed (1 hr) CAPS patient PBMC in the presence or absence of MCC950, n=5. (B) TNFα quantification in the supernatant of LPS-primed (1 hr) CAPS patient PBMC in the presence or absence of MCC950 for 3 hr, n=7. (C) IL-6 quantification in the supernatant of LPS-primed (1 hr) CAPS patient PBMC in the presence or absence of MCC950 for 3 hr, n=6. (D) IL-1β quantification in the supernatant of LPS-primed (1 hr) MWS n=12, FCAS n=6. (E) Plasma quantification of IL-1β in CAPS patients, canakinumab or anakinra treated indicated where known. P values calculated using one-way ANOVA for multiple comparisons or two-tailed Student’s t-test for paired comparisons. Data represent mean ± SEM. Each n represents an individual donor.
acquire an infection where immune suppression would prove detrimental\textsuperscript{9}. An orally available alternative with a relatively short half-life would be preferable. An additional property that confers superiority of small molecule inhibitors over biologics is the potential to cross the blood brain barrier, which could facilitate inhibition of NLRP3 in the CNS. There is also a clear requirement for an improved therapeutic arsenal in certain cases e.g. severe somatic mutations when conventional therapy may not be efficacious\textsuperscript{14}.

In this study we have explored the efficacy of a specific NLRP3 inhibitor MCC950 on \textit{ex vivo} samples from CAPS patients. We have found inhibition against a range of different patient mutations. In spite of the CAPS patients maintaining anti-IL-1β therapy, elevated IL-1β was detected in the serum of the patients. The trend towards elevated IL-1β in the MWS patients should be viewed as both a validation of a diseased state and also an indication that these patients may benefit from further IL-1β blockade.

Patients with both high penetrance and low penetrance mutations were included as part of this study. In particular, 4 patients with the low penetrance V198M mutation were included as part of the MWS cohort. While a previous study using frozen CAPS PBMC indicated an intermediate disease state for low penetrance mutations such as V198M, falling between healthy donors and full penetrance variants in terms of aberrant IL-1β production, our patients did indeed exhibit elevated levels of IL-1β production, as demonstrated by IL-1β production in response to LPS stimulation alone. The study in question utilised frozen PBMCs and the authors concede that a blunted stimulatory effect occurs following a freeze thaw cycle of the cells. Possibly the use of fresh PBMCs in our experimental set up allows for a more accurate and sensitive means to detect the increased IL-1β production in response to LPS alone in the mutations tested\textsuperscript{8}.

The FCAS cohort of patients provided the weakest data in support of the inhibition of IL-1β by MCC950. However, IL-1β production from individual CAPS patients following stimulation with LPS was found to be highly variable. This necessitated the use of normalised maximum production values of IL-1β in order to quantify the inhibitory effect. It is important to note that the FCAS patients studied had lower levels of IL-1β both in serum and in response to LPS treatment, leading to a lower level baseline of inhibitory potential.

Pelegrin demonstrated that certain mutations may result in an impaired inhibition by MCC950. Evidence for this centred on the NLRP3 303 site which is located in the NACHT domain\textsuperscript{9}. Our study included patients harbouring a mutation at the D303N site that could be fully inhibited by MCC950, which was confirmed in our \textit{in vitro} assay to model NLRP3 mutations. Further investigation into this is required with additional patients with relevant NLRP3 mutations in order to establish the inhibitory efficacy of MCC950 in this patient cohort.

LDHA assays were also used to quantify cell death, a process that can be initiated by NLRP3 activation. However, elevated cell death was not apparent in the PBMCs of the CAPS patients relative to healthy donors. While in mouse monocytes NLRP3 activation has been shown to lead to increased pyroptosis, in human monocytes this process has not been detected to date\textsuperscript{10}. Systemic pyroptosis of activated cells in patients harbouring NLRP3 mutations could lead to catastrophic inflammation.

