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Inflammasome Biology in Drug Discovery: **Overcoming Selectivity and Safety Challenges**

Overview

Since chronic inflammation is a key contributor to inflammatory, metabolic, neurodegenerative and autoimmune disorders, inflammasome inhibitors have a broad therapeutic potential, making them highly attractive to drug developers. Recent breakthroughs in inflammasome biology have led to a new generation of therapeutics designed to target the proteins that drive release of inflammatory cytokines. A key milestone in successfully bringing this promising new class of therapeutics to market lies in ensuring that these drugs are effective *in vitro* and *in vivo,* on target at a suitable dose, and display no off-target toxicity.

Selection of the most appropriate assays, reference controls and output measures is critical to ensure that the efficacy of candidate therapeutics is quantified accurately and the best leads are progressed into clinical trials.

Explore how a complete understanding of the inflammasome is necessary to support pre-clinical assay selection and overcome safety concerns, and learn more about our suite of inflammasome assays designed to interrogate the efficacy of your candidate therapeutics.

The Inflammasome is a Dynamic Target for Inflammatory Disease Therapy

Inflammatory responses are essential to prevent harmful infections from spreading within the human body while promoting repair. Although inflammation is usually protective, excessive responses lead to immune-mediated pathology, exacerbating existing conditions or driving new ones. A key mediator of inflammation is IL-1β.

Since 2002, scientists have known that macrophages are a crucial source of IL-1β, and that caspase-1 is the main catalyst for converting pro-IL-1β into active IL-1β, but the mechanism that drives IL-1β secretion remained elusive. That was until a caspase-activating complex – the inflammasome – was identified in the monocyte-like THP-1 cell line [1]. Within the inflammasome, assembly of a sensor protein (such as NLRP1), an adaptor protein (such as ASC), and an effector protein (such as caspase-1) leads to catalytical activation of the effector protein and subsequent IL-1β secretion.

Since then, our understanding of the inflammasome has grown at a phenomenal rate (Figure 1). Inflammasomes are large intracellular protein complexes that assemble in response to danger signals and function as key components of the innate immune system. Caspase-1 converts pro-IL-1β and pro-IL-18 to their active forms and cleaves gasdermin D into fragments that assemble into membrane pores, initiating pyroptosis, an inflammatory form of programmed cell death [2]. It is now understood that different pattern recognition receptors (PRR), including those from the NLR, TLR, CLR and RLR families, can drive formation of different inflammasomes. Furthermore, while the canonical inflammasome activation pathway is associated with caspase-1 activation, non-canonical activation via caspase-4 and caspase-5 can also mediate release of IL-1β and IL-18 and cleavage of gasdermin D [3]. With each of these findings came promising new targets for biologics or chemotherapy.

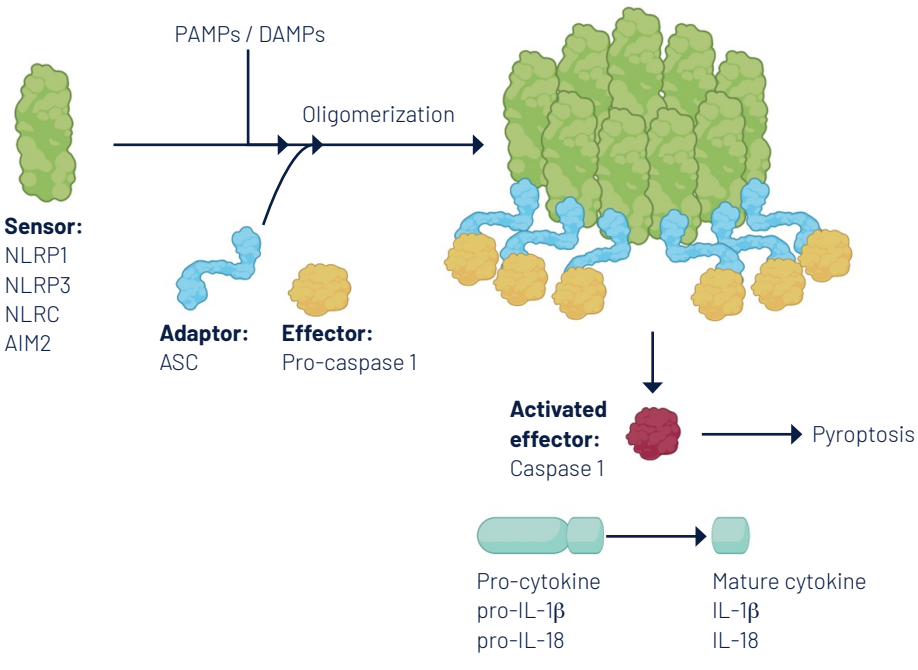


Figure 1. Inflammasome Structure

Inflammasomes assemble within cells in response to danger signals such as Pathogen-Associated Molecular Patterns (PAMPs) or Damage-Associated Molecular Patterns (DAMPs). Inflammasomes have three main components: a sensor protein, which recognizes the danger signal, an adaptor protein that recruits and activates the effector protein, and the effector protein itself. Upon activation, via detection of danger signals, these components undergo structural changes and assemble to form the inflammasome. The outcome of this is the activation of the effector protein, of which caspase-1 is the most prominent example. In turn, caspase-1 acts by cleaving the inactive cytokine precursors IL-1β and IL-18, releasing their active forms in the extracellular environment. It also triggers an inflammatory type of cell death called pyroptosis.

