

Dehydroisohispanolone as a promising NLRP3 inhibitor agent. Bioevaluation and Molecular Docking.

Laura González-Cofrade¹, Irene Cuadrado¹, Angel Amesty², Ana Estévez-Braun^{2,*}, Beatriz de las Heras^{1,*}, Sonsoles Hortelano^{3,*}

¹Departamento de Farmacología, Farmacognosia y Botánica, Facultad de Farmacia, Universidad Complutense de Madrid (UCM), Plaza Ramón y Cajal s/n-28040, Madrid, Spain; lagonz11@ucm.es (L.G-C); icberrocal@ucm.es (I.C.); lasheras@ucm.es (B. de las H.).

²Departamento de Química Orgánica. Instituto Universitario de Bio-Organica Antonio González, Universidad de La Laguna, Avda. Astrofísico Francisco Sánchez Nº 2, 38206, La Laguna, Tenerife, Spain; aarnesty@ull.es (A.A.); aestebra@ull.es (A. E-B.)

³Unidad de Terapias Farmacológicas, Área de Genética Humana, Instituto de Investigación de Enfermedades Raras (IIER), Instituto de Salud Carlos III, Carretera de Majadahonda-Pozuelo Km 2-28220, Madrid, Spain; shortelano@isciii.es (S.H.)

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Preparation of deshydroisohispanolone (DIH).

Deshydroisohispanolone was obtained from the natural diterpene hispanolone following the procedure described in reference [21]. Thus, to 3.0 g (9.45 mol) of hispanolone in 175 mL of EtOH were added 10 mL of concentrated HCl and the reaction mixture was heated under reflux for 18 h. Next, this was treated with 100 mL of H₂O and extracted with CH₂Cl₂ (3 x 30 mL). The organic phases were collected, dried over anhydrous MgSO₄, filtered, and the solvent removed. The residue was purified by column chromatography with hexanes-EtOAc (95:5) to yield 0.14 g (1.0 %) of deshydroisohispanolone (DIH) and 1.3 g of deshydroisohispanolone (46%) as yellow oils.

DIH: $[\alpha]_D^{20}$ -91 (*c* 0.9, CHCl₃); IR (neat) $\nu_{\max}$ 3150, 2970, 2850, 1665, 1505, 1465, 1305, 1160, 1030, 995, 765 cm⁻¹; ¹H NMR (CDCl₃, 500 MHz) δ 7.26 (1H, bs, H-15), 7.11 (1H, bs, H-16), 6.18 (1H, s, H-14), 3.53 (1H, d, *J* = 14.8 Hz, H-6a), 3.25 (1H, d, *J* = 14.8 Hz, H-6b), 2.33 (2H, m, H-12), 1.86 (1H, dd, *J* = 13.7, 1.4 Hz, H-5), 1.59 (3H, m, H-2, H-1), 1.43 (1H, dd, *J* = 13.2, 1.4 Hz, H-3), 1.32 (1H, td, *J* = 13.4, 5.1 Hz, H-8), 1.19 (1H, td, *J* = 13.0, 5.1 Hz, H-8), 1.07 (3H, t, *J* = 7.7 Hz, H₃-17), 0.99 (3H, s, H₃-20), 0.98 (3H, s, H₃-19), 0.91 (3H, s, H₃-18); ¹³C NMR (CDCl₃, 100 MHz) δ 204.7 (C, C-9), 158.6 (C, C-11), 142.5 (CH, C-15), 139.1 (CH, C-16), 130.8 (C, C-7), 124.0 (C, C-13), 111.2 (CH, C-14), 48.4 (CH, C-5), 44.2 (C, C-10), 41.7 (CH₂, C-3), 33.7 (CH₂, C-1), 33.7 (C, C-4), 32.3 (CH₃, C-19), 28.1 (CH₂, C-6), 27.8 (CH₂, C-12), 22.2 (CH₃, C-20), 20.8 (CH₂, C-8), 18.3 (CH₂, C-2), 17.2 (CH₃, C-18), 12.4 (CH₃, C-17); EIMS *m/z* 300 ([M⁺], 100) 285 (8), 271 (11), 267 (10), 229 (15), 149 (32), 147 (30), 81 (54); HRESIMS *m/z* 323.1981 [M+Na]⁺ (calcd for C₂₀H₂₈O₂Na, 323.1987).

