Supporting Information

A probe for NLRP3 inflammasome inhibitor MCC950 identifies carbonic anhydrase 2 as a novel target

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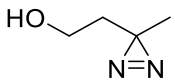
Supplementary Methods

Materials

Chemicals were purchased from Sigma-Aldrich, Fluorochem, Acros Organics, TCI, Alfa Aesar or Fisher Scientific and used without further purification. AzTB and AzRB were synthesized in-house as previously reported. NeutrAvidin agarose resin and PreScission™ Protease were purchased from Thermo Fisher Scientific. CA2 protein was purchased from Sigma-Aldrich.

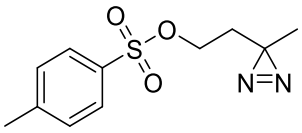
2-(3-Methyl-3H-diazirin-3-yl)ethan-1-ol (1)



1

Ammonia (100 mL) was condensed at -78 °C into a 250 mL round bottom flask fitted with a cold finger of dry ice-acetone and a 6 M $HCl_{(aq)}$ trap. 4-Hydroxybutan-2-one (10.0 g, 113 mmol) dissolved in dry MeOH (15.4 mL) was added to the reaction vessel and stirred at -78 °C. After 5 h, hydroxylamine-O-sulphonic acid (16.7 g, 159 mmol) dissolved in anhydrous MeOH (108 mL) was added dropwise at -78 °C and the reaction stirred and allowed to warm to RT overnight. The reaction mixture was then filtered, and the solids washed with anhydrous MeOH (2 × 30 mL). The filtrate was concentrated under reduced pressure at 30 °C and the residue re-dissolved in anhydrous MeOH (77 mL), cooled to 0 °C, and DIPEA (23 mL, 136.2 mmol) was added. I_2 was added portion-wise until a dark brown colour persisted for 1 h. The reaction mixture was diluted with Et_2O (230 mL), $HCl_{(aq)}$ (1 M, 230 mL) and phases separated. The aqueous phase was extracted with Et_2O (2 × 150 mL) and the combined organic phases washed successively with $HCl_{(aq)}$ (1 M, 230 mL), $Na_2S_2O_{3\ (aq)}$ (10%, 230 mL) and brine (230 mL). The organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure to give title compound **1** as a pale-yellow oil (3.90 g, 39.0 mmol, 34%). R_f 0.53 (10% DCM in MeOH); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.50 (t, *J* = 6.4 Hz, 2H), 2.64 – 2.38 (m, 1H), 1.60 (t, *J* = 6.4 Hz, 2H), 1.04 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 65.82, 57.53, 37.50, 20.16.

2-(3-Methyl-3H-diazirin-3-yl)ethyl 4-methylbenzenesulfonate (2)

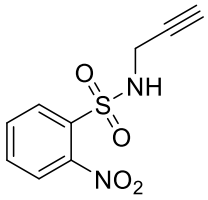


2

4-Methylbenzenesulfonyl chloride (11.7 g, 61.5 mmol) was added portion-wise to a solution of compound **1** (4.09 g, 41.0 mmol) in pyridine (33 mL) at 0 °C, allowed to warm to RT and stirred. After 3 h, the reaction mixture was diluted with DCM (600 mL) and $HCl_{(aq)}$ (1 M, 600 mL), separated, and the organic phase washed successively with $HCl_{(aq)}$ (1 M, 200 mL), saturated $NaHCO_{3\ (aq)}$ (600 mL), brine (600 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by automated flash column chromatography (8-66% EtOAc in hexane) to give title compound **2** as a colourless oil (6.75 g, 26.5 mmol, 65%). R_f 0.57 (100% DCM); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.86 – 7.79 (m, 2H), 7.38 (d, *J* = 7.9 Hz, 2H), 3.97 (t, *J* = 6.4 Hz, 2H), 2.47 (s, 3H), 1.69 (t, *J* = 6.4 Hz, 2H),

1.02 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 145.05, 132.77, 129.92, 127.98, 65.10, 34.18, 23.37, 21.65, 19.76.