Overcoming Selectivity Challenges is Essential for Success

While inflammasomes are essential for host defense, their aberrant activation is a key contributor to the chronic inflammation that underlies metabolic, neurodegenerative, and autoimmune disorders. Therefore, the broad applicability of therapeutics that target inflammasomes is an attractive treatment opportunity for pharmaceutical research.

The best characterized of all the inflammasomes is the one containing the sensor protein NLRP3. The NLRP3 inflammasome is associated with responses to various damage- and pathogen-associated molecular patterns and is proven to drive chronic inflammation in a broad range of human diseases. Therapeutics designed to target NRLP3, unlike approaches utilizing anti-IL-1, have been shown to inhibit pyroptosis, the critical endpoint of inflammasome activation [4]. Front-runners in early clinical trials were small molecules that directly inhibit NLRP3, such as MCC950, RRx-001 and Dapansutrile [5]. Amongst these early drug candidates, RRx-001 produced encouraging results in a small cell lung cancer trial, while Dapansutrile reduced the symptoms of gout in over 80% of trial participants [6,7]. More recently, Ofirnoflast, which prevents NLRP3 inflammasome formation indirectly by inhibiting the NEK7 modulator protein, has moved to Phase 2a clinical trials for myelodysplastic syndrome [8].

While clinical trials have been largely limited to drugs that target NLRP3, alternative strategies that target different inflammasome components are in pre-clinical development. IC100 targets ASC and has shown promise in neurodegenerative diseases such as multiple sclerosis [9], and MM01, which interferes with ASC speck formation, has been shown to reduce peritoneal inflammation in an animal model of peritonitis [10].

One of the challenges facing drug developers is to prove that their treatments are highly selective for their targets. MCC950 has been shown to reverse inflammation in over fifty models of inflammatory disease including neurodegenerative disease [11]. Targeting specialized brain macrophages, microglia, with MCC950 has ameliorated disease progression in animal models of multiple sclerosis and Alzheimer's disease [12,13]. Despite this success, off-target effects such as inhibition of carbonic anhydrase-2 and associated toxicity in liver hepatocytes, alongside evidence of drug resistance to some NLRP3 variants, has led to delays in drug development [11,14]. These challenges are not limited to MCC950; Glyburide

inhibits the NLRP3 inflammasome, but the doses required have been shown to induce hypoglycemia [15]; Parthenolide inhibits capsase-1 activation but has poor solubility and bioavailability [16]; VX-740 and VX-765 both inhibit caspase-1 but induce hepatic toxicity after long-term use [17]. Aside from potential safety concerns, challenges caused by these issues can increase development timeframes, delay regulatory approvals, and inflate costs.

As work progresses to refine these compounds, and new therapeutics emerge, rigorous testing is essential to support each asset through to clinical trials. A carefully considered selection of appropriate preclinical studies can exclude poor candidates early and reduce attrition in Phase 1 and Phase 2 clinical trials. These include:

- Screening the effects of different compounds at different concentrations on a disease-relevant cell line
- Confirming on-target effects in primary human macrophages and microglia
- Performing a dose response analysis and identifying IC50 or EC50 values in comparison to competitor drugs
- Identifying reproducibility across human donors
- Determining any off-target toxicity

Effective Screening of Therapeutic Candidates that Target the Inflammasome: Selecting the Right Model is Key