Figure S1: ^1H NMR (CDCl_3 , 500 MHz) of deshydroisohispanolone.

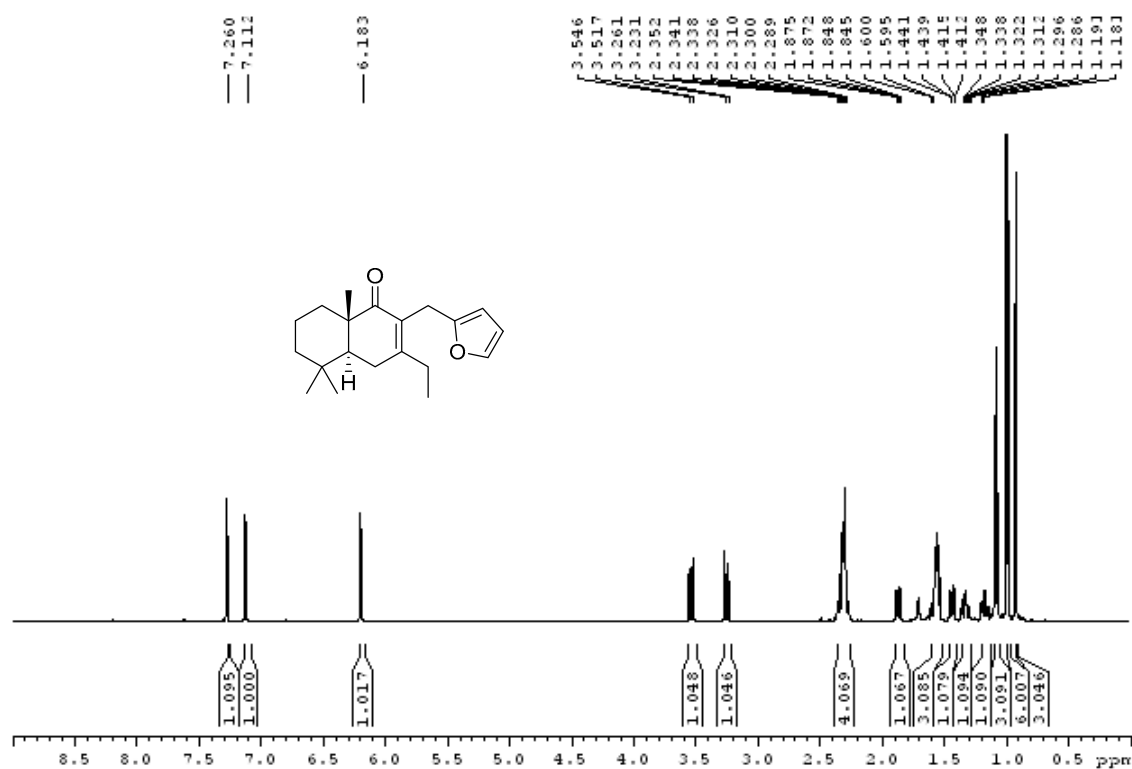


Figure S2: ^{13}C NMR (CDCl_3 , 100 MHz) of deshydroisohispanolone.

