The NLRP3 inflammasome is a critical component of the innate immune system. NLRP3 activation is induced by diverse stimuli associated with bacterial infection or tissue damage, but its inappropriate activation is involved in the pathogenesis of inherited and acquired inflammatory diseases. However, the mechanism by which NLRP3 is activated remains poorly understood. In this study, we explored the role of kinases in NLRP3 activation by blocking assembly of the NLRP3 inflammasome by inhibiting NLRP3 ATPase activity. Notably, MNS did not affect the activation of the NLRC4 or AIM2 (absent in melanoma 2) inflammasome. Mechanistically, MNS specifically prevented NLRP3-mediated ASC speck formation and oligomerization without blocking potassium efflux induced by NLRP3 agonists. Surprisingly, Syk kinase, the reported target of MNS, did not mediate the inhibitory activity of MNS on NLRP3 inflammasome activation. We also found that the nitrovinyl group of MNS is essential for the inhibitory activity of MNS. Immunoprecipitation, mass spectrometry, and mutation studies suggest that both the nucleotide binding oligomerization domain and the leucine-rich repeat domain of NLRP3 were the intracellular targets of MNS. Administration of MNS also inhibited NLRP3 ATPase activity in vitro, suggesting that MNS blocks the NLRP3 inflammasome by directly targeting NLRP3 or NLRP3-associated complexes. These studies identified a novel chemical probe for studying the molecular mechanism of NLRP3 inflammasome activation.
EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies against mouse caspase-1, Asc, Nlrp3, and Nlrc4 have been described previously (16, 17). Murine IL-1β antibody (AF-401-NA) was purchased from R&D Systems. IL-18 antibody (5180R-100) was purchased from BioVision. Antibodies against Syk, phosphotyrosine (Tyr(P)-100), and GST were from Cell Signaling. Antibodies against actin and GAPDH were from Genescript. The InhibitorSelect 384-well protein kinase Library I, MNS, Bay 11-7082, and nigericin were purchased from Millipore. Anti-FLAG antibody, ATP, 3,4-(methylenedioxy)cinnamic acid, 1,2-methylenedioxy-4-propenylbenzene, trans-β-nitrostyrene, benzoylnitro- methane, and trans-4-hydroxy-3-methoxy-β-nitrostyrene were from Sigma. Biotinyl-6-aminohexanoic acid (C16H27N3O4S) was purchased from Chem-Impex (Wood Dale, IL). Biotinylation of trans-4-hydroxy-3-methoxy-β-nitrostyrene with biotinyl-6-aminohexanoic acid was performed as described (18). The purity of biotinylated product was 97.3% as determined by HPLC. Ultrapure LPS from Escherichia coli 0111:B4 and poly(dA:dT)/lyovec were purchased from Invivogen. Salmonella enterica sv. typhimurium strain SL1344 was a gift from Denise Monack (Stanford University, Stanford, CA). Streptavidin magnetic beads were from Pierce. Recombinant GST-NLRP3 was purchased from Abnova. P. ColorLock Gold phosphate detection system was purchased from Innova Bioscience. All other reagents if not specified were from Sigma.

Cells and Treatments—Bone-marrow derived macrophages (BMDMs) were prepared and cultured as described previously (19). For screening with a kinase inhibitor library, 5 × 10⁶ cells were plated on 96-well plates overnight. Cells were primed with 100 ng/ml LPS for 4 h in serum-free Iscove’s modified Dulbecco’s medium. Cells were incubated with each inhibitor (10 μM) for 15 min before being pulsed with 5 mM ATP for 30 min. The release of IL-1β in culture supernatants was determined by ELISA. For the detection of inflammasome activation by Western blotting, 1 × 10⁶ cells were plated on 12-well plates overnight. Inhibitors were added to medium in the last 15 min of LPS priming. Inflammasome activation was induced by adding respective stimuli: 5 mM ATP (30 min), 10 μM nigericin (1 h), 500 μg/ml silica (4 h), 2 μg/ml poly(dA:dT) (4 h), and S. enterica sv. typhimurium (m.o.i. = 10, 1 h).