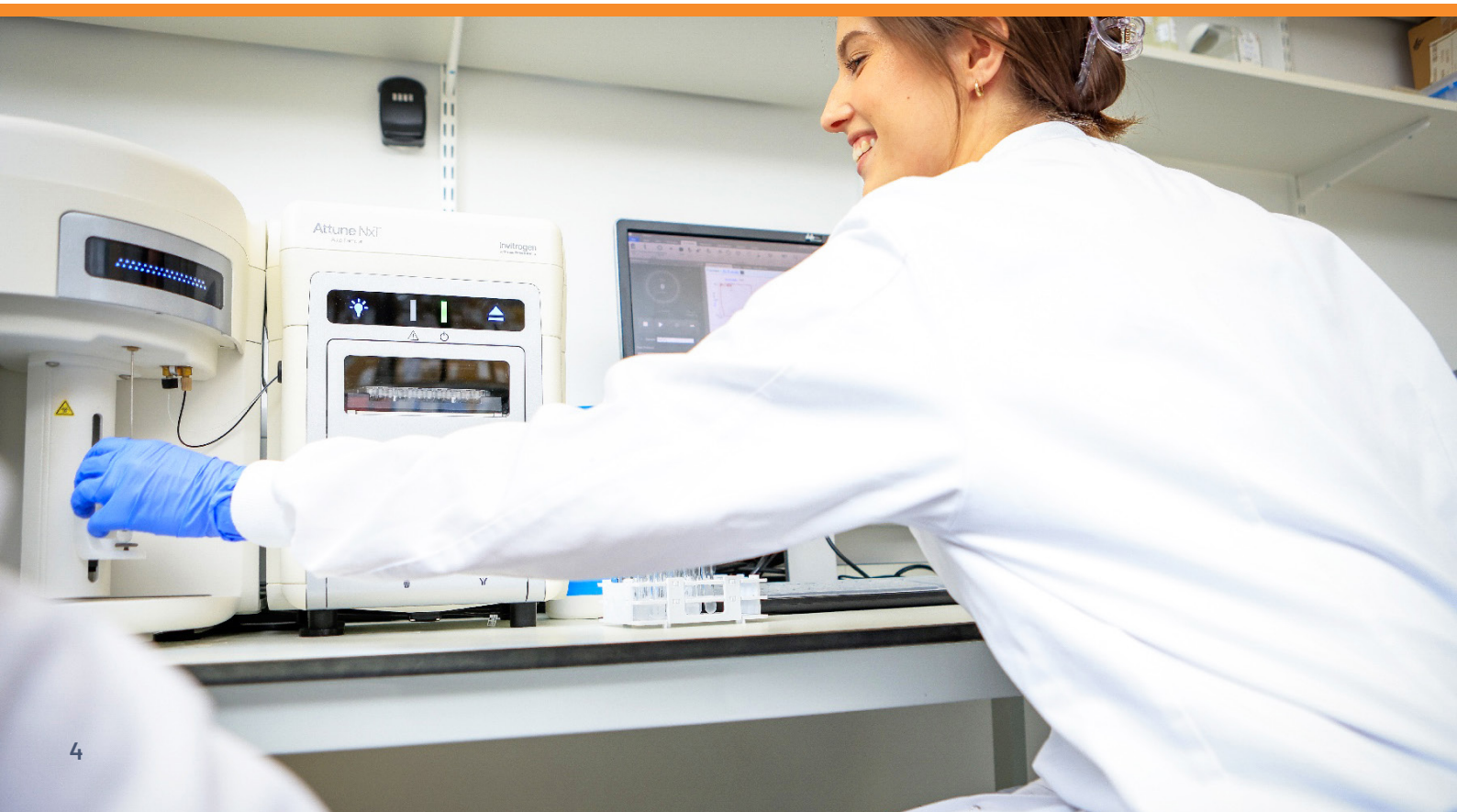
While many cell types can induce the expression of inflammasome components following exposure to a priming stimulus, macrophages are commonly studied because they are key mediators of the inflammatory response, readily form inflammasomes with appropriate activation and are an important source of IL-1β and IL-18. By selecting the appropriate PRR ligand, different inflammasomes can be activated and studied. Importantly, macrophages orchestrate the inflammatory response making them a primary treatment target.

In vitro cell-based assays provide the optimal system with which to determine the efficacy of candidate compounds. Primary human macrophages closely resemble disease-associated cells but are difficult to obtain in large numbers. Therefore, cell lines, such as THP-1 monocytes, are the ideal choice for screening assays aimed at quickly and efficiently comparing the efficacy of many candidates.

While cell lines support large-scale studies, they do not allow the assessment of the effect of biological variability on the drugs' efficacy, which can only be achieved with primary cell cultures. This is crucial to understanding whether a candidate therapeutic might fail during clinical trials. Once a lead candidate is identified, it is important to select the primary macrophage assay that most closely resembles the disease microenvironment. Ideally, macrophages from multiple donors should be tested to confirm biological reproducibility and efficacy, providing a good estimation of potency and effective dose by comparison to reference therapeutics.

Specialized Preclinical Models to Advance Neurodegeneration Therapeutic Discovery

Microglia – specialized tissue-resident macrophages within the central nervous system (CMS) – are important targets for neurodegenerative disorders. While microglia share characteristics with macrophages, they are a different cell lineage. Therefore, to provide confidence in inflammasome-



targeting candidates designed for CNS indications, it is crucial to perform additional preclinical assays to confirm their efficacy in microglia.

Unlike primary human macrophages, primary human microglia are difficult to source and are affected by many confounding factors that are virtually impossible to control for, such as the variable nature of the underlying pathology that led to brain tissue resection. Due to these limitations, iPSC-derived microglia have become the cell model of choice to assess the efficacy of candidate drugs designed to modulate neuroinflammatory pathways, including inflammasome activation. These cells offer excellent reproducibility and scalability, coupled with the ability to model a range of genetic backgrounds (if sourced from different individuals) or to assess the impact of specific disease-associated mutations, which can be introduced by genetic manipulation of cells from healthy donors. Whilst extremely valuable to quickly evaluate candidates at scale, monocultures of iPSC-derived microglia may not accurately reproduce the complexity of cell-cell interactions that take place in the intact brain. Therefore, to derisk the transition to *in vivo* efficacy studies, it is important to confirm the efficacy of candidate inflammasome therapeutics in more complex 3D CNS models, such as organotypic brain slice cultures.