Patients with a non-NLRP3-mediated auto-inflammatory disease, Schnitzler’s Syndrome, were included as part of the \textit{ex vivo} study. Interestingly, some of these patients exhibited striking NLRP3 activity and overproduction of IL-1β relative to healthy donors. While increased donor numbers are required to explore this further, in the three patients MCC950 inhibited IL-1β production, indicating that specific inhibition of NLRP3 in this patient cohort may be beneficial. In particular, one Schnitzler’s Syndrome patient produced IL-1β in a range comparable with the upper end of production of IL-1β in the CAPS patients under the same conditions, this activation was inhibited by MCC950 and therefore provides novel evidence for the potential involvement of NLRP3 in this disorder. A further Schnitzler’s patient exhibited a profile that resembled a CAPS patient and the final patient showed little to no IL-1β production, similar to a healthy donor. Like FCAS, the symptoms of these auto-inflammatory conditions are known to flare, and they exhibit variable responses to the current therapies. The efficacy of MCC950 in inhibiting IL-1β production in these patients is testament to the potential value of investigating small molecule inhibition of NLRP3 in this cohort during flares.

Published literature continues to support the involvement of NLRP3 in a plethora of other conditions, from cardiovascular indications to Alzheimer’s Disease\textsuperscript{16}. Here we provide evidence for modulation of a disease-relevant pathway by a small molecule in autoinflammatory patients. MCC950 can effectively block aberrant IL-1β production \textit{ex vivo} in 19 patients with active NLRP3-mediated disease. In summary our investigation is an informative study providing insight into the potential efficacy of a small molecule NLRP3 inhibitor in the largest cohort of CAPS patients to date.

Clinical implications
Inhibition of NLRP3 using small molecule inhibitors is a possible novel therapeutic strategy for the treatment of autoinflammatory diseases that present with elevated serum IL-1β.

Methods
Study population
All CAPS patients had confirmed NLRP3 mutations and symptoms consistent with FCAS, MWS or NOMID. Patients with clinical diagnoses of Schnitzler’s syndrome were also included in the study. Recruitment was carried out by members of the patient’s own clinical team between November 2017 and April 2019. Patients were approached to provide a
voluntary blood sample for the study. Patients were provided with an information leaflet 24 hours in advance of sample donation. All patients or their parents/legal guardians gave informed written consent. Sample collection was carried out in the Clinical Research Facility, St. James’s Hospital or Children’s Health Ireland, Dublin. Direct travel costs were covered but no incentive or compensation was offered. Detailed patient demographics were not recorded. Patients were receiving standard anti-IL-1 therapy (Anakinra). This treatment was not discontinued prior to blood sampling. Healthy blood donations were obtained by means of voluntary donation from staff and students in the Trinity Biomedical Science Institute, Dublin. No incentive or compensation was offered for these donations.

Ethical statement/study approval
The study was conducted in accordance with the Declaration of Helsinki and approved by Institutional Review Board at Trinity College Dublin, the Joint Research Ethics Committee of St James Hospital and Tallaght Hospital (REF 2017-11), the Ethics (Medical Research) Committee of Our Lady’s Children’s Hospital, Crumlin (Now Children’s Health Ireland) (REF: GEN/577/17) and the School of Biochemistry and Immunology Research Ethics Committee (REF: BI-SC-010118).

Collection of PBMC and serum
45 ml whole blood was collected from adult patients into Lithium Heparin tubes (Greiner, Cat. no. 455084), and a volume appropriate to the patient age and weight from the paediatric patients. Following donation, blood samples were maintained at room temperature and processed for PBMC isolation within 90 min of blood donation. Whole blood was diluted 1:1 in PBS and PBMCs were isolated using a Lymphoprep™ (STEMCELL Technologies, Cat. no. 07861) density gradient. Serum was removed from the top of the density gradient and stored at -80°C. PBMCs were removed and washed twice in PBS and counted using a haemocytometer. Isolated PBMC were plated at 2x10⁵ cell/mL in a 12 well plate in RPMI (Gibco Cat. no. 61870-010) supplemented with 10% Penicillin/Streptomycin and MCC950 at 37°C 5% CO₂ before experimental treatment commenced.

Inflammasome assay
PBMC were either left untreated or stimulated with 1μg/mL LPS (Enzo, Cat. no ALX-581-010-L002) and incubated for one hour at 37°C 5% CO₂. The media was then replaced with RPMI supplemented with 10% Penicillin/Streptomycin and MCC950 (Sigma, Cat. no. P4333). Cells were left to rest for 2 hours at 37°C 5% CO₂, for one hour prior to the addition of 20 μM nigericin for 20 minutes. The supernatants were then harvested cells were lysed.