Western Blots—Cells were lysed in ice-cold PBS buffer containing 1% Nonidet P-40 supplemented with complete protease inhibitor mixture (Roche Applied Science). The proteins from cell-free supernatants were precipitated by choloform/methanol method. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes by electroblotting (Bio-Rad), and membranes were immunoblotted with respective antibodies.

Measurement of Cytokines—Mouse IL-1β and TNF-α in culture supernatants were measured by ELISA kits (R&D Systems) according to manufacturer’s manual. Mouse IL-18 in culture supernatants was measured with an ELISA kit from eBioscience. Assays were performed in triplicate for each independent experiment.

Generation of Syk−/− Chimeras—Fetal livers were harvested at day 16 of gestation from both WT and syk−/− embryos generated by intercrossing syk−/− transgenic mice (S129). 5 × 10⁶ cells were injected into the lateral tail vein of 8-week-old recipients that had been lethally irradiated with two doses of 550 rads of x-irradiation separated by 3 h. The recipient animals received antibiotics (sulfamethoxazole and trimethoprim) for 8 weeks after reconstitution. Bone marrows were harvested from chimeras and differentiated for macrophages as described above.

Potassium Efflux Assay—Intracellular K⁺ measurements were performed as described previously (20). Briefly, macrophages were plated on 96-well plates. Culture medium was thoroughly aspirated after stimulation. Cells were lysed with 3% ultrapure HNO₃. Intracellular K⁺ was then determined by inductively coupled plasma optical emission spectrometry with an Optima 2000 DV spectrometer (PerkinElmer Life Sciences) using yttrium as internal standard.

ASC Speck Staining and ASC Oligomer Cross-linking—BMDMs were plated on an 8-well chamber slide overnight. Cells were primed and pretreated with inhibitor or DMSO before being stimulated with ATP or nigericin, transfected with poly(dA:dT), or infected with S. enterica sv. typhimurium. After stimulation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with PBS buffer containing 3% BSA. Cells were stained with anti-Asc antibody and Alexa Fluor 488-conjugated secondary antibody. DAPI was used to stain nuclei. Cell images were taken with Zeiss fluorescent microscope.

For ASC oligomer cross-linking, cells were lysed with PBS buffer containing 0.5% Triton X-100, and the cell lysates were centrifuged at 8000 rpm for 15 min at 4 °C. The Triton X-100-insoluble pellets were washed with PBS twice and then suspended in 200 μl of PBS. The pellets were then cross-linked at room temperature for 30 min by adding disuccinimidyl suberate (2 mM). The cross-linked pellets were spun down at 8000 rpm for 15 min and dissolved directly in SDS sample buffer.

HEK293T Cell Transfection—2.5 × 10⁶ HEK293T cells were plated on a 100-mm Petri dish a day before transfection. Cells were transfected for 16 h with triple FLAG-tagged full-length NLRP3 (FL, 1.25 μg), pyrin deletion mutant ΔPyrin (amino acids 94–1036, 1.25 μg), LRR deletion mutant ΔLRR (amino acids 1–741, 2.5 μg), pyrin domain (amino acids 1–93, 1.25 μg), NOD domain (amino acids 220–536, 5 μg), and LRR domain (amino acids 742–991, 2.5 μg) by Lipofectamine LTX (Invitrogen). Cell culture medium was replaced with prewarmed serum-free DMEM. Cells were incubated with biotinylated compound biotin-trans-4-hydroxy-3-methoxy-β-nitrostyrene (biotin-HMNS) (10 μM) for 30 min at 37 °C and then lysed in 1.5 ml of ice-cold lysis buffer (50 mM Tris, pH 7.4, 2 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1× EDTA-free proteinase inhibitor mixture (Roche Applied Science)). Cell lysates were clarified by centrifugation (14,000 × g) at 4 °C for 10 min. Cell lysates were incubated with streptavidin magnetic beads (25-μl slurry) overnight. The proteins bound by biotin-HMNS were pulled down and subjected to Western blotting.