Selecting Appropriate Output Measures

A potential pitfall in preclinical testing of inflammasome inhibitors is the absence of orthogonal approaches that confirm suppression of downstream effectors. In addition to standard caspase-1 output measures, Concept Life Sciences provide measurement of cell viability to demonstrate inhibition of pyroptosis, quantification of ASC speck formation to confirm mechanism of action, and measurement of downstream IL-1β and IL-18 production.

Endpoint pyroptosis can be quantified by assays that measure cell death, such as LDH release. Importantly, by measuring LDH release in non-activated macrophage cultures, it is possible to assess off-target toxicity. Furthermore, we are validating this assay to assess toxicity in other cells such as hepatocytes.

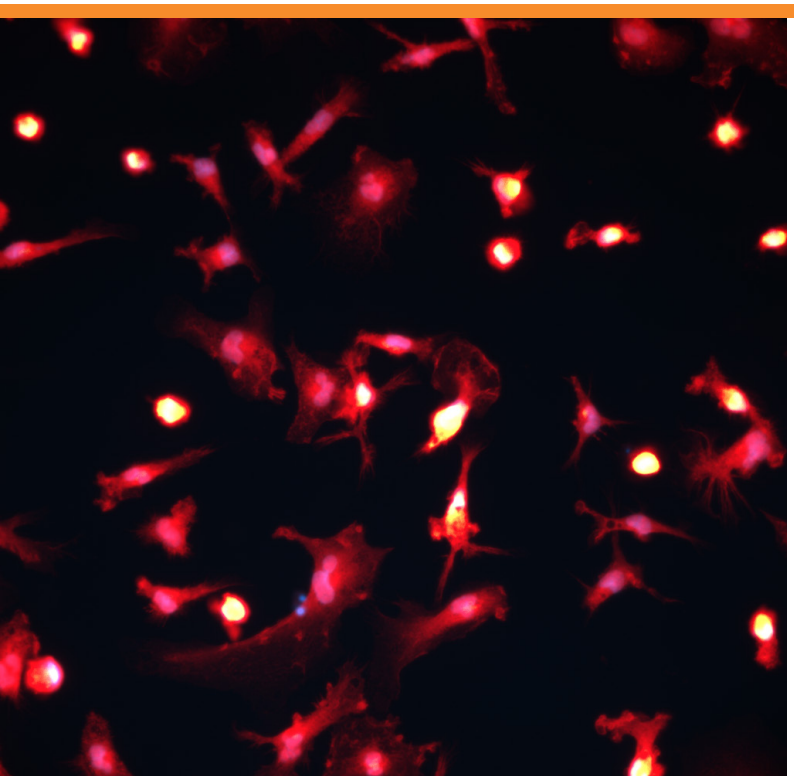
Additionally, flow cytometry and genetic analysis to address further phenotypic questions can be performed after mechanical dissociation of cells from the culture plates or brain tissue.

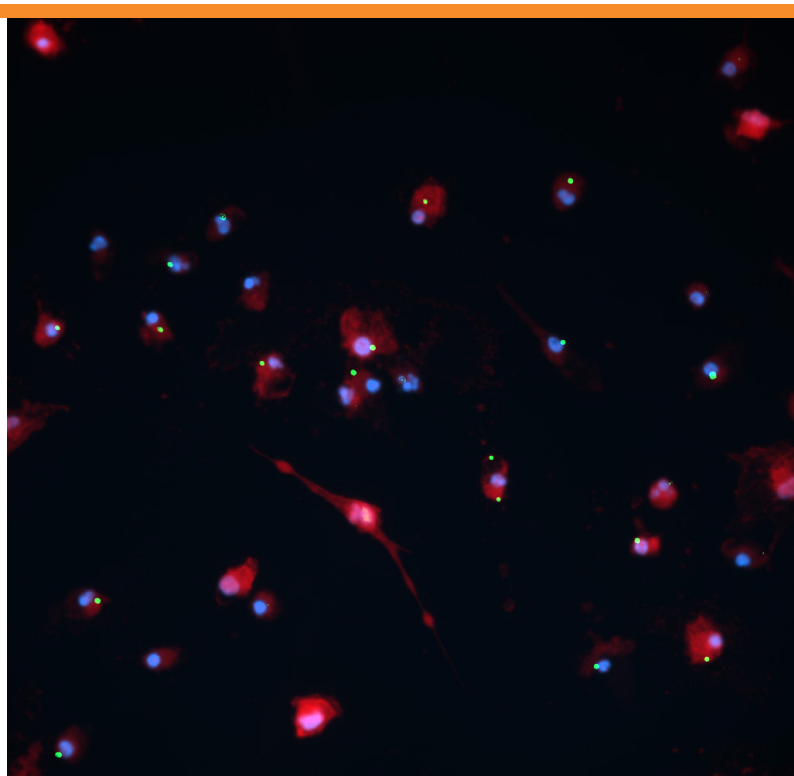
Within all these assays, the primary effector output from cells following caspase-1 activation is an increase in secretion of IL-1β and IL-18, which can be measured in culture supernatants using ELISA, multiplex, or MSD technologies. The approach is determined by the number of cytokines of interest, the culture model, and the anticipated concentration of each cytokine.