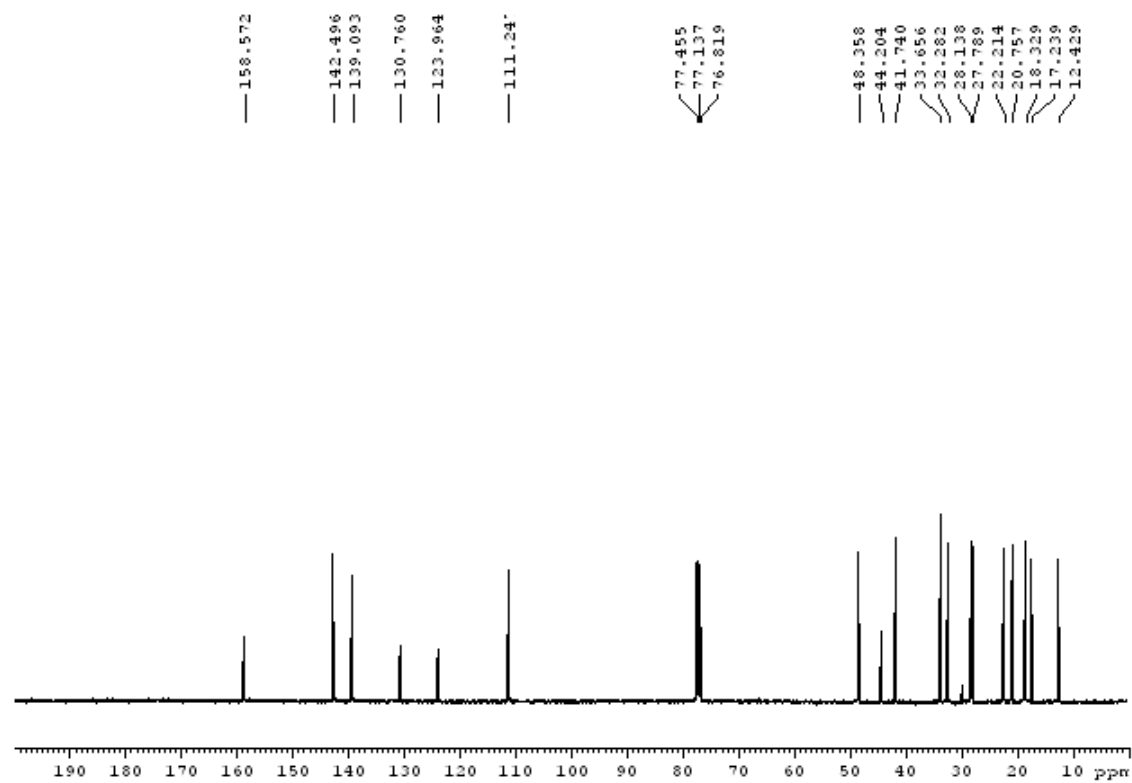


Figure S3: NLRP3 inflammasome inhibition by MCC950. MCC950 was used as a positive control for NLRP3 inflammasome inhibition. (A, B, C) LPS-primed J774A.1 macrophages were stimulated with Nig in presence of DIH (10, 20 μ M) or MCC950 (10 μ M) and IL-1 β release (A), Caspase-1 activity (B) and LDH release (C) were then measured as described. (D) LPS-primed BMDMs were stimulated with Nig in presence of DIH (10, 20 μ M) or MCC950 (10 μ M) and IL-1 β release was measured. Results are expressed as means $\pm$ SD (n = 3). *p < 0.05 and ***p < 0.001 vs. LPS + Nig treatment.

