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Letters

A Probe for NLRP3 Inflammasome Inhibitor MCC950 Identifies Carbonic Anhydrase 2 as a Novel Target

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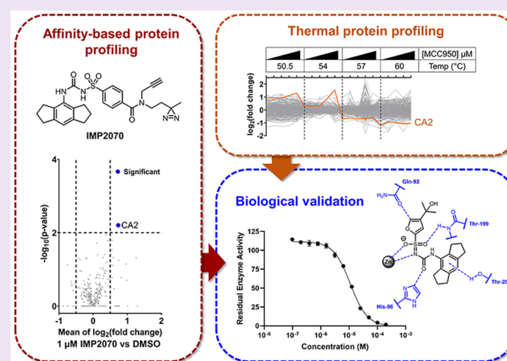
ABSTRACT: Inhibition of inflammasome and pyroptotic pathways are promising strategies for clinical treatment of autoimmune and inflammatory disorders. MCC950, a potent inhibitor of the NLR-family inflammasome pyrin domain-containing 3 (NLRP3) protein, has shown encouraging results in animal models for a range of conditions; however, until now, no off-targets have been identified. Herein, we report the design, synthesis, and application of a novel photoaffinity alkyne-tagged probe for MCC950 (**IMP2070**) which shows direct engagement with NLRP3 and inhibition of inflammasome activation in macrophages. Affinity-based chemical proteomics in live macrophages identified several potential off-targets, including carbonic anhydrase 2 (CA2) as a specific target of **IMP2070**, and independent cellular thermal proteomic profiling revealed stabilization of CA2 by MCC950. MCC950 displayed noncompetitive inhibition of CA2 activity, confirming carbonic anhydrase as an off-target class for this compound. These data highlight potential biological mechanisms through which MCC950 and derivatives may exhibit off-target effects in preclinical or clinical studies.



The inflammasome is a multiprotein signaling complex that serves as a platform for caspase-1 activation in response to infection, cellular damage, or stress.[1] Active caspase-1 proteolyzes the biologically inert pro-IL-1β and pro-IL-18 cytokines into their bioactive, inflammatory forms. In addition, the proteolysis of the pore-forming protein Gasdermin D (GSDMD) by caspase-1 results in pyroptotic cell death, a pro-inflammatory, lytic form of cell death. While the inflammasome signaling pathway is vital in immune responses, when dysregulated it can lead to heightened cell death and cytokine release, ultimately resulting in uncontrolled inflammation and autoimmune disorders. Currently, clinical inhibitors of inflammasome pathways include the IL-1β monoclonal antibody therapy, e.g., Canakinumab, and inhibitor proteins, e.g., Anakinra and Rilonacept. However, these agents are not specific to particular stimuli or to cell death driven by a specific class of inflammasome.[2]

The NLRP3 (nucleotide binding and oligomerization domain, leucine-rich repeat and pyrin containing protein 3) inflammasome is among the most versatile cytosolic sensors of microbial infection or endogenous sterile signals (Figure 1A).[3] Activating mutations in NLRP3 are directly linked to autoinflammatory fever syndromes, and deregulated NLRP3/caspase-1 signaling is linked to inflammation. Therefore, there is much interest in clinical development of specific small molecule inflammasome inhibitors.

MCC950 is a small molecule NLRP3 ligand which locks NLRP3 in a "closed" conformation, preventing ATP hydrolysis to ADP and thus inhibiting oligomerization and activation of the NLRP3 inflammasome (Figure 1A,B).[4−7] MCC950 blocks NLRP3-driven cytokine processing and pyroptosis including during bacterial infection of human macrophages.[8] MCC950 has shown promise in more than 50 animal disease models, including models of multiple sclerosis,[9] traumatic brain injury (TBI),[10−12] cryopyrin-associated periodic syndromes (CAPS),[4] and Alzheimer's and Parkinson's diseases.[13,14] While MCC950 as a clinical candidate was reportedly halted in Phase II clinical trials, possibly due to off-target effects at high doses, derivatives of MCC950 remain in clinical trials, and it is critical to understand the off-target mechanisms of this class of molecules. MCC950, also reported as CRID3 and CP-456,773, was previously extensively screened for off-target activity on a range of proteins, with no significant targets discovered.[15] Direct engagement of NLRP3 by MCC950 has been demonstrated only in the context of overexpressed NLRP3 protein or in cell lysates and potential MCC950 targets beyond NLRP3 remain uncharacterized, leaving open