ELISA
Human IL-1β ELISA kits (R&D, Cat. no. SLB50) were used to quantify IL-1β in cell supernatants and serum. Human TNF alpha and IL-6 were quantified by ELISA (R&D, Cat. no. DY206 and DY210) in cell supernatants. Free ASC was measured by ELISA in serum samples (Cusabio, Cat. no. CSB-EL019114HU). These kits utilised a microplate reader set at 450 nm to determine the optical density of the wells. Human AlphaLISA Detection Kits (Perkin Elmer, Cat. No. AL220) were used to quantify IL-1β in whole blood when read on an AlphaLISA-enabled spectrophotometer at 615 nm.

Western blot
Cells were lysed in SDS sample loading buffer and boiled for 5 minutes. Sample lysates were run on a SDS page gel, transferred to Immobilon-P PVDF (Millipore, Cat. no. IPVH09010) membrane and probed for pro-IL-1β (R&D, Cat. no. AF-201-NA, polyclonal goat IgG), IL-1β p17 (R&D, Cat. no. AF-201-NA, polyclonal goat IgG), IL-1β (Abcam, Cat. no. ab71495, polyclonal rabbit IgG), Caspase-1 (Cell Signalling Cat. no. D57A2, monoclonal rabbit IgG) and beta actin (Sigma Cat. no. A5316 monoclonal mouse IgG). All antibodies were used at a dilution of 1:1000 in 5% powered milk for 2 hours at room temperature, with the exception of beta actin which was used under the same conditions but at a dilution of 1:5000. Peroxidase-conjugated AffiniPure goat anti-rabbit (Jackson ImmunoResearch Cat. no. 111-035-144), bovine anti-goat (Jackson ImmunoResearch Cat. no. 805-035-180), and goat anti-mouse (Jackson ImmunoResearch Cat. no. 115-035-146) IgG were used as secondary antibodies at a dilution of 1:2000 in 5% powered milk, prior to development with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Cat. no. WBKLS0500) using a ChemiDoc Imaging System (Bio-Rad). The study was conducted in accordance with the Declaration of Helsinki and approved by Immunology Research Ethics Committee (REF: BI-SC-010118) and the School of Biochemistry and Immunology Research Ethics Committee (REF: BI-SC-010118).

Expression of human NLRP3 mutants in NLRP3-deficient mouse macrophages
NLRP3-deficient iBMDM expressing Tet3G transactivator were transduced with amphotropic retrovirus encoding specific human NLRP3 mutant NLRP3-deficient iBMDM were a kind gift from Kate Fitzgerald (UMass, MA) and the recipient Tet3G cells are described elsewhere. To produce the retrovirus 1.5–2x10⁶ cells of packaging cell line (Gryphon Amrho, Allele Biotech) per well were plated in a 6 well plate and left overnight at 37°C 5% CO₂. The next day, cells were transfected with 4 μg of the plasmid DNA (synthetic gene encoding NLRP3 mutant was prepared by Twist Biosciences) using 10 μL of Lipofectamine 2000 (Invitrogen). The next day, the medium was exchanged. 4x10⁵ recipient cells were seeded per well of 6-well plate. The following day the media of recipient NLRP3-deficient iBMDM was changed to DMEM +10% FBS+ polybrene (2 μg/ml, Sigma). The retroviral supernatant from the packaging cells was filtered through sterile 0.45 μm syringe filter. The retroviral supernatant was added dropwise to the recipient NLRP3 deficient iBMDM. After 24 hours, the medium was removed and the cells transferred to a 9 cm Petri dish.
1.5 mg/ml G418 and 6 μg/ml puromycin were added to the culture for selection. The cells were maintained in this manner until use in an inflammasome assay.

Statistics
Statistical analysis and IC\textsubscript{50} calculations were carried out using GraphPad Prism 8 (GraphPad Inc.). P values calculated using one-way ANOVA for multiple comparisons or two-tailed Student’s t-test for paired comparisons. Data represent mean ± SEM. Each n represents an individual donor. P < 0.05 was considered statistically significant.