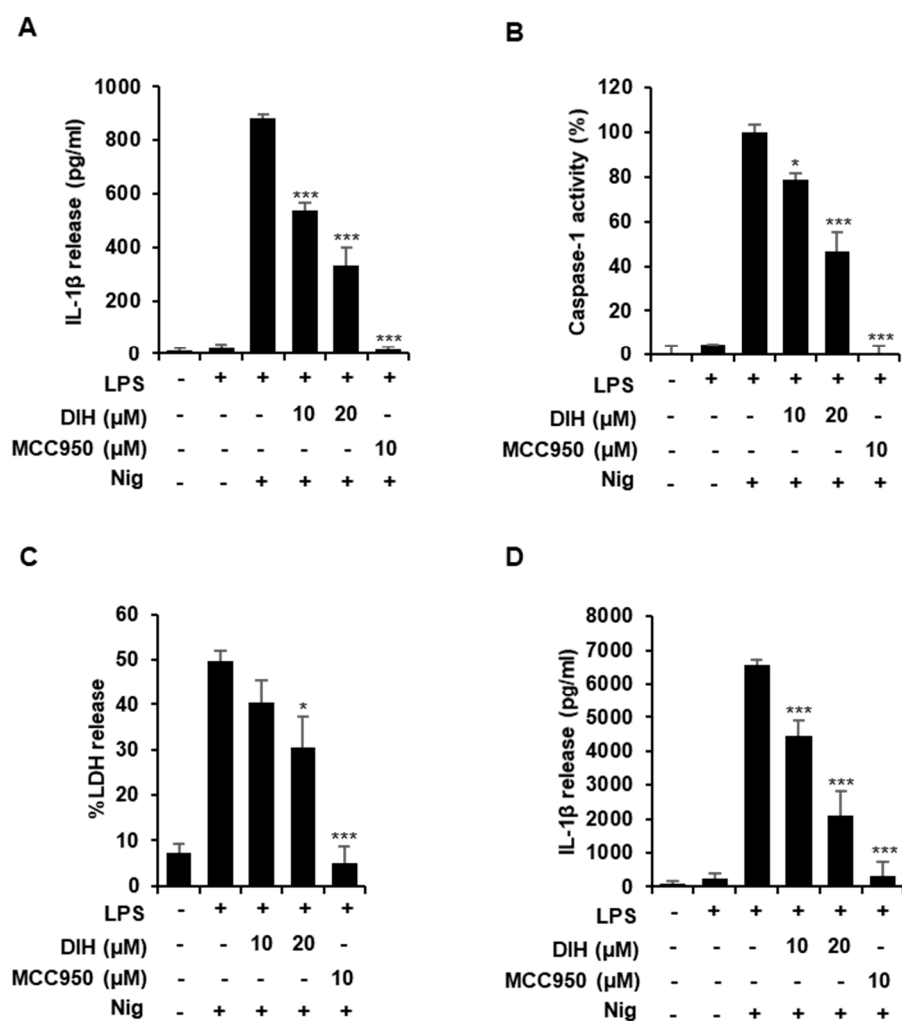


Figure S4. Original western blots for two repeats of Figure 2D. Whole blot of cell lysates after cutting membrane at molecular weight 35 kDa and 28 kDa for β -actin (42 kDa) and pro-IL-1 β (31 kDa); and supernatants after cutting membrane at 28 kDa for cleaved IL-1 β (17 kDa).