Pulldown Assay by Biotin-HMNS—LPS primed BMDMs were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 2 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1× Roche EDTA-free
proteinase inhibitor mixture). 1 ml of cell lysates with total 1 mg protein were incubated with bixin (1 mM) or bixin-HMNS (1 mM) at 4 °C for 1 h. 50 µl of streptavidin magnetic beads (slurry) was added to cell lysates, followed by further incubation at 4 °C for 1 h. Magnetic beads were pulled down with a magnetic stand and washed with lysis buffer four times. Bound proteins were dissolved in SDS sample buffer. Proteins pulled down by bixin-HMNS were fractionated by SDS-polyacrylamide gel and subjected to mass spectrometry with whole lane analysis. When cells were used for the binding assay, cells were pretreated with bixin-HMNS (10 µM) for 30 min before lysis. For binding assay of GST-NLRP3 by bixin-HMNS, glutathione was removed from the solution of recombinant GST-NLRP3 with Slide-A-Lyzer Mini Dialysis Devices (Pierce) before incubation. For competition binding assay, 5 × 10^6 LPS-primed BMDMs were lysed in 1 ml of lysis buffer. Cell lysates were pretreated with 100 µM Bay 11-7082 or L-cysteine for 30 min at 4 °C before adding 1 µM bixin-HMNS.

Measurement of NLRP3 ATPase Activity—Recombinant NLRP3 was incubated with the indicated concentrations of MNS inhibitor in reaction buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM PMSF, and 1x proteinase inhibitor) for 15 min. DMSO was used as a vehicle control. The reaction mixtures were further incubated for 1 h at 37 °C after the addition of 250 µM ATP. The hydrolysis of ATP by NLRP3 was measured by P, ColorLock Gold phosphate detection system according to the manual.

Cytotoxicity Assay—The percentage of cell death was determined using the lactate dehydrogenase release assay (Promega). The absorbance at 490 nm was measured, and the percentage of cytotoxicity was calculated relative to the 100% release value obtained by lysis of cells with a solution of 0.1% Triton X-100.

Statistical Analysis—Student’s t test was used to determine statistically significant difference between two groups. One-way analysis of variance was used to analyze differences among multiple groups. A p value <0.05 was considered significant.

RESULTS

Identification of MNS as a Potent Inhibitor for ATP-induced NLRP3 Inflammasome Activation—Given the universal role of kinases in signal transduction, we sought to investigate the signaling mechanism of NLRP3 inflammasome activation by screening a kinase inhibitory library containing 160 selected kinase inhibitors. As Toll-like receptor ligands or cytokines are required to induce the expression of Nlrp3 and pro-IL-1β through NF-κB signaling in mouse bone marrow macrophages (21), we primed macrophages with LPS before the treatment with inhibitors to exclude their potential inhibitory effects on the NF-κB signaling pathway. Macrophages were treated with each kinase inhibitor (10 µM) for 15 min before stimulation with the NLRP3 activator ATP. We used ELISA to measure the level of IL-1β in the culture supernatant as a readout for NLRP3 inflammasome activation. The compound Bay 11-7082 inhibited Nlrp3 inflammasome activation in accordance with a previous report (22). We identified an additional chemical compound, MNS, that consistently abolished IL-1β secretion in our screening experiments (Fig. 1A).

To further investigate the inhibitory activity of MNS, we examined ATP-induced IL-1β secretion by LPS-primed mouse macrophages in the presence of different concentrations of the compound. As shown in Fig. 1B, MNS inhibited IL-1β secretion with an approximate IC50 of 2 µM. In contrast, the levels of TNF-α secretion between untreated and MNS-treated macrophages were comparable (Fig. 1C). Consistent with the ELISA results, MNS inhibited the production of mature IL-1β in the cell supernatant as shown by immunoblotting (Fig. 1D). Furthermore, IL-18 secretion and caspase-1 cleavage were also inhibited in the presence of MNS. Notably, MNS did not affect the protein levels of Nlrp3, Asc, or caspase-1 in mouse macrophages (Fig. 1D). As a comparison, MNS was slightly more potent than Bay 11-7082 in inhibiting NLRP3-mediated caspase-1 cleavage (Fig. 1E). In addition, treatment with MNS also inhibited ATP-induced NLRP3 inflammasome activation in Nlrp3-null immortalized macrophages reconstituted with Nlrp3, which do not need priming for Nlrp3 induction (Fig. 1F). These results indicate that chemical compound MNS potently inhibits ATP-induced NLRP3 inflammasome activation.