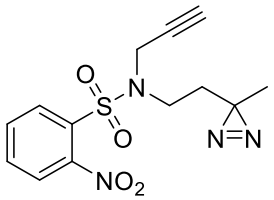
2-Nitro-N-(prop-2-yn-1-yl)benzenesulfonamide (3)



3

Et_3N (7.59 mL, 54.5 mmol) was added to a solution of prop-2-yn-1-amine **2** (3.00 g, 54.5 mmol) in DCM (45 mL) and cooled to 0 °C under nitrogen. 2-Nitrobenzenesulfonyl chloride (11.4 g, 51.4 mmol) was added and the reaction stirred at RT. After 3 h, the reaction mixture was diluted with DCM (150 mL) and $HCl_{(aq)}$ (2 M, 150 mL) and separated. The aqueous phase was extracted with DCM (150 mL) and the combined organic phases washed successively with $HCl_{(aq)}$ (2 M, 225 mL), H_2O (225 mL), and brine (75 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by automated flash column chromatography on silica gel (10 - 50% EtOAc in hexane) to give title compound **3** as a pale-orange solid (11.1 g, 46.3 mmol, 90%). R_f 0.46 (50 % EtOAc in hexane); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.31 – 8.17 (m, 1H), 7.98 – 7.91 (m, 1H), 7.82 – 7.74 (m, 2H), 5.71 (t, J = 6.3 Hz, 1H), 4.04 (dd, J = 6.1, 2.7 Hz, 2H), 2.00 (t, J = 2.5 Hz, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 134.04, 133.80, 132.90, 131.59, 125.77, 125.53, 77.21, 73.26, 33.40.

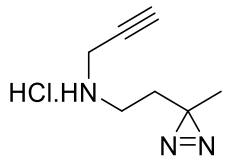
N-(2-(3-Methyl-3H-diazirin-3-yl)ethyl)-2-nitro-N-(prop-2-yn-1-yl)benzenesulfonamide (4)



4

K_2CO_3 (18.8 g, 136.0 mmol) and compound **3** (10.9 g, 45.34 mmol) were added sequentially to a stirred solution of **7** (11.5 g, 45.3 mmol) in DMF (92 mL) and the reaction stirred at 80 °C. After 3 h, the reaction mixture was diluted with EtOAc (450 mL) and H_2O (450 mL) and separated. The aqueous phase was extracted with EtOAc (2 × 300 mL) and the combined organic phases washed successively with $LiCl_{(aq)}$ (5%, 2 x 600 mL) and brine (600 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography on silica gel (33% EtOAc in hexane) to give title compound **4** as a pale yellow oil (9.50 g, 29.5 mmol, 65%). R_f 0.22 (33% EtOAc in hexane); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.08 – 8.03 (m, 1H), 7.75 – 7.69 (m, 2H), 7.67 – 7.63 (m, 1H), 4.18 (d, *J* = 2.5 Hz, 2H), 3.46 – 3.40 (m, 2H), 2.18 (t, *J* = 2.4 Hz, 1H), 1.67 – 1.60 (m, 2H), 1.06 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 148.31, 133.94, 132.34, 131.74, 130.94, 124.26, 76.37, 74.30, 60.38, 42.18, 36.69, 33.30, 19.34.

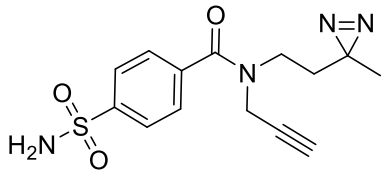
N-(2-(3-Methyl-3H-diazirin-3-yl)ethyl)prop-2-yn-1-amine hydrochloride (5)