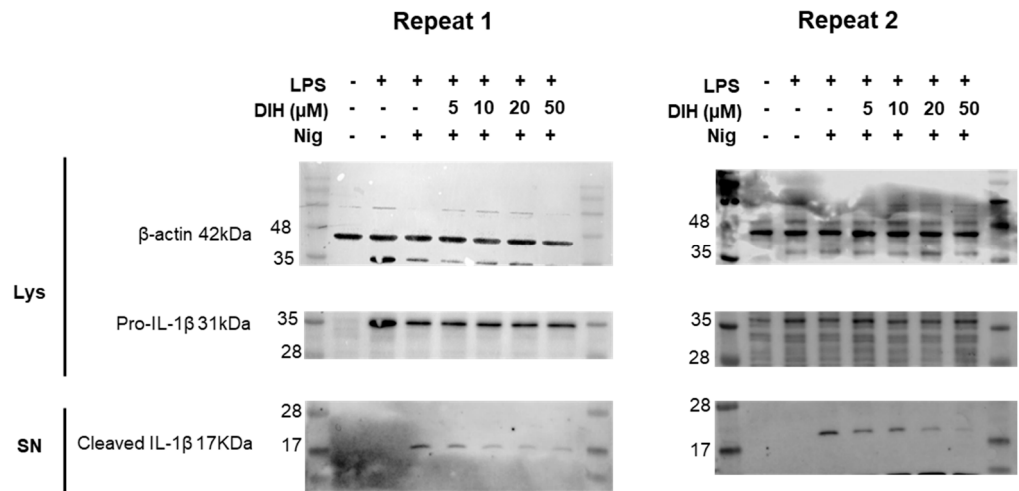


Figure S5. Original western blots for two repeats of Figure 2E. Whole blot of cell lysates after cutting membrane at molecular weight 35 kDa and 28 kDa for β -actin (42 kDa) and pro-IL-1 β (31 kDa); and supernatants after cutting membrane at 28 kDa for cleaved IL-1 β (17 kDa).

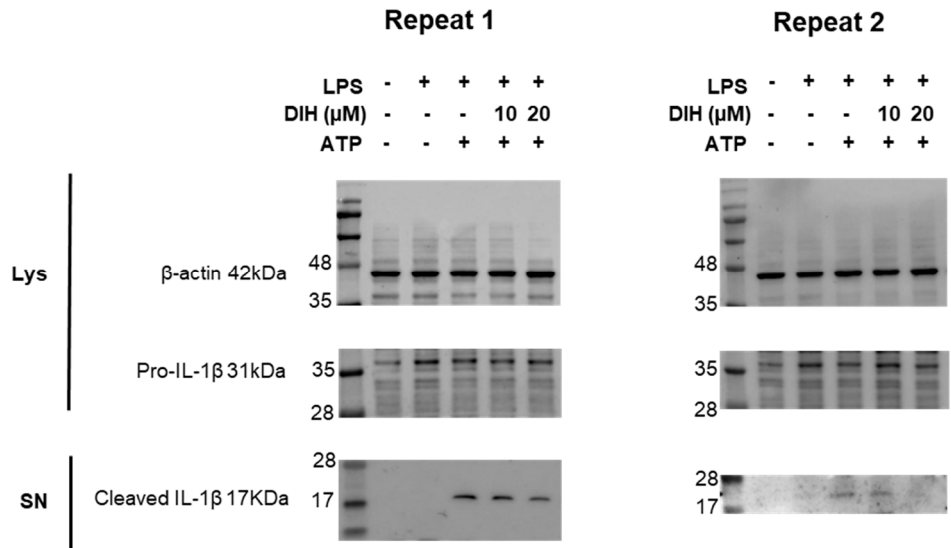


Figure S6. Original western blots for two repeats of Figure 2F. Whole blot of cell lysates after cutting membrane at molecular weight 35 kDa and 28 kDa for β -actin (42 kDa) and pro-IL-1 β (31kDa); and supernatants after cutting membrane at 28 kDa for cleaved IL-1 β (17 kDa).