MNS Specifically Inhibits NLRP3 Inflammasome Activation—ATP activates the NLRP3 inflammasome through P2X7 receptor signaling (23, 24). To determine whether MNS specifically targets P2X7 receptor signaling or a common signaling component(s) for NLRP3 inflammasome activation, we tested the inhibitory activity of MNS on inflammasome activation induced by two additional NLRP3 activators, nigericin and silica, which act independently of the P2X7 receptor. As it was observed with ATP, treatment with MNS inhibited IL-1β secretion induced by both NLRP3 activators in a dose-dependent manner (Fig. 2, A and B). Consistent with reduced secretion of IL-1β, immunoblotting of culture supernatant revealed that the cleavage of caspase-1 was abolished in the presence of MNS (Fig. 2C). In contrast, MNS treatment did not affect caspase-1 activation induced by Salmonella, a pathogen known to trigger NLRC4 inflammasome activation (Fig. 2D) (25, 26). Transfection of synthetic poly(dA:dT) induces AIM2 inflammasome activation in macrophages (27, 28). Likewise, MNS did not affect AIM2 inflammasome activation by poly(dA:dT) (Fig. 2E). Caspase-1 activation triggers pyropotosis in macrophages, which can be revealed by measuring the release of lactate dehydrogenase (29). Consistently, MNS prevented ATP- and nigericin-induced lactate dehydrogenase release, but not that induced by Salmonella and poly(dA:dT) (Fig. 2F). These data indicate that MNS is a specific inhibitor of the NLRP3 inflammasome.

Syk Kinase Contributes to Pro-IL-1β Production but Is Dispensable for NLRP3 Inflammasome Activation in Macrophages—MNS is categorized as a Syk kinase inhibitor in our screening kinase inhibitor library. A previous study has shown that Syk was not required for NLRP3 inflammasome activation by nigericin but was essential for Candida albicans-induced NLRP3 inflammasome activation in dendritic cells (30). To examine further the role of Syk in NLRP3 inflammasome activation, we generated Syk-deficient BMDMs from chimeric mice. Syk depletion was confirmed in Syk–/– BMDMs by immunoblotting (Fig. 3A). There was minimal or no impairment in caspase-1 cleavage in response to the NLRP3 activators ATP,
nigericin and silica in Syk\textsuperscript{−/−} macrophages compared with wild-type macrophages (Fig. 3B). However, IL-1β secretion was reduced in Syk\textsuperscript{−/−} macrophages, consistent with the reduced levels of pro-IL-1β in Syk\textsuperscript{−/−} macrophages (Fig. 3, B and C). In contrast, IL-18 secretion was slightly elevated in Syk\textsuperscript{−/−} macrophages as well as the intracellular levels of pro-IL-18 (Fig. 3B). This elevated secretion of IL-18 in Syk\textsuperscript{−/−} macrophages was also confirmed by ELISA (Fig. 3D). The secretion of cytokine TNF-α was not affected by Syk deficiency (Fig. 3E). These data suggest that the Syk kinase contributes to pro-IL-1β induction by LPS but is largely dispensable for MNS-mediated inhibition of the NLRP3 inflammasome.