Data availability
Open Science Framework: The NLRP3 inhibitor MCC950 inhibits IL-1β production in PBMC from 19 patients with Cryopyrin-Associated Periodic Syndrome and in 2 patients with Schnitzler’s Syndrome. https://doi.org/10.17605/OSF.IO/2WBGE

This project contains the following underlying data:
- Data summary.xlsx (summary of underlying data)
- Blot_Beta_actin.tif (Raw gel image for beta actin)
- Blot_Casp1_p20.tif (Raw gel image for Caspase 1)
- Blot_IL1B.tif (Raw gel image for supernatant IL1B)
- Blot_pro-Il1b.tif (Raw gel image for lysate IL1B)
- CAPS Patient PBMC LDHA.xlsx (Raw data for LDH assays)
- CAPS Patient PBMC Raw data ELISA.xlsx (Raw data for IL1B ELISA)
- CAPS Patient PBMC Raw data IL6 and TNFa ELISA.xlsx (Raw data for IL-6 and TNFa ELISA)
- Healthy donor whole blood Raw data alphaELISA.xlsx (Raw data for IL1B ELISA in healthy whole blood)
- In vitro mutagenesis D303N.xlsx (Raw data for IL1B ELISA from mutagenesis assay)
- Patient Plasma ASC Raw data ELISA.xlsx (Raw data for ASC ELISA)
- Patient Plasma IL1B Raw data ELISA.xlsx (Raw data for IL1B plasma ELISA)

Acknowledgements
We would like to thank the staff of the Clinical Research Facility in St. James’s Hospital and Children’s Health Ireland at Crumlin for their assistance. We would also like to thank the clinicians (including Drs. Helen Lachmann, Lisa Devlin, Ronan Leahy, Sonia Melo Gomes, Claire Sheehy, John Ryan, Sinisa Savic, Dennis McGonagle and John McConville) that assisted us with recruitment for this study by means of patient referral. We would like to thank Snygature Discovery, Nottingham for their contribution to this work. Prof. Kate Fitzgerald (UMass, MA) kindly provided the murine BMDM which were used to produce the NLRP3-deficient iBMDM cell line. Finally, we would like to express our gratitude to the patients and legal guardians who participated in this study.

References
11. Corcoran S: The NLRP3 inhibitor MCC950 inhibits IL-1β production in PBMC from 19 patients with Cryopyrin-Associated Periodic Syndrome and in 2 patients with Schnitzler’s Syndrome. Open Science Framework. 2020. [PubMed Abstract] [Publisher Full Text] [Publisher Full Text]
Open Peer Review

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Sannula Kesavardhana

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Cryopyrin-associated periodic syndromes (CAPS) are autoinflammatory conditions caused by NLRP3 inflammasome activating mutations promoting IL-1b mediated systemic inflammatory responses. Anakinra (IL-1R antagonist) and Canakinumab (antibody against IL-1b) target IL-1b function and alleviate inflammatory responses. Canakinumab is tested for mitigating CAPS associated clinical symptoms. However few observations like relapse after withdrawal of the Canakinumab and sensitivity to microbial infections are concerning. MCC950 is a selective small molecule inhibitor of NLRP3 with a wide exploration of its effect in various disease models in recent times. Here, the authors address the efficacy of MCC950 on inhibiting NLRP3 activation and IL-1b release in a broad range of CAPS mutations by employing CAPS patient samples (ex vivo). The results in this manuscript demonstrate that MCC950 blocks the IL-1b release in ex vivo cultured PBMCs from CAPS patients with distinct NLRP3 mutations.

Although not comprehensive, this work is appropriate and makes a clear contribution to bringing advancement in MCC950 usage in autoinflammatory diseases. CAPS patient PBMCs representing a wide range of NLRP3 mutations and freshly prepared PBMCs for conducting experiments support this work's conclusions.

Specific comments

1. Very few number of samples of NOMID and Schnitzler’s Syndrome patients were used to study the MCC950 effect and need to increase the sample size for supporting conclusions.

2. Variations in MCC950 efficacy with distinct CAPS mutations need further discussion.

3. MCC950 treatment-induced inhibition of IL-1b production is less in FCAS-PBMCs than MWS. Also, overall ASC specks and IL-1b in plasma samples of FCAS patients is much less than MWS. Is this due to mutations at different positions of NLRP3? Although the authors mentioned this in the discussion, providing specific reasons for these variations is necessary.
4. Why high LPS concentration (1mg/ml) was used for PBMC stimulations.