Developing your Therapeutic with the Support of Concept Life Sciences

The problem facing scientists is finding an experimental approach that provides a complete understanding of inflammasome activity for efficient progress in a drug discovery program. Concept Life Sciences offer a suite of immune-based assays to evaluate drug candidates that target the inflammasome. From the selection of the appropriate cell model, to optimizing your study to provide the most informative outputs, experts at Concept Life Science will guide you through all our available options to support your therapeutic through to clinical trials.

In our validated cell-based assays, we use NLRP3-selective triggers to induce inflammasome formation, in a macrophage cell line (THP-1), primary human monocyte-derived macrophages, iPSC-derived microglia or mouse organotypic brain slices.

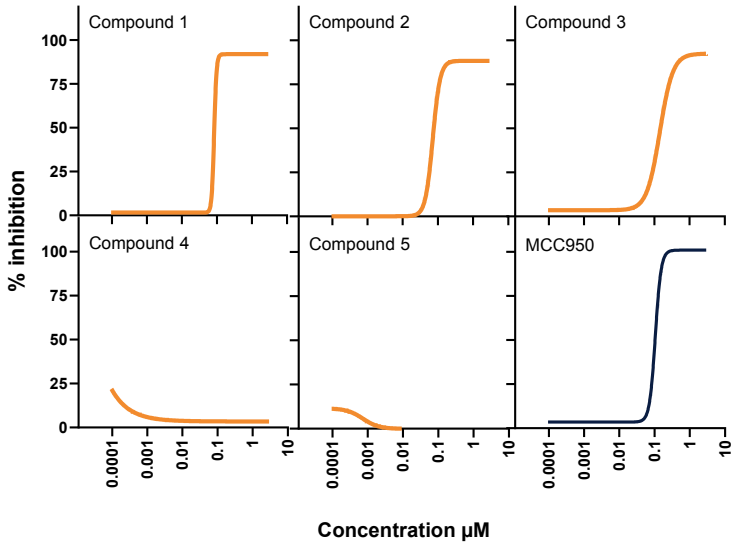




Our suite of assays:

THP-1 Cell Line Assay: Identifying lead candidates and concentrations for testing in primary cell assays: A 'work horse' assay for screening larger number of treatments and concentrations. The monocytic THP-1 cell line is driven by PMA to a macrophage-like phenotype that constitutively expresses inflammasome components. Nigericin, a potassium ionophore originally identified as an antibiotic from Streptomyces hygroscopicus, is used to initiate inflammasome activation [18]. Treating THP-1-derived macrophages with nigericin promotes caspase-1 activation and subsequent pyroptosis, which results in LDH release. The reference NLRP3 inhibitor MCC950 induces a concentration-dependent decrease in caspase-1 activation and LDH release (Figure 2A). To confirm cells have undergone pyroptosis, cell death is measured by LDH release in THP-1 macrophages activated with nigericin. Non-specific toxicity induced by treatments in the absence of inflammasome activation can be investigated in parallel cultures that are not treated with nigericin (Figure 2B).

A



Treatment	R_2	Hill Slope	IC50 (uM)
Compound 1	0.99	12.7	0.083
Compound 2	0.98	4.15	0.072
Compound 3	0.95	2.35	0.143
Compound 4	0.42	-0.86	Unreliable
Compound 5	0.51	-1.92	Unreliable
MCC950	0.98	5.33	0.105

B

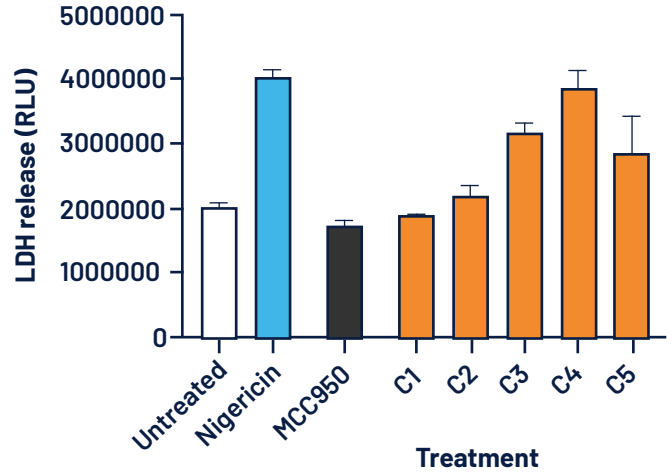


Figure 2. THP-1 Screening Assay

A THP-1 screening assay was performed to identify therapeutic leads from candidate compounds. Cells were polarized towards a macrophage phenotype with PMA, then stimulated with nigericin to promote inflammasome formation. Macrophages were treated with a reference control substance known to inhibit inflammasome formation (MCC950) or different candidate therapeutics at 10 concentrations of which five exemplar compounds are shown (Compounds 1-5). (A) Caspase-1 levels were determined and the percent inhibition of the maximal response calculated. The figure shows the curve fit used to calculate the IC50 for each test substance. Compounds 1 and 2 were found to have higher potency than the reference control. (B) In a separate experiment, direct toxicity (off target effect) was determined by measuring LDH-release in THP-1 macrophage cultures treated with test or reference compounds in the absence of nigericin. The figure shows that compound 4 induced levels of toxicity similar to the levels of pyroptosis induced by nigericin. n=2 technical replicates.