MNS Prevents NLRP3 Agonist-induced ASC Speck Formation without Blocking Potassium Efflux—Potassium efflux is required for NLRP3 inflammasome activation in response to ATP, bacterial toxins, and particulate matter (20, 23, 24, 31). To test whether MNS inhibits NLRP3 inflammasome activation by preventing potassium efflux, we compared the levels of intracellular potassium after ATP stimulation in the presence or absence of MNS. ATP stimulation induced the drop of intracellular potassium in DMSO-treated macrophages, and this was unaffected by MNS pretreatment, suggesting that MNS acts downstream of potassium efflux to inhibit NLRP3 activation (Fig. 4A). A common event associated with inflammasome activation is the formation of large intracellular ASC aggregates called ASC specks that can be observed by microscopy or detected as oligomers after chemical cross-linking by immunoblotting (29). MNS prevented ASC speck formation induced by NLRP3 agonists, but did not affect ASC speck formation induced by stimuli that activate NLRC4 or AIM2 (Fig. 4, B and C). Biochemically, the presence of MNS abolished ASC oligomerization induced by activation of NLRP3, but not of the
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In contrast, YVAD, an inhibitor of caspase-1, blocked caspase-1 cleavage but did not affect ASC oligomerization (Fig. 4D). These results indicate that MNS specifically inhibits NLRP3-mediated ASC speckle formation.

Structure-Activity Assay of MNS Analogues—To explore the structure-activity relationship of MNS and the NLRP3 inflammasome, we examined the inhibitory activity of MNS analogues on NLRP3 inflammasome activation. We selected five ana-
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MNS Binds Directly to NLRP3 and Inhibits Its ATPase Activity—As MNS was previously reported to inhibit tyrosine kinases (32), we examined the pattern of tyrosine phosphorylation in BMDMs stimulated by ATP or nigericin in the absence or presence of inhibitors. Cells treated with MNS and C4 had comparable patterns of tyrosine phosphorylation as detected by immunoblotting with anti-Tyr antibody (data not shown). To determine the mechanism by which MNS inhibits NLRP3 inflammasome, we examined MNS-targeted proteins in mouse macrophages. The compound HMNS was biotinylated through hydrogen oxide group (biotin-HMNS) (Fig. 6A). Biotin-HMNS retained inhibitory activity on NLRP3 inflammasome, albeit at a higher concentration (Fig. 6B). Macrophage proteins pulled down by biotin-HMNS were fractionated by SDS-PAGE and analyzed by mass spectrometry (Fig. 6C and supplemental Table). Notably, NLRP3 protein was one of the most abundant proteins pulled down by biotin-HMNS. The association between biotin-HMNS and NLRP3 protein was further confirmed by immunoprecipitation followed by immunoblotting (Fig. 6D). Consistent with the finding that MNS does not inhibit NLRC4 inflammasome, we could not detect biotin-HMNS associated with the NLRC4 protein by mass spectrometry or immunoprecipitation assay (Fig. 6D and supplemental Table). Furthermore, biotin-HMNS could pull down recombinant NLRP3, and this was abolished in the presence of excess HMNS (Fig. 6E). These data suggest that NLRP3 is directly targeted by MNS. To map the targeting domains of NLRP3 by MNS, we treated HEK293 cells expressing full-length wild-type NLRP3 (FL) or mutants lacking the pyrin domain (ΔPyrin) or the leucine-rich repeat domain (ΔLRR) with biotin-HMNS. Biotin-HMNS could pull down WT NLRP3 and NLRP3 mutants with deletion of either the pyrin or LRR domain (Fig. 6F). Moreover, biotin-HMNS could pull down the NOD and the LRR domain, but not the pyrin domain (Fig. 6G). Furthermore, MNS inhibited the ATPase activity of NLRP3 in a concentration-dependent manner, as measured by the release of free phosphate (Fig. 6H). Bay 11-7082 has been reported to inhibit the ATPase activity of NLRP3 possibly through the mechanism of cysteine modification (22). We
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examined whether Bay 11-7082 and l-cysteine could compete off the pulldown of NLRP3 by biotin-HMNS. Addition of excess amounts of Bay 11-7082 and l-cysteine abolished the pulldown of NLRP3 by biotin-HMNS (Fig. 6I). Taken together, these results suggest that MNS directly binds to NLRP3 and inhibit its ATPase activity.