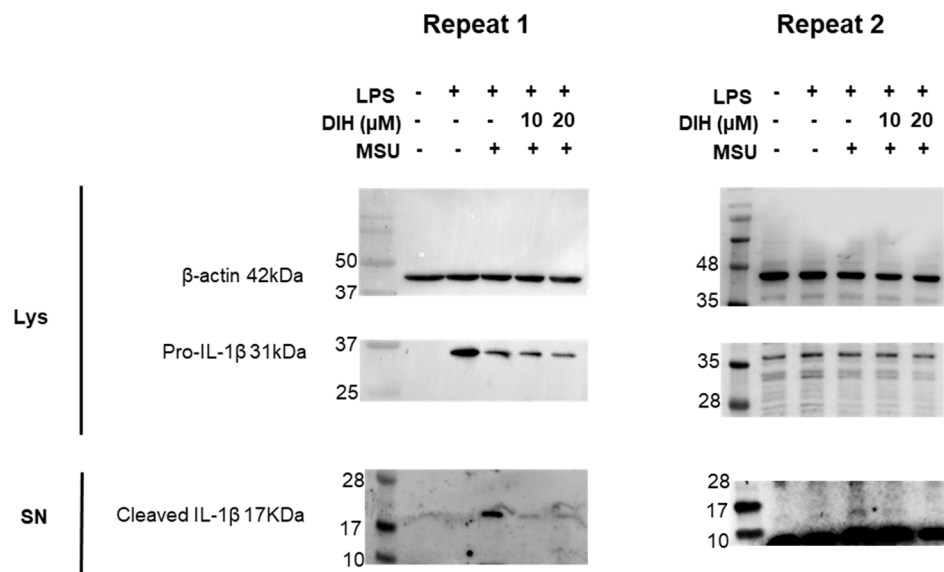


Figure S7. Original western blots for two repeats of Figure 3B. Whole blot of cell lysates after cutting membrane at molecular weight 48 kDa for pro-caspase-1 (45 kDa) and β-actin (42 kDa); and supernatants after cutting membrane at 10 kDa for caspase-1 p10 (10 kDa).

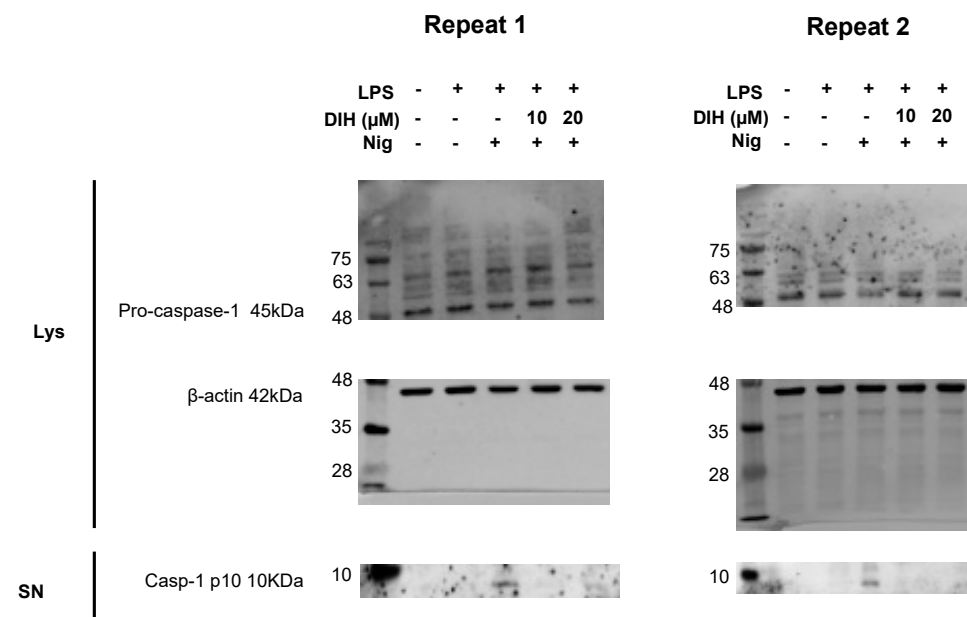


Figura S8. Original western blots for two repeats of Figure 4B. Whole blot of cell lysates after cutting membrane at molecular weight 48 kDa and 35 kDa for GSDMD (53 kDa), β-actin (42 kDa) and GSDMD-N (32 kDa).