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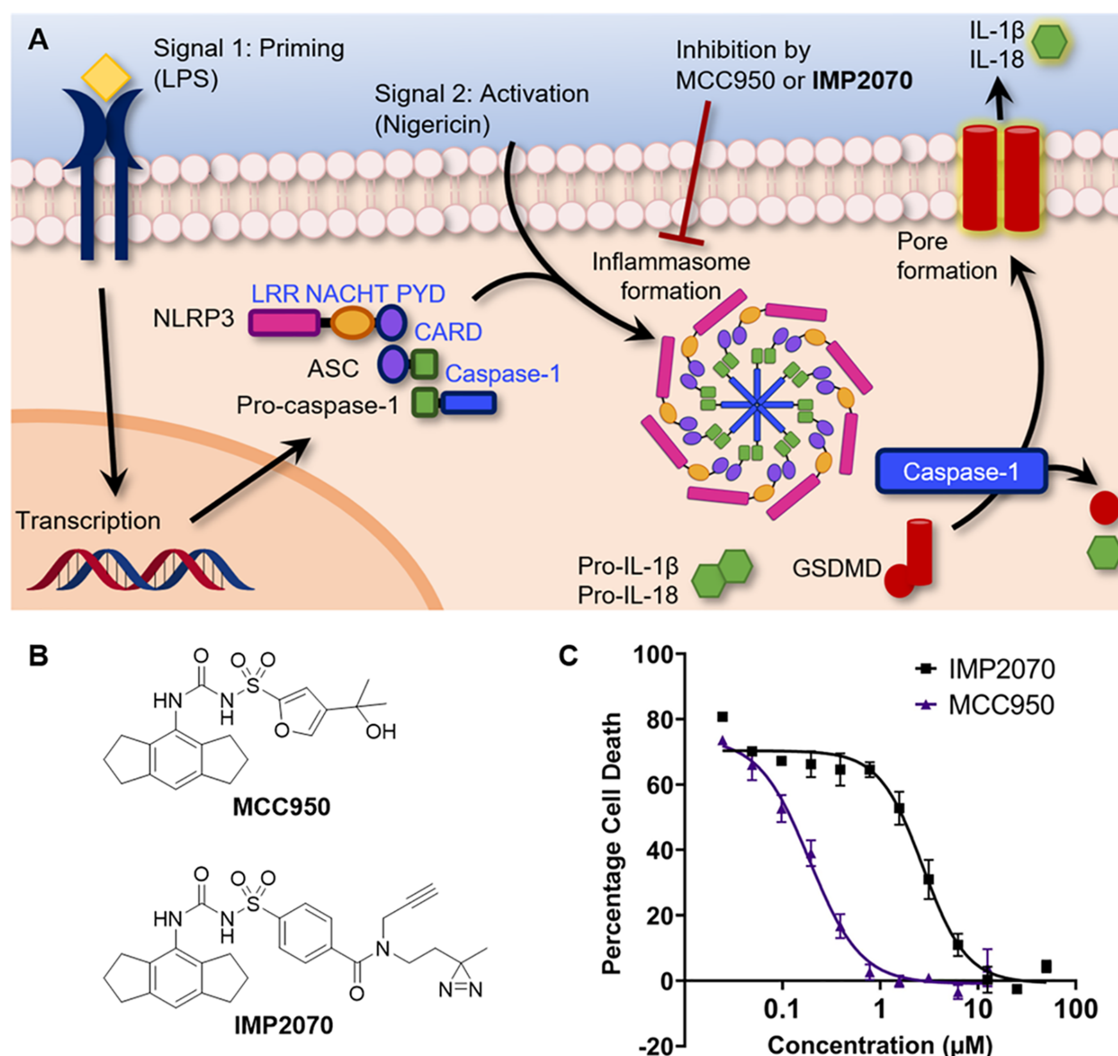

Figure 1. Design of **IMP2070**, a photoaffinity probe for target identification for MCC950, a potent inhibitor of NLRP3 inflammasome activation. (a) NLRP3 canonical inflammasome activation in macrophages occurs through a two-step process. A priming stimulus triggers up-regulation of key inflammatory genes before an activating signal triggers inflammasome formation, which enables activation of caspase-1. Caspase-1 cleaves pro-IL-1*β*, pro-IL-18, and GSDMD into their active forms, triggering GSDMD pore formation, inflammatory cytokine release, and cell lysis. (b) Structures of NLRP3 inflammasome inhibitor MCC950 and **IMP2070** AfBP (affinity-based probe). (c) Inhibition of LPS and nigericin induced cell death in THP1-derived macrophages by MCC950 and **IMP2070**. Results are representative of two experiments; error bars represent SD (n = 3). MCC950: IC_{50} = 0.2 μM; slope = −1.66; R^2 = 0.98. **IMP2070**: IC_{50} = 2.7 μM; slope = −1.94; R^2 = 0.97.

the question of what additional MCC950 activities may contribute to efficacy or toxicity.