5

$LiOH{\cdot}H_2O$ (4.95 g, 118.0 mmol) and 3-mercaptopropanoic acid (5.26 mL, 59.0 mmol) were added to a stirred solution of compound **4** (9.50 g, 29.5 mmol) in DMF (73 mL) and the reaction stirred at RT. After 5 h, the reaction mixture was diluted with EtOAc (600 mL) and H_2O (600 mL) and separated. The aqueous phase was extracted with EtOAc (2 × 600 mL) and the combined organic phases washed successively with $LiCl_{(aq)}$ (5%, 1200 mL), saturated $NaHCO_{3\ (aq)}$ (1200 mL) and brine (600 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. Hydrochloric acid (4 N in 1,4-dioxane, 7.97 mL) was added to the resulting residue at 0 °C and stirred. After 20 min, the mixture was diluted with Et_2O (200 mL), warmed to RT and filtered. The solids were washed with Et_2O (20 mL) and collected to give title compound **5** as a white powder (3.000 g, 17.3 mmol, 59%). R_f 0.22 (50% EtOAc in hexane); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.24 (s, 2H), 3.89 (d, *J* = 2.6 Hz, 2H), 3.73 (t, *J* = 2.5 Hz, 1H), 2.96 – 2.87 (m, 2H), 1.71 – 1.65 (m, 2H), 1.05 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 79.95, 76.23, 65.97, 41.60, 36.07, 31.03, 23.03.

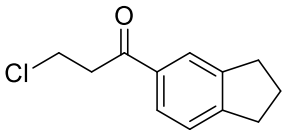
N-(2-(3-methyl-3H-diazirin-3-yl)ethyl)-N-(prop-2-yn-1-yl)-4-sulfamoylbenzamide (6)



6

To a solution of N-(2-(3-methyl-3H-diazirin-3-yl)ethyl)prop-2-yn-1-amine hydrochloride **5** (100 mg, 0.576 mmol) in DMF (15 mL) was added 4-sulfamoylbenzoic acid (116 mg, 0.576 mmol) followed by propanephosphonic acid anhydride (700 µL, 367 mg, 1.15 mmol; 50% in EtOAc), and DIPEA (301 µL, 1.73 mmol). The reaction was stirred overnight at RT and diluted with EtOAc (50 mL). The organic phase was washed with saturated $NaHCO_{3\ (aq)}$ (50 mL), 5% $LiCl_{(aq)}$ (2 × 50 mL) and brine (50 mL) and dried over $MgSO_4$, filtered, and EtOAc evaporated under reduced pressure. The crude product was dry-loaded onto a C-18 reverse-phased column and purified using automated column chromatography, eluting with a gradient of MeCN /H_2O (50-98%) to afford the title compound **6** as a white solid (80 mg; 0.25 mmol; 43%). ^{1}H NMR (400 MHz, DMSO-d_6, 100 °C) δ 7.92 (d, J = 8.7 Hz, 2H), 7.58 (d, J = 8.7 Hz, 2H), 7.18 (s, 2H), 4.11 (d, J = 2.4 Hz, 2H), 3.44 (t, J = 7.5 Hz, 2H), 3.13 (t, J = 2.4 Hz, 1H), 1.70 (t, J = 7.6 Hz, 2H), 1.00 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6, 100 °C) δ 168.64, 144.79, 138.48, 126.39, 125.38, 78.65, 74.35, 41.26, 31.79, 23.62, 18.60, one quaternary carbon was not observed; LC-MS 18 minutes, 50-98% MeCN in H_2O, retention time 9.09 minutes, ESI m/z $[M+H]^+$ = 321.17; HRMS found 319.0869 ($C_{14}H_{15}N_4O_3S$, $[M-H]^-$ requires 319.0870); IR vmax/cm−1 1624 (C=O).