CONCLUSION

Inappropriate activation of the NLRP3 inflammasome has been implicated in the pathogenesis of inflammation-associated diseases, such as gouty arthritis, silicosis, type 2 diabetes, Alzheimer disease, and cryopyrin-associated periodic fever syndromes (2, 33). Thus, inhibition of NLRP3 inflammasome activation could have therapeutic potential for treating these diverse inflammatory diseases. Based on research in mouse macrophages, a two-step mechanism has been proposed for NLRP3 inflammasome activation. In the first step, known as priming, NLRP3 is transcriptionally up-regulated by TLR ligands or cytokines through the NF-κB signaling pathway (21). However, NLRP3 induction is not sufficient for activation of the NLRP3 inflammasome in that a second step triggered by diverse stimuli such as ATP, nigericin, or particulate matter, is necessary for NLRP3 activation (7, 34). Although kinases such as IkK and TAK1 are required for the priming step of NLRP3 induction, it is not clear whether kinases are also involved in the activating step. A previous study showed that the kinase PKR was required for the activation of all known inflammasomes including the NLRP3 inflammasome (35). However, these results could not be confirmed by a different study (36). Furthermore, PKCθ was reported to regulate NLRC4 inflammasome activation by phosphorylating NLRC4 (37). In this study, we sought to explore the role of kinases in NLRP3 inflammasome activation by screening a kinase inhibitor library. We identified a Syk kinase inhibitor, MNS, as an inhibitor of NLRP3 inflammasome activation. MNS did not affect the activation of the NLRC4 and AIM2 inflammasomes at concentrations that abrogated NLRP3 inflammasome activation. Surprisingly, deletion of the Syk kinase in macrophages did not affect NLRP3 inflammasome activation induced by ATP, nigericin and silica, indicating that Syk is dispensable for NLRP3 inflammasome activation. These results are consistent with a previous study that Syk was required for caspase-1 activation induced by ATP, nigericin and silica. MNS was reported to inhibit other tyrosine kinases such as Src and FAK at higher concentrations (IC_{50} > 27.3 μM) (32). Thus, it is possible that those tyrosine kinase(s) may play a redundant role in NLRP3 inflammasome activation in macrophages. However, we found that MNS treatment had patterns of tyrosine phosphorylation comparable with those of C4 treatment after ATP or nigericin stimulation. Alternatively, MNS may inhibit NLRP3 inflammasome activation through a novel kinase-independent fashion (see below). Further studies are needed to understand how MNS inhibits the NLRP3 inflammasome.

The blockade of IL-1β function by anakinra is currently used in the clinic to treat cryopyrin-associated periodic fever syndrome patients (38). In addition to IL-1β maturation, NLRP3 inflammasome activation induces other cell responses such as cell death and IL-18 maturation, suggesting that therapeutic strategies that directly target NLRP3 inflammasome activation would be more effective than blocking IL-1β alone. Previous studies have identified several inhibitors of NLRP3 inflammasome activation, including glyburide, parthenolide, Bay 11-7082, bromoxone, CRID3, and deubiquitinase inhibitors (22, 39–44). These inhibitors have been suggested to target the NLRP3 ATPase activity, ASC oligomerization, or upstream signaling events required for NLRP3 activation. Here, we have identified MNS as a novel inhibitor of NLRP3 inflammasome activation. Previously MNS was reported to inhibit platelet aggregation, tumor cell growth, and apoptosis (45–47). Effects of nitrostyrene on tyrosine kinases, protein phosphatases, and telomerase activity have been suggested to account for those inhibitions. Structurally, the nitrovinyl group was essential for MNS inhibitory activity as tested compounds with this group retained inhibitory activity for NLRP3 inflammasome activation in dendritic cells (30). MNS was identified as a novel inhibitor of NLRP3 inflammasome activation, including glyburide, parthenolide, Bay 11-7082, bromoxone, CRID3, and deubiquitinase inhibitors (22, 39–44). These inhibitors have been suggested to target the NLRP3 ATPase activity, ASC oligomerization, or upstream signaling events required for NLRP3 activation. Here, we have identified MNS as a novel inhibitor of NLRP3 inflammasome activation. Previously MNS was reported to inhibit platelet aggregation, tumor cell growth, and apoptosis (45–47). Effects of nitrostyrene on tyrosine kinases, protein phosphatases, and telomerase activity have been suggested to account for those inhibitions. Structurally, the nitrovinyl group was essential for MNS inhibitory activity as tested compounds with this group retained inhibitory activity for NLRP3 inflammasome activation. In contrast, the dioxole group was dispensable for NLRP3 inhibitory activity. One possibility is that the first carbon of the nitrostyrene on tyrosine kinases, protein phosphatases, and telomerase activity have been suggested to account for those inhibitions. Structurally, the nitrovinyl group was essential for MNS inhibitory activity as tested compounds with this group retained inhibitory activity for NLRP3 inflammasome activation. In contrast, the dioxole group was dispensable for NLRP3 inhibitory activity. One possibility is that the first carbon of
This compound. Further biochemical studies on the mechanism of action of nitrostyrene on NLRP3 may provide additional insight for the development of new therapeutic strategies for blocking abnormal NLRP3 inflammasome activation.