Here, we describe the first proteome-wide unbiased target profiles for MCC950. We show that a novel photoaffinity-based probe (AfBP) for MCC950, **IMP2070**, retains inhibitory activity against the NLRP3 inflammasome, and exhibits direct engagement of endogenous NLRP3 in intact macrophages primed for pyroptosis. *De novo* target identification with **IMP2070** and thermal proteome profiling further identified multiple potential MCC950 off-targets including carbonic anhydrase 2 (CA2), and biochemical assays confirmed MCC950 as a bona fide noncompetitive CA2 inhibitor. We anticipate that these novel MCC950 target profiles will prove useful in interpretation of both prior and future *in vitro* and *in vivo* studies using MCC950.

Photoaffinity labeling (PAL) is a powerful chemical biology technique which, when paired with proteomics, enables *de novo* identification of noncovalent protein binders to a small molecule; for example, we recently applied proteomics-coupled PAL to identify target and off-target proteins of the drug Olaparib.[16] Directed, single-target PAL was previously used to support assignment of NLRP3 as the main target of MCC950, utilizing benzophenone and alkyne functionalities, however, without the application of proteomics to discover novel binding proteins.[5,7] Successful photoaffinity probes mimic the parent compound as closely as possible, with minimal disruption from the addition of photo-cross-linking and alkyne handles. MCC950 (Figure 1B) bears a sulfonyl urea linked to a hydrophobic hexahydro-*s*-indacene ring system which is required for NLRP3 inhibition, while the isopropyl furan may be replaced with a range of substituents with only modest loss of activity.[4,17] Based on these structure−activity relationship (SAR) data for MCC950, we designed and synthesized a photoaffinity probe for MCC950, **IMP2070** (Figure 1B), which maintains the essential features for activity along with diazirine and alkyne functional groups for photo-cross-linking and bioorthogonal ligation, respectively.

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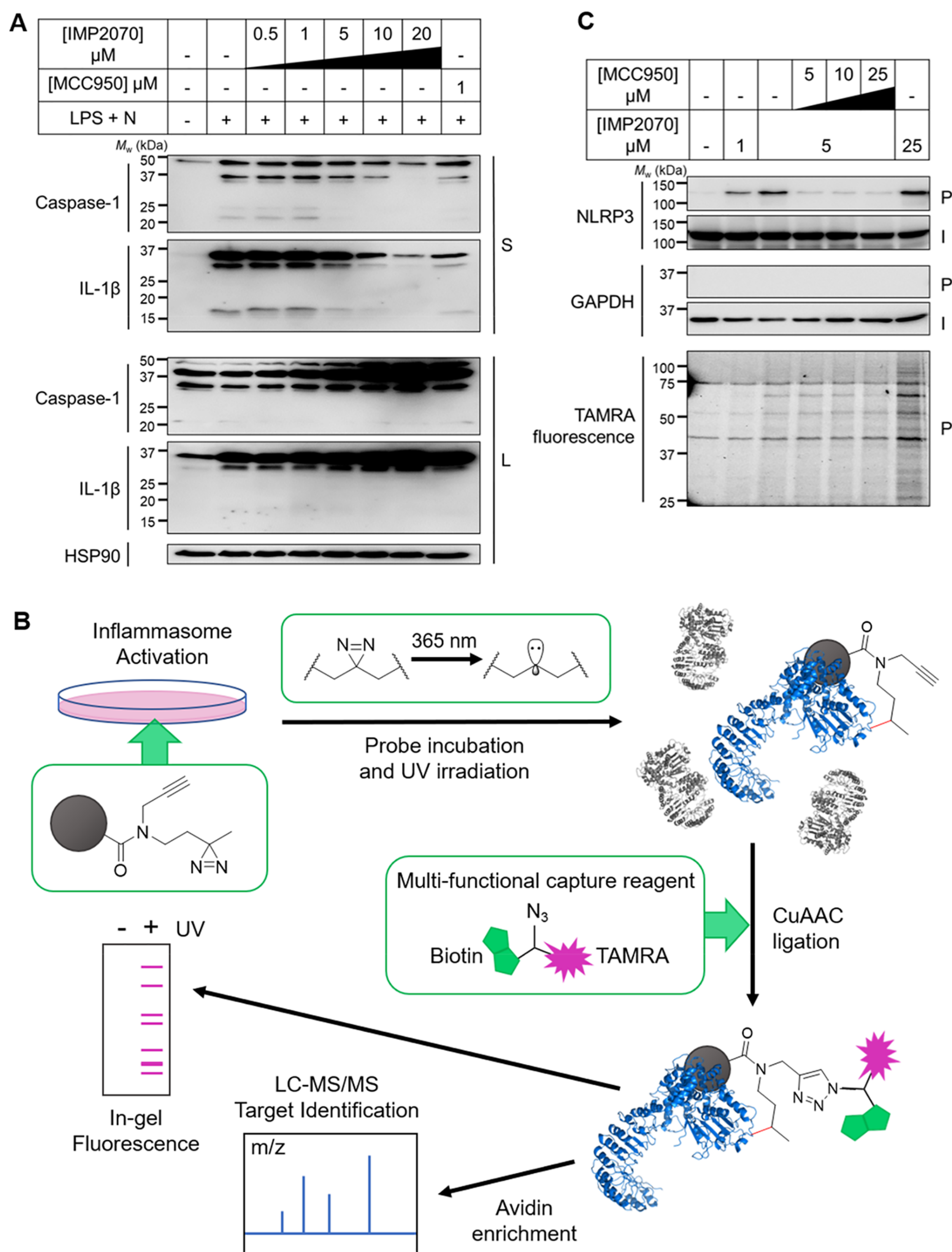