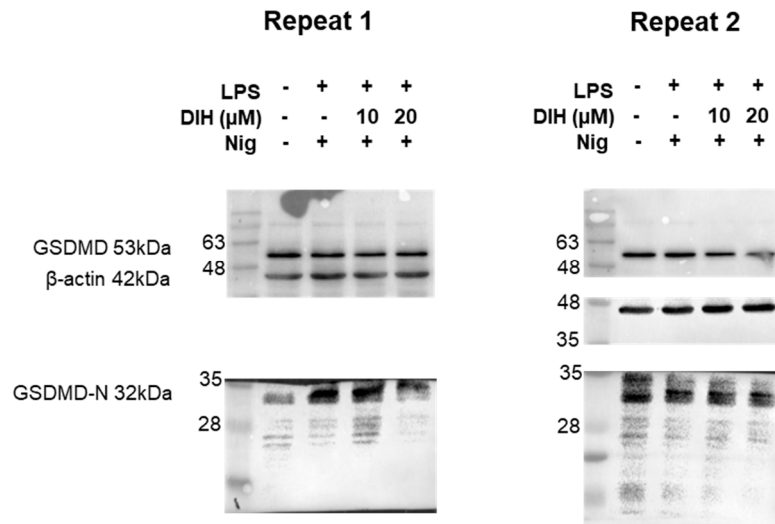


Figure S9. Original western blots for two repeats of Figure 5B. Whole blot of cell lysates after cutting membrane at molecular weight 63 kDa, 35 and 28 kDa for NLRP3 (103 kDa), β -actin (42 kDa) and ASC (24 kDa).

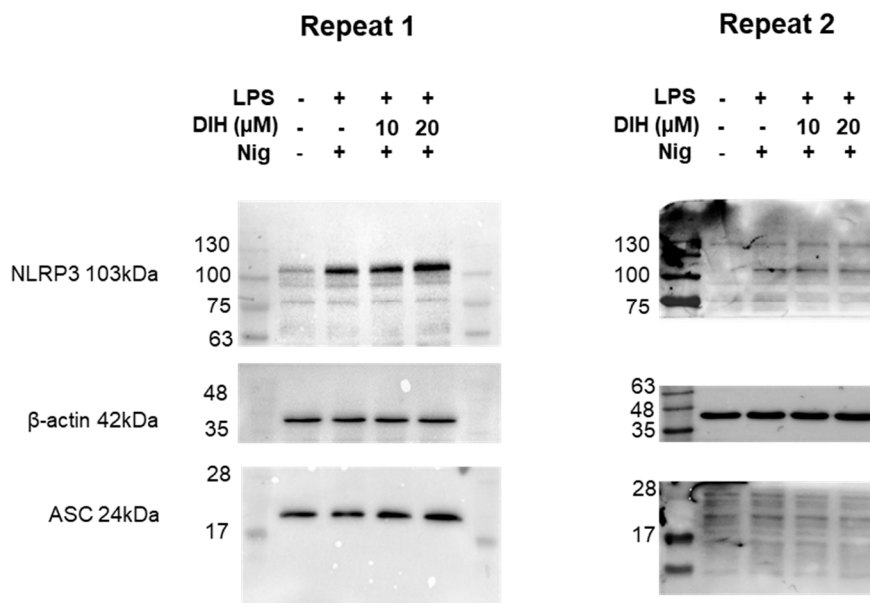


Figure S10. Original western blots for two repeats of Fig. 6B. Whole blot of cell lysates after cutting membrane at molecular weight 63 kDa, 48, 35 and 28 kDa for NLRP3 (103 kDa), pro-caspase-1 (45 kDa), β -actin (42 kDa), pro-IL-1 β (31kDa) and ASC (24 kDa).