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REFERENCES

1. Dinarello, C. A. (2009) Immunological and inflammatory functions of the interleukin-1 family. Annu. Rev. Immunol. 27, 519–550
2. Martinon, F., Mayor, A., and Tschopp, J. (2009) The inflammasomes: guardians of the body. Annu. Rev. Immunol. 27, 229–265
3. Franchi, L., Muñoz-Planillo, R., and Núñez, G. (2012) Sensing and reacting to microbes through the inflammasomes. Nat. Immunol. 13, 325–332
4. Schroder, K., and Tschopp, J. (2010) The inflammasomes. Cell 140, 821–832
5. Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., Fitzgerald, K. A., and Latz, E. (2008) Silica crystals and aluminium salts activate the NALP3 inflammasome through phagosomal destabilization. Nat. Immunol. 9, 847–856
6. Halle, A., Hornung, V., Petzold, G. C., Stewart, C. R., Monks, B. G., Reinheckel, T., Fitzgerald, K. A., Latz, E., Moore, K. J., and Golenbock, D. T. (2008) The NALP3 inflammasome is involved in the innate immune response to amyloid-β. Nat. Immunol. 9, 857–865
7. Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A., and Tschopp, J. (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 440, 237–241
8. Cassel, S. L., Eisenbarth, S. C., Iyer, S. S., Sadler, J. J., Flavell, R. A., and Sutterwala, F. S. (2008) The Nalp3 inflammasome is essential for the development of sili
cosis. Proc. Natl. Acad. Sci. U.S.A. 105, 9035–9040
9. Duewell, P., Kono, H., Rayner, K. J., Sirois, C. M., Vlam, G., Bauernfeind, F. G., Abela, G. S., Franchi, L., Nuñez, G., Schurmann, M., Espévak, T., Lien, E., Fitzgerald, K. A., Rock, K. L., Moore, K. J., Wright, S. D., Hornung, V., and Latz, E. (2010) NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 464, 1357–1361
10. Masters, S. L., Dunne, A., Subramanian, S. L., Hull, R. L., Tannahill, G. M., Sharp, F. A., Becker, C., Franchi, L., Yoshihara, E., Chen, Z., Mullolooy, N., Mielke, L. A., Harris, J., Coll, R. C., Mills, K. H., Mok, K. H., Newsholme, P., Nuñez, G., Yodoi, J., Kahn, S. E., Lavelle, E. C., and O’Neill, L. A. (2010) Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β in type 2 diabetes. Nat. Immunol. 11, 897–904
11. Heneka, M. T., Kummer, M. P., Stutz, A., Delisate, A., Schwatz, S., Vieira-Saecker, A., Grieb, A., Axt, D., Remus, A., Tseng, T. C., Gelpi, E., Halle, A., Korte, M., Latz, E., and Golenbock, D. T. (2013) NLRP3 is activated in Alzheimer’s disease and contributes to pathology in APP/PS1 mice. Nature 493, 674–678
12. Ting, J. P., Kastner, D. L., and Hoffman, H. M. (2006) CATERPILLERS,
Nitrostyrene Inhibits NLRP3 Inflammasome

pyrin and hereditary immunological disorders. Nat. Rev. Immunol. 6, 183–195

13. Hoffman, H. M., Mueller, I. L., Brodie, D. H., Wanderer, A. A., and Kolodner, R. D. (2001) Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat. Genet. 29, 301–305