Figure 2. IMP2070 is an MCC950-competitive probe and inhibitor of NLRP3-mediated pyroptosis, and directly cross-links to NLRP3 in intact cells during inflammasome activation. (a) Western blot analysis of caspase-1 and IL-1β in the supernatants and lysates of THP1-derived macrophages. (b) Workflow diagram for photoaffinity based protein profiling with proteomics analysis. Macrophages were treated with **IMP2070**, and inflammasome activators before UV irradiation generate a carbene from the diazirine. Probe-cross-linked proteins can be further functionalized through copper(I)-catalyzed alkyne−azide cycloaddition before analysis by fluorescence or mass spectrometry. The gray ball represents the specificity region of the AfBP. (c) In-gel fluorescence and western blot analysis of **IMP2070**-enriched proteins. Results are representative of three

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Figure 2. continued

experiments. S = supernatant; L = lysate; I = input (whole lysate); P = pull down (enriched fraction). Uncropped blots and gels can be seen in Figures S3 and S4.

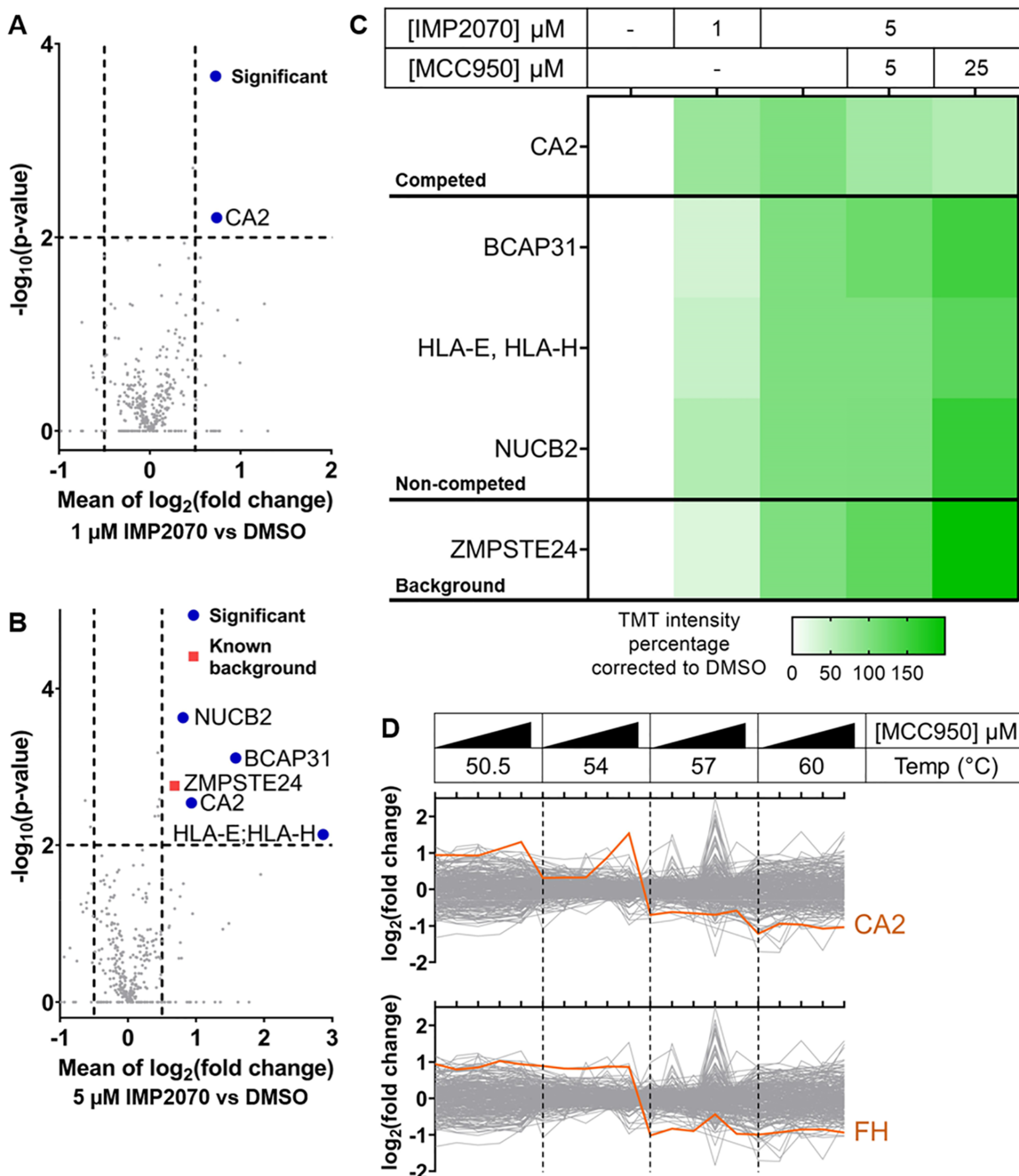

Figure 3. Global target identification for MCC950 combining photoaffinity and thermal proteome profiling, and identification of carbonic anhydrase 2 (CA2) as a major MCC950 off-target. Photoaffinity labeling experiments with **IMP2070** highlighted