Primary Macrophage Assay: Confirming concentration-response kinetics and reproducibility across different human donors: This assay is used to confirm the lead therapeutics' ability to inhibit inflammasome activation in a highly physiologically relevant and translational human model. Monocytes are isolated from the blood of healthy donors and matured in the presence of growth factors into macrophages over a period of several days. Macrophages can be further polarized to a M1, M2 or TAM-like phenotype to more closely resemble disease-associated macrophages. Once primed and activated, macrophages will upregulate inflammasome formation, which can be inhibited with MCC950.

iPSC-derived Microglia Assay: Confirming efficacy and concentration-response kinetics in the key cell type for CNS indications. To determine whether lead therapeutics designed for CNS indications inhibit the key drivers of inflammasome responses in the brain. Human iPSC-derived microglia are recovered from cryopreserved stocks and stabilized in culture before being exposed to specific inflammasome-activating stimuli, in the presence or absence of reference inflammasome inhibitors, such as MCC950 (Figure 3).

Organotypic Brain Slice Assay: supports testing lead therapeutics in a complex 3D ex vivo brain model. Coronal forebrain slices are cultured for 14 days prior to the addition of treatments, then culture supernatant is analyzed for changes in cytokine production. In the presence of LPS and either ATP or nigericin, there is a significant increase in IL-1β, gasdermin D and HMGB1 production, which can be inhibited in a concentration-dependent manner with MCC950 (Figure 4).

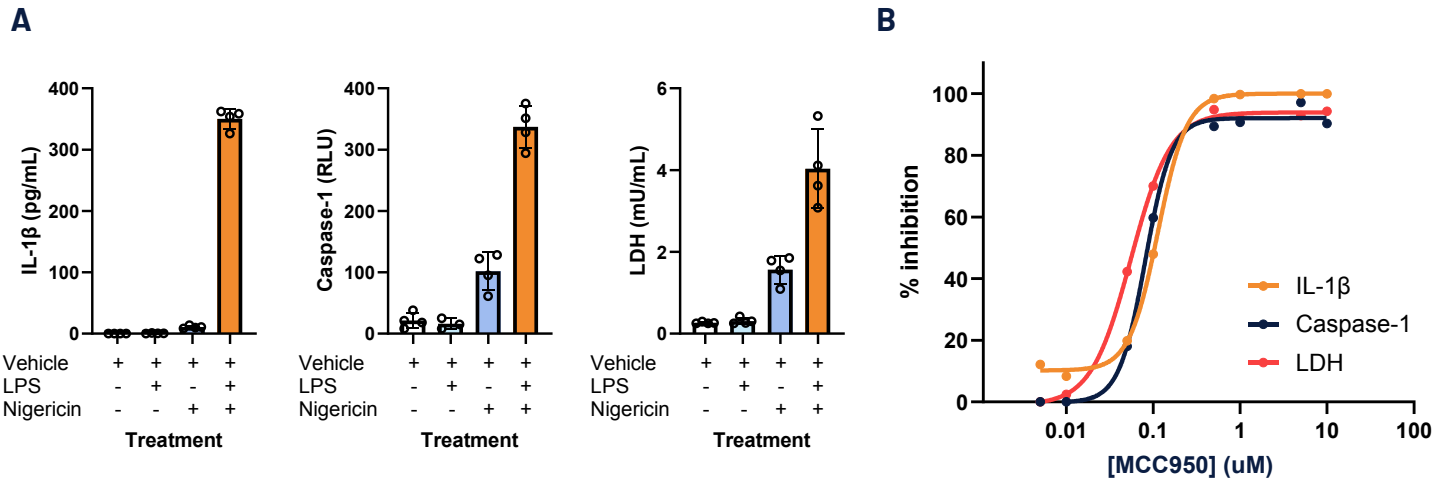
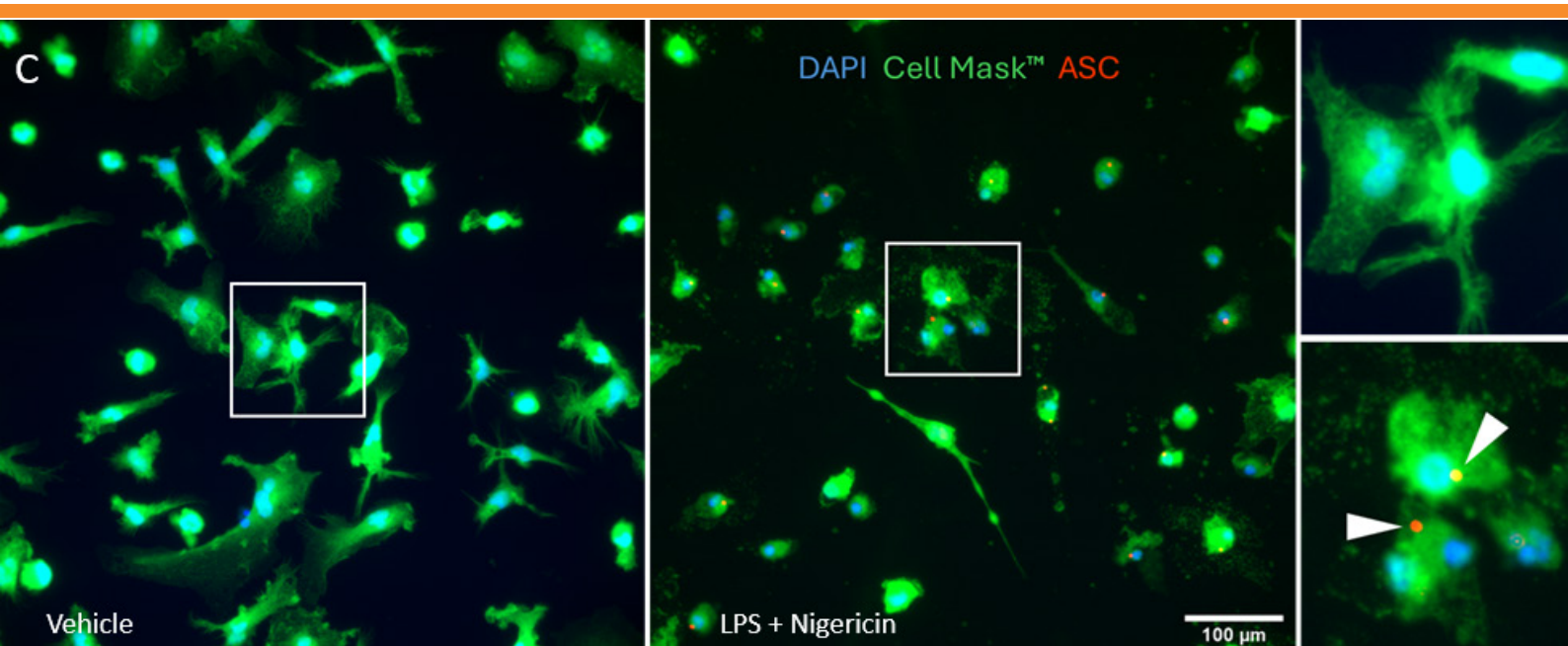