14. Feldmann, J., Priew, A. M., Quarter, P., Berquin, P., Certain, S., Cortes, E., Teillac-Hamel, D., Fischer, A., and de Saint Basile, G. (2002) Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. Am. J. Hum. Genet. 71, 198–203

15. Rathinam, V. A., Vanaja, S. K., and Fitzgerald, K. A. (2012) Regulation of inflammasome signaling. Nat. Immunol. 13, 333–342

16. He, Y., Franchi, L., and Núñez, G. (2013) TLR agonists stimulate NLRP3-dependent IL-1β production independently of the purinergic P2X7 receptor in dendritic cells and in vivo. J. Immunol. 190, 334–339

17. Franchi, L., Kamada, N., Nakamura, Y., Burberry, A., Kuffa, P., Suzuki, S., Shaw, M. H., Kim, Y. G., and Núñez, G. (2012) NLRC4-driven production of IL-1β discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. Nat. Immunol. 13, 449–456

18. Giubellino, A., Shi, Z. D., Jenkins, L. M., Worthy, K. M., Bindu, L. K., Thaunat, Y., Peruzzi, B., Fisher, R. J., Ippolita, E., Burke, T. R., and Bottaro, D. P. (2008) Selectivity and mechanism of action of a growth factor receptor–bound protein 2 SRC homology 2 domain binding antagonist. J. Med. Chem. 51, 7459–7468

19. Franchi, L., Eigenbrod, T., and Núñez, G. (2009) Cutting edge: TNF-α mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. J. Immunol. 183, 792–796

20. Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendran, T. M., and Núñez, G. (2013) K+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity 38, 1142–1153

21. Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B. G., Fitzgerald, K. A., Hornung, V., and Latz, E. (2009) Cutting edge: NF-κB is not required for the activation of the NLRP3 inflammasome. Nat. Immunol. 10, 183, 787–791

22. Juliana, C., Fernandez-Alnemri, T., Wu, J., Datta, P., Solorzano, L., Yu, J. W., Meng, R., Quong, A. A., Latz, E., Scott, C. P., and Alnemri, E. S. (2010) Anti-inflammasome compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome. J. Biol. Chem. 285, 9792–9802

23. Franchi, L., Kanneganti, T. D., Dubby, G. R., and Núñez, G. (2007) Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. J. Biol. Chem. 282, 18810–18818

24. Kahlenberg, J. M., and Dubyak, G. R. (2004) Mechanisms of caspase-1 activation in dendritic cells and in vivo. J. Immunol. 170, 995–1007

25. Lamkanfi, M., Mueller, J. L., Vitari, A. C., Misaghi, S., Fedorova, A., Deshayes, K., Wehrli, N., Lassus, Y., Liu, J., Córdova, S., Kopp, J. C., and Arntz, D., Monack, D., and Dixit, V. M. (2006) Glyburide inhibits the cryopyrin/NALP3 inflammasome. J. Biol. Chem. 281, 2721–2733

26. Lopez-Castejón, G., Luheshi, N. M., Compan, V., High, S., Whitehead, R. C., and O’Neill, L. A. (2011) The cytokine release inhibitory drug CRD1 targets ASC oligomerization in the NLRP3 and AIM2 inflammasomes. PLoS One 6, e29539

27. Wang, Y. W., Wu, Y. C., and Wu, C. C. (2006) Prevention of platelet glycoprotein Ib/IIIa activation by 3,4-methylenedioxy-flavonoids: a novel tyrosine kinase inhibitor. Mol. Pharmacol. 70, 1380–1389

28. Kim, J. H., Lee, G. E., Lee, J. E., and Chung, I. K. (2003) Potent inhibition of human tolleromerase by nitrostyrene derivativ. Mol. Pharmacol. 63, 1117–1124

29. Kaap, S., Quentin, L., Tamiru, D., Shaheen, M., Eger, K., and Steinfeld, H. J. (2003) Structure activity analysis of the pro-apoptotic, autitumor effect of nitrostyrene adducts and related compounds. Biochem. Pharmacol. 65, 603–610