several proteins as significant hits (blue) with (a) 1.0 μM (FC = 1.41; p-value = 0.01), and (b) 5.0 μM **IMP2070** (FC = 1.41; p-value = 0.01). Background from known diazirine binders is shown in red. (c) Competition photoaffinity labeling experiments with **IMP2070** and MCC950 identified CA2 as an MCC950 binding protein. (d) Thermal protein profiling in whole cells with 0−10 μM MCC950 showed CA2 stabilization with MCC950 at 50.5 and 54 °C. Fumarate hydrase (FH) is shown as an example of a protein not stabilized by the presence of MCC950.

In macrophages, NLRP3 activation follows transcriptional up-regulation and post-translation licensing by pro-inflammatory signals, such as bacterial lipopolysaccharide (LPS) (Figure 1A). A second signal triggers NLRP3 inflammasome assembly, leading to efflux of K^+ ions from the cell due to membrane damage. The adaptor protein ASC recruits multiple caspase-1 proenzyme proteins to the NLRP3 inflammasome, where they are activated by proximity-induced autoproteolysis. **IMP2070** was first tested for its ability to inhibit cell death in THP1-derived macrophages treated with LPS and nigericin, for inflammasome priming and activation, respectively (Figure 1C), and was found to have an IC_{50} of 2.7 μM. Western blot

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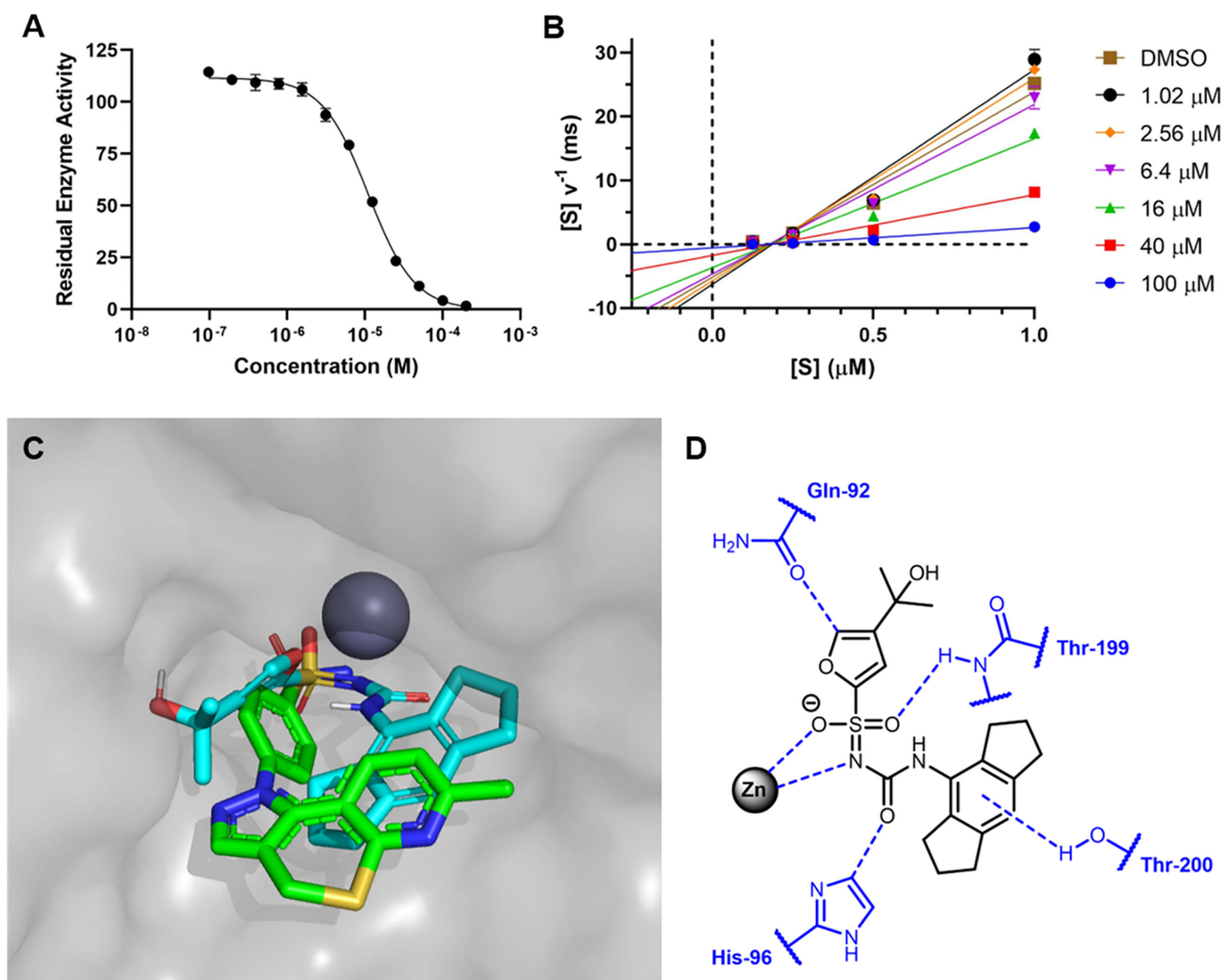