Figure 3. Primary Microglia Inflammasome Assay

Microglia were grown from cryopreserved stocks, primed with vehicle or LPS, and stimulated with either vehicle or nigericin to promote inflammasome formation. The figures show the mean of technical replicates and SD. (A) Upregulation of IL-1β production, increased expression of caspase-1 and LDH release confirms that the cells have activated the NLRP-3 inflammasome pathway leading to inflammatory cytokine production and pyroptosis. n=4 technical replicates (B) Treating microglia with increasing concentrations of MCC950 inhibits IL-1β production, caspase-1 activation and LDH release. (C, below) ASC specks (arrows) can be quantified by high content imaging of microglia.



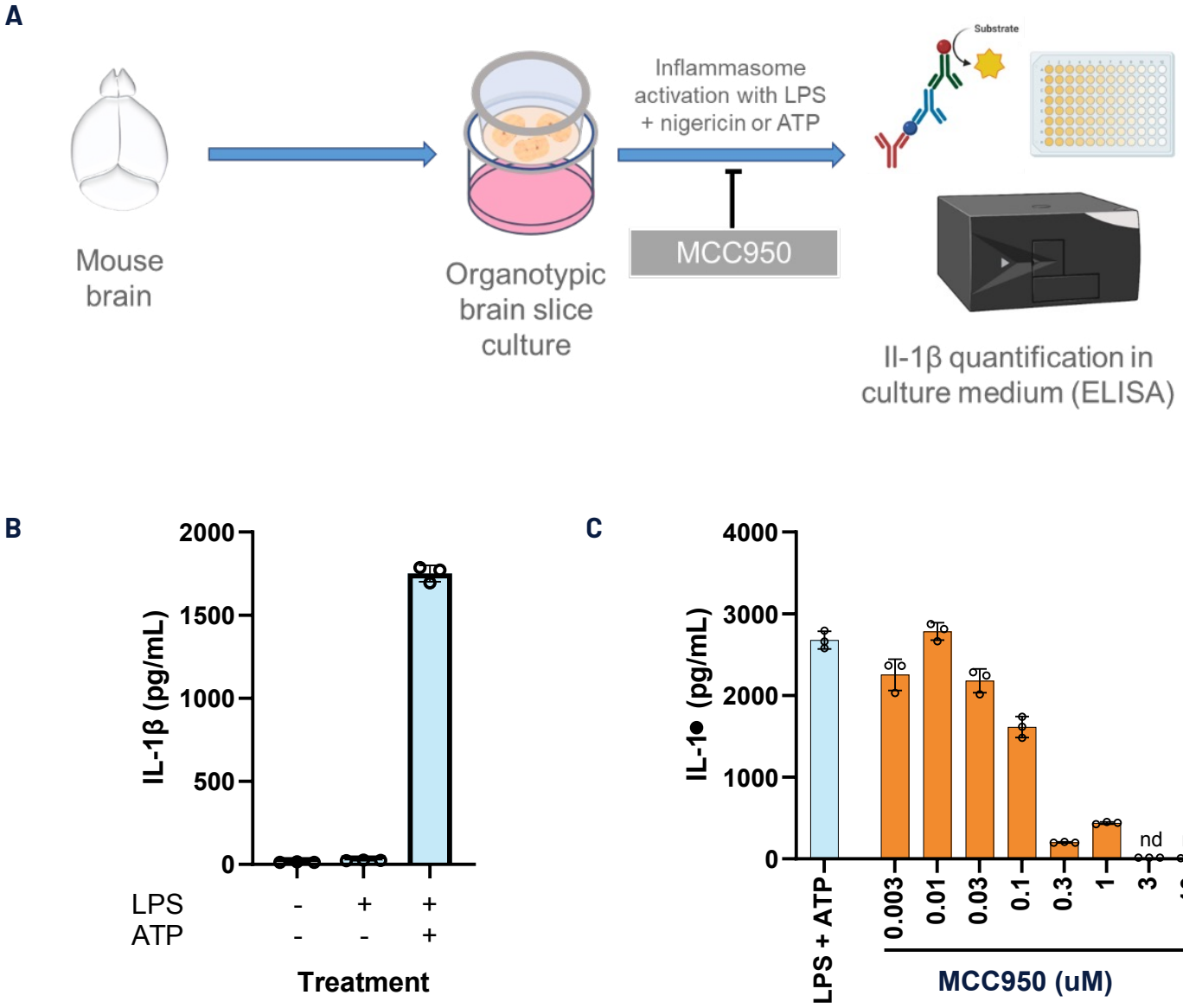


Figure 4. Organotypic Brain Slice Assay

(A) Coronal forebrain slices were generated from C57BL/6 mice and cultured for 14 days prior to the addition of LPS and ATP to activate inflammasome formation. Slices were also treated with MCC950 to inhibit inflammasome formation. After treatment, supernatants were collected and the concentration of IL-1β determined by ELISA. (B) Brain slices were primed and activated with a combination of LPS and ATP. The figure shows the mean (+/- SEM) IL-1β concentration identified in culture supernatants (n=3). (C) Inflammasome activation was induced by LPS and ATP in the presence of increasing concentrations of reference control MCC950. The figure shows the mean (+/- SEM) IL-1β concentration in culture supernatants (n=3). nd = not detected

Working with Concept Life Sciences

The inflammasome has proven to be a promising and druggable target. At Concept Life Sciences we understand that advancing a small molecule or biologic to Phase I clinical trials is a monumental task – and stakes are high. But success is by no means out of reach. For your inflammasome therapeutics, we offer:

- A selection of *in vitro* assays that streamlines candidate selection.
- Early confirmation of selectivity and mechanism of action with a range of biologically relevant readouts, de-risking further development.
- Assays that provide mechanistic clarity, from screening to validation, with increasing complexity to avoid pitfalls often identified in later clinical testing.
- Experienced scientists who will guide you through a comprehensive selection of physiologically relevant assays.
- Extensive experience in assay development, and the ability to provide tailored solutions to suit your goals and timeframe.