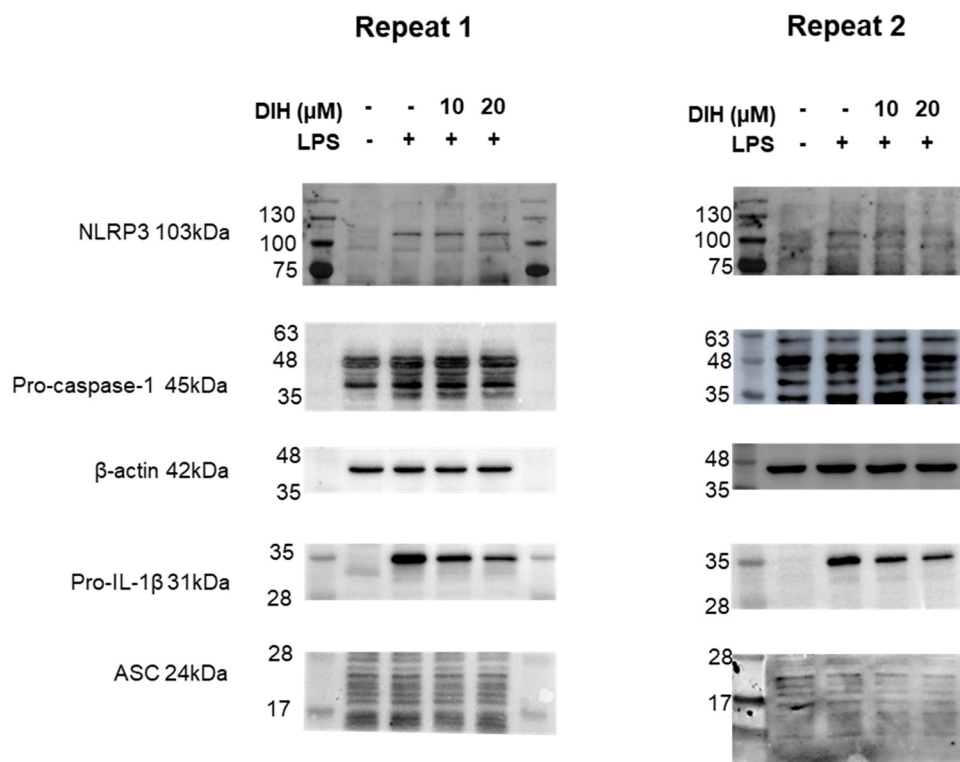


Table S1. Primers used and their sequences.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
NOS-2	TCCACAGTATGTGAGGATCAAAAC	ATGTGGCCTTGTTGGAAGAGT
COX-2	GCTGTACAAGCAGTGGCAAAG	GCGTTTGCGGTACTCATTGAGA
TNFα	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTTCATACA
Pro-IL-1β	TCTTTGAAGTTGACGGACCC	TGAGTGATACTGCCTGCC TG
NLRP3	AGCCTTCCAGGATCCTCTTC	CTTGGGCAGCAGTTTCTTTC
IL-18	TGGTTCCATGCTTTCTGGACTCCT	TTCCTGGGCCAAGAGGAAGTGATT
Pro-caspase-1	AGATGGCACATTTCAGGAC	GATCCTCCA GCAGCAACTTC
36B4	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC

Table S2. Antibodies used for Western Blot analysis.

Antibody	Source	Identifiers	Dilution
Anti-mouse IL-1 β (goat polyclonal)	R&D systems	AF-401-NA	1:500
Anti-pro Caspase-1 + p10 + p12 (rabbit monoclonal)	Abcam	ab179515	1:1000
Anti-NLRP3/NALP3 (Cryo-2) (mouse monoclonal)	AdipoGen	AG-20B-0014	1:1000
Anti-mouse ASC/TMS1 (D2W8U) (rabbit monoclonal)	Cell Signaling Technology	67824T	1:1000
Anti- β -actin (mouse monoclonal)	Sigma	A5541	1:5000
Anti-GSDMD (rabbit monoclonal)	Abcam	ab209845	1:1000
Goat Anti-Rabbit IgG H&L (HRP)	Abcam	ab205718	1:10000
Anti-goat IgG HRP	R&D systems	HAF017	1:1000
Anti-mouse IgGk BP-HRP	Santa Cruz	sc-516102	1:5000