Figure 4. Biochemical validation of MCC950 as a noncompetitive inhibitor of carbonic anhydrase 2 (CA2). (a) Residual esterase enzyme activity of CA2 when treated with MCC950. Error bars represent SEM, $N = 3$. $IC_{50} = 11$ μM; slope = −1.47, $R^2 = 0.99$. (b) Hanes-Woolf plot assessing CA2 (85 nM) esterase activity in the presence of MCC950 (1−100 μM) and *p*-NPA (0.125−1 mM). Error bars represent SEM, $N = 3$. (Slope, R^2) for each MCC950 concentration: 100 μM (3.17, 0.94); 40 μM (9.51, 0.95); 16 μM (20.19, 0.96); 6.4 μM (26.55, 0.95); 2.56 μM (31.74, 0.96); 1.02 μM (33.59, 0.95); DMSO (29.03, 0.96). (c) Molecular docking of MCC950 against CA2 (PDB 3QYK) predicted MCC950 (cyan) interactions with the catalytic zinc (gray), and occupation of the protein pocket similar to 4-(7-methylpyrazolo[3′,4′:4,5]thiopyrano[2,3-*b*]pyridin-1(4*H*)-yl)benzenesulfonamide (green). Oxygens are shown in red, sulfur in yellow, and nitrogen in dark blue. (d) Predicted interactions between MCC950 (black) and CA2 residues (blue) and zinc (gray).

analysis showed inhibition of cleavage of pro-caspase-1 and pro-IL-1β by **IMP2070** and MCC950 (Figure 2A). In line with previous data on substitution of the sulfonylurea,[17,18] **IMP2070** is approximately 10-fold less potent than MCC950 (IC_{50} 0.2 μM), and this ratio is maintained across inhibition of pyroptosis and caspase substrate cleavage, confirming that **IMP2070** also inhibits inflammasome-induced cell death and cytokine maturation.

A photoaffinity strategy was developed in intact THP1-derived macrophage cells stimulated with LPS concurrently with **IMP2070** treatment, followed by in-cell UV irradiation to induce covalent photo-cross-linking between target proteins and probe (Figure 2B). Labeled proteins can then be further functionalized through copper-catalyzed alkyne−azide cycloaddition (CuAAC) to multifunctional capture reagents to enable analysis by in-gel fluorescence or enrichment for western blot or proteomics analysis.[19−22] We explored the potential of **IMP2070** to directly engage endogenous NLRP3 in intact cells, a phenomenon demonstrated previously only for overexpressed protein or in cell lysates.[5,7] THP1-derived macrophages treated with LPS and **IMP2070** were UV-irradiated, proteins ligated to an azido-TAMRA/biotin capture reagent (AzTB, Figure S1),[16,23] and labeled proteins enriched on NeutrAvidin agarose beads (Figure 2C). Western blot of enriched labeled proteins demonstrated direct engagement of **IMP2070** with NLRP3, which furthermore could be potently competed by MCC950.