- Options for a fully integrated approach utilizing medicinal chemistry, prior to biological profiling, to:
  - Computer model candidate drugs
  - Synthesize chemicals
  - Analyze DMPK

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About Concept Life Sciences

Concept Life Sciences is a leading contract research organisation (CRO) serving the global life sciences industry. For over 25 years, the company, and its heritage companies, have provided consultative and collaborative drug discovery and development services. Our approach, supported by passionate scientists and world-leading capabilities, enables clients to overcome complex scientific challenges across a broad range of therapeutic areas, improving program success rates. The company has successfully helped 29 candidates advance to the clinic.

The company offers sophisticated translational biology services coupled with exceptional end-to-end chemistry capabilities across all modalities including small molecules, biologics, peptides and cell & gene therapies, with the ability to seamlessly integrate capabilities and provide bespoke solutions to address client needs.

Collectively, the company's high-quality services and commitment to customer service across the drug development pathway enhances efficiency in drug discovery, helping clients advance their drugs to clinic in as little as 32 months, well ahead of the industry average of 60 months.

Driven by a passion for science, Concept Life Sciences has around 230 employees, with around 70% holding PhDs. The company operates from state-of-the-art UK facilities, headquartered near Manchester, with additional operations in Edinburgh, Dundee, and Sandwich. The headquarters is one of the UK's largest medicinal chemistry CRO sites with key discovery services all under one roof.

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Glossary

ASC: Adapter protein apoptosis-associated speck-like protein containing a CARD

CARD: Caspase recruitment domain

CLR: C-type lectin receptor

NALP: Nucleotide-binding oligomerization domain, Leucine-rich Repeat, and Pyrin domain-containing protein

NLR: Nod-like receptor

NLRP: Nod-like receptor protein (also known as NALP)

PMA: Phorbol 12-myristate 13-acetate

PRR: Pattern recognition receptor

RLR: RIG-1-like receptor

TAM: Tumour Associated Macrophage

TLR: Toll-like Receptor

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