5. Figure-3d and 3e – MCC950 concentration is mentioned as 500uM instead of 500nM.

6. Figure 2d: MCC_950 instead of MCC950.

7. Figure-2e: In manuscript text, figure-2e is mentioned as 1e.

Is the work clearly and accurately presented and does it cite the current literature?  
Yes

Is the study design appropriate and is the work technically sound?  
Yes

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
Partly

Are all the source data underlying the results available to ensure full reproducibility?  
Partly

Are the conclusions drawn adequately supported by the results?  
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Inflammasomes and cell death

I confirm that I have read this submission and believe that I have an appropriate level of 
expertise to confirm that it is of an acceptable scientific standard, however I have 
significant reservations, as outlined above.

Reviewer Report 11 November 2020
https://doi.org/10.21956/wellcomeopenres.17680.r41174

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The authors have examined whether a small molecule NLRP3 inhibitor, MCC950, could inhibit
IL-1β production *ex vivo* in a cohort of patients with autoinflammatory disease (19 with CAPS, and 2 with Schnitzler’s Syndrome (SchS)). They used plasma free ASC levels and mature IL-1β as readouts of inhibition. The authors conclude the MCC950 blocked constitutive activation of NLRP3 in the PBMCs of 19 patients with CAPS and in 2 patients with SchS.

**General comments**

This is timely study due to the current interest in the use of small molecule inhibitors of the NLRP3 inflammasome in clinical practice. Overall, MCC950 appears to have good inhibition of the NLRP3 inflammasome, based on the IL-1β data, particularly in CAPS patients (Figure 3d). However, these effects are variable in extent and very high doses of both LPS (1mg/mL) and of the drug MCC950 (500nM) were used. MCC950 also blocked NLRP3 activity in an *in vitro* mutation reconstitution assay. Similar results were observed with PBMC from two patients with SchS; one SchS patient did not produce an excess of IL-1β and unsuitable for the study. Therefore, question remains regarding potential use of MCC950 compared to the current effective anti-IL-1 therapies, based on this report.

They acknowledge the limitations of the study and have not provided clinical information as to the degree of response in the patients from whom the samples were taken. A number of factors might be taken into consideration regarding this study.

1. A very high dose of LPS (1mg/mL, compared to the usual 10ng/mL, or maximally 100ng/mL) was used for the stimulations (with potential for artifacts). It would be interesting to see similar experiments with lower doses of LPS and nigericin/ATP as a second stimulus rather than with LPS alone.

2. The dose of the drug MCC950 (500nM) is also quite high (20 mM or less may be used in *ex vivo* work) but may have been used in the context of establishing dose-response curves or maximal responses. Considering the high IC50 established in whole blood, and the implications that has for treatments, may be useful to have the corresponding LDHA release data for the higher dose. These points could be justified and discussed.

3. The data from Figure 2a and 2e do not correlate well; in Figure 2a the 50nM concentration of MCC950 shows a significant increase in % of IL-1β inhibition; however, this isn't replicated in the western blot.

4. Figure 1b; Difficult to see how the NOMID data be can non-significant, compared to Healthy Donors, as there is a very large detection of ASC specks in the NOMID patient?

5. Access to treatment data for patients would be helpful in Fig 1B; if the diseases were labelled as in fig 1C it would make the overall figure clearer i.e. labeling the X axis on Fig 1B would make it more consistent.

6. Similar clinical information could improve figures such as Fig 2G, where the IL-1β release between the patients is hugely variable - could this be due to existing medications?

7. Standard errors of the mean (SEM) are large but perhaps may be expected with these patients' samples due to variation between patients.

**Specific comments**

Page 4 the statement ‘Elevated plasma free ASC (Figure 1b) and IL-1β Figure 1c) were detected in
patients samples relative to healthy donor donations”; can this statement be made, definitively, on data presented as only CAPS patients Figure 1c) show significant increase in IL-1β levels.

Page 4; there's a typo in reference to the western blot; it should say Fig 2e instead of 1e.

There's also a typo in “LPS-treated PBMCs was showed a trend” change to “PBMCs showed a trend”

Page 8 – suggest changing “Pelegrin demonstrated ..” to either “Tapia-Abellán et al demonstrated ..”. or “Pelegrin's group have demonstrated ..”

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Autoinflamamation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.