With a validated MCC950 affinity-based probe in hand, we turned to *de novo* chemical proteomic identification of potential MCC950 targets. THP1-derived macrophages were treated with LPS and either DMSO, **IMP2070** (1 or 5 μM) alone, or **IMP2070** (5 μM) in combination with MCC950 (5, 10, or 25 μM). Cells were irradiated at 365 nm, lysed, and lysates subjected to CuAAC with an azido-arginine-biotin trypsin-cleavable capture reagent (AzRB, Figure S1).[16,23] Labeled proteins were enriched on NeutrAvidin agarose

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beads, subjected to reduction and alkylation, and enzymatic digestion with trypsin. The resulting peptides were then tandem mass tag (TMT) labeled, combined, fractionated, and analyzed by nanoscale liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) on a high-resolution QExactive orbitrap mass spectrometer. Comparing DMSO vehicle-treated and **IMP2070**-treated samples identified probe-binding proteins at 1 μM (Figure 3A) and 5 μM (Figure 3B). At 1 μM **IMP2070**, one protein was significantly enriched: carbonic anhydrase 2 (CA2) (Figure 3A); and at 5 μM significant enrichment of CA2, B Cell Receptor Associated Protein 31 (BCAP31), Nucleobindin-2 (NUCB2), and human leukocyte antigen (HLA) was seen (Figure 3B). In addition, one frequently identified binder of diazirine motifs was enriched at 5 μM: Zinc Metallopeptidase STE24 (ZMPSTE24).[16,24] Proteins enriched by 5 μM **IMP2070** were further analyzed for dose-dependent enrichment by **IMP2070**, and for evidence of competition of **IMP2070** labeling by MCC950 (Figure 3C). These analyses showed dose-dependent enrichment of the 5 identified proteins with **IMP2070**, from which one, CA2, showed significant competition by MCC950 by quantitative chemical proteomics (Figure 3C).

Although chemical proteomic approaches are powerful tools to enrich and identify interacting proteins in cells, probe design strongly influences target profiles, and MCC950 offers little scope for modification around the essential sulfonyl urea hexahydro-*s*-indacene motif making alternate probe designs problematic. We therefore looked to thermal protein profiling (TPP) as a complementary *de novo* target identification approach to correlate targets against profiles obtained with **IMP2070**. TPP has recently emerged as a potentially powerful technique for target discovery when coupled with proteomics,[25] with notable examples including quinine on-target[26] and panobinostat off-target identifications.[27] Putative MCC950-binding proteins were profiled through two-dimensional intact cell TPP (Figures S2A–D and 3D) in THP1-derived macrophages. Briefly, cells were treated with LPS and either DMSO vehicle or MCC950 (0.1, 1, 5, or 10 μM), resuspended in PBS and heat treated between 50 and 60 °C, a temperature range across which denaturation of the majority of proteins is observed (Figure S2A), for 3 min. Following lysis, insoluble protein was removed by centrifugation, and residual protein subjected to quantitative proteomic analysis by TMT labeling and nanoLC-MS/MS. Putative MCC950-binding proteins were identified by dose-dependent stabilization in the presence of MCC950 (Table S1), by normalizing protein abundances to DMSO levels for each temperature (Figure S2B–C) to create pseudo melting curves for each protein, enabling identification of melt curves right-shifted by higher MCC950 concentrations. Separately, protein abundances were sorted by temperature sets, enabling identification of proteins with abundance increased in a dose-responsive manner (Figure 3D); this manner of analysis was more suitable for proteins with a melt temperature (T_m) at the lower end of the measured range (Figure S2D). In total, 26 proteins were identified as potential binding partners for MCC950 (Table S1). From these, two proteins overlapped with the potential MCC950 binders previously identified by affinity-based protein profiling, CA2 and RPL27A.

The target identification data above suggest multiple potential off-targets for MCC950; we selected CA2 for further validation, since in addition to direct stabilization by MCC950, **IMP2070** was outcompeted by MCC950 at this target in intact cells. CA2 is one of five cytosolic α-carbonic anhydrases that catalyze the conversion of carbon dioxide to bicarbonate, and therefore regulate physiological pH.[28] They also catalyze ester hydrolysis, utilizing the same catalytic zinc ion for CO_2 hydration and esterase activity.[29] α-CAs play important roles in multiple diseases, with inhibitors of α-CAs used clinically for the treatment of glaucoma, epilepsy, and altitude sickness.[28] CA2 esterase activity was assessed in the presence of DMSO or MCC950 in an assay in which CA2 hydrolyzes *para*-nitrophenol acetate (p-NPA) to *para*-nitrophenol (p-NP) (Figure 4A),[30] yielding an IC_{50} of 11 μM. Carbonic anhydrases are notably susceptible to noncompetitive inhibition by sulfonamides, such as acetazolamide (AZA, Figure S2D), methazolamide, dorzolamide, brinzolamide, topiramate, zonisamide, and sultiame.[28] We hypothesized that sulfonylurea-containing MCC950 could inhibit CA2 through an analogous mechanism. Inhibition of CA2 was further assessed across a range of *p*-NPA and MCC950 concentrations, and Hanes-Woolf analysis confirmed that MCC950 exhibits noncompetitive inhibition against CA2 (Figure 4B). In order to eliminate the possibility that inhibition of CA2 was a result of sulfonamide impurities or degradation products, MCC950 purity was carefully confirmed by LC-MS and NMR. Molecular docking of MCC950 against human CA2 (PDB 3QYK) provided further insight into how this inhibition may occur, with complexation around the catalytic zinc ion of CA2, and occupation of space similar to that of the previously cocrystallized CA2 ligand 4-(7-methylpyrazolo[3′,4′:4,5]-thiopyrano[2,3-*b*]pyridin-1(4*H*)-yl)benzenesulfonamide (Figure 4C). In addition to the analogous sulfonamide N–H to zinc interaction, interactions were predicted with the sulfonamide oxygen to Thr-199 and zinc, H-bonding between His-96 and the urea oxygen, arene stacking with Thr-200, and between Gln-92 and furan C–H (Figure 4D).

Interestingly, no NLRP3 peptides were observed in any of our proteomic data sets, even though the macrophages were primed for pyroptosis. At first sight, this is surprising since NLRP3 is predicted to generate proteotypic tryptic peptides suitable for identification by mass spectrometry, with a maximum theoretical protein coverage of 90% according to Proteomics DB.[31] However, in an extensive MS-based human proteome draft in 2014, just 45 peptide–spectrum matches (PSMs) were made for NLRP3 covering only 7.82% of the NLRP3 sequence,[31] compared to an average of 9651 per protein (~175 million PSMs across 18,097 proteins).[32] It remains unclear why NLRP3 presents such a significant challenge for proteomics, particularly when it is readily identified by western blotting as seen in our study through direct engagement by **IMP2070**.

Here, we have developed **IMP2070** as the first diazirine-based photoaffinity-based probe (AfBP) for MCC950, and the first MCC950 AfBP to be used for *de novo* chemical proteomic identification of MCC950-binding proteins. **IMP2070** mimics MCC950 in cellular assays, inhibiting NLRP3 inflammasome activation and pyroptosis, and shows direct target engagement with endogenous NLRP3 protein in human macrophages. A combination of chemical proteomic and thermal protein profiling experiments in intact THP1 cells identified a spectrum of novel MCC950 binders through competition and protein stabilization at endogenous levels. These approaches individually identified CA2 as an important off-target protein for MCC950, which we further confirmed as a novel noncompetitive inhibitor of CA2 in an independent

activity assay. The identification of CA2 as the only target identified in both label-based and label-free proteomic methods is likely due to a combination of abundance and behavior under thermal unfolding conditions, together with affinity for MCC950 and a binding mode which favors cross-linking. This highlights the added stringency of multiple orthogonal target engagement approaches in order to focus subsequent experiments on targets with a high probability of confirmation; however, it does not preclude the possibility to confirm targets found in only one of the approaches in future experiments.

MCC950 is ubiquitously used as a specific inflammasome inhibitor *in vitro*, and despite a reported pyroptosis IC_{50} of 7.5 nM, it is frequently applied in the micromolar range, suggesting the potential for CA2 inhibition in these experiments.[4] Given that pyroptosis is typically complete within a few hours, off-target phenotypic impacts are likely to be minimized by the short timeframes of most *in vitro* experiments, including those performed here. However, the impact of CA2 inhibition may be more significant for mouse models of inflammasome-associated diseases where doses up to 50 mg kg^{-1} MCC950 are used routinely over many days, with serum concentrations predicted to exceed 60 μM for much of the dosing period.[4] The pharmacological significance of CA2 inhibition will depend on the context, including drug concentration, target organ, and CA2 expression levels. However, CA2 is a widely expressed cytosolic CA involved in maintaining pH and fluid balance. Genetic deficiency of CA2 in humans is linked to osteopetrosis, a bone disease wherein osteoclasts (a type of myeloid cell related to macrophages) are unable to perform bone resorption normally.[33] Therefore, it is plausible that CA2 inhibition during long-term use of MCC950 in clinical settings in inflammatory disease could have had unwanted effects. Moreover, given the active site sequence similarity between CA isoforms and broad expression in tissues,[34] MCC950 could potentially also inhibit other CA enzymes. We suggest that CA2 and potentially other carbonic anhydrases should be considered when addressing the safety and on-target efficacy of MCC950 and related sulfonylurea derivatives as clinical NLRP3 inhibitors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00218.

Methods and materials used, and additional figures. Proteomics data are available via ProteomeXchange with identifiers PXD024913 and PXD024915. (PDF)

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Notes

The authors declare no competing financial interest.
The data and compounds reported in this study are available upon request from the corresponding author.

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