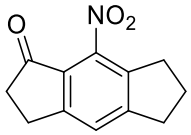
3-Chloro-1-(2,3-dihydro-1H-inden-5-yl)propan-1-one (7)



7

Aluminium chloride (6.76 g, 50.8 mmol) was added portion-wise to a solution of indan (5.18 mL, 42.3 mmol) and 3-chloropropionyl chloride (4.44 mL, 46.5 mmol) in DCM (60 mL) at room temperature (RT). The reaction quenched after two hours by addition of ice-cold 2 M $HCl_{(aq)}$ (60 mL). The organic layer was extracted with DCM (2 × 200 mL), washed with brine (100 mL), dried ($MgSO_4$) and concentrated under reduced pressure to yield a brown oil. Addition of 15% ethyl acetate in hexane resulted in precipitate formation which was filtered to give the title compound **7** as pale brown crystals (7.62 g, 36.6 mmol, 72%). R_f 0.48 (10% EtOAc in hexane); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.85 – 7.72 (m, 2H), 7.34 – 7.27 (m, 1H), 3.92 (t, J = 6.9 Hz, 2H), 3.44 (t, J = 6.9 Hz, 2H), 2.96 (t, J = 7.5 Hz, 4H), 2.19 – 2.06 (m, 2H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 196.83, 150.97, 145.13, 135.09, 126.75, 124.63, 124.12, 41.48, 39.11, 33.20, 32.70, 25.49; HRMS found 208.0660 ($C_{12}H_{13}ClO$, [M+•] requires 208.0655).

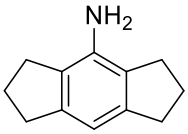
4-Nitro-3,5,6,7-tetrahydro-s-indacen-1(2H)-one (8)



8

Compound **7** (3.0 g, 14.4 mmol) was added portion-wise to concentrated sulphuric acid (15 mL) at RT. The mixture was heated to 55 °C and stirred for 48 hours. The reaction mixture was not purified and taken forwards crude. The reaction was cooled on ice, and a 1:1 mixture of concentrated nitric and sulphuric acid (3 mL) added. The solution was then stirred at 0-5 °C for 1 hour. The reaction was quenched by dropwise addition to a mixture of water (125 mL) and DCM (125 mL) on ice. The organic layer was collected, and the aqueous layer extracted with DCM (2 × 125 mL). Combined organic layers were washed with saturated $NaHCO_{3\ (aq)}$ (100 mL), before drying with magnesium sulphate and concentrated *in vacuo* to give a brown oil. The crude reaction was purified using silica, eluting with a gradient of EtOAc/hexane (15-20%) to afford three isomers. The title compound **8** was isolated as pale yellow crystals (1.640 g, 52.4%). R_f 0.19 (20% EtOAc in hexane); HRMS found 217.0739 ($C_{12}H_{11}NO_3$, [$M^{+\bullet}$] requires 217.0743); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.44 (s, 1H), 3.12 (t, J = 5.8 Hz, 2H), 3.04 (t, J = 7.4 Hz, 2H), 2.99 (t, J = 7.6 Hz, 2H), 2.77 (m, 2H), 2.20 (p, 121 J = 7.4, 7.6 Hz, 2H).

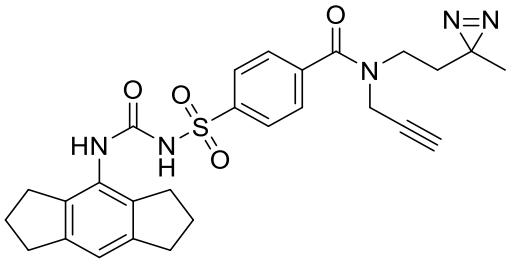
1,2,3,5,6,7-Hexahydro-s-indacen-4-amine (9)



9

To a solution of 4-Nitro-3,5,6,7-tetrahydro-s-indacen-1(2H)-one **8** (250 mg, 1.15 mmol) in methanol (12.5 mL) were added Pearlman's catalyst (62.5 mg, 20% wt on carbon) and methanesulfonic acid (120 µL, 70% wt in H_2O) before agitation under a hydrogenous atmosphere at 50 psi. The reaction was monitored by TLC and after four hours the reaction was poured over Celite, and the filtrate was washed with MeOH before concentration under reduced pressure. The crude solid was purified on silica gel, eluting with 10% EtOAc/hexane to afford the title compound **9** as brown wax (147 mg, 0.85 mmol, 74%). R_f 0.35 (10% EtOAc in hexane); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.63 (s, 1H), 3.50 (s, 2H), 2.87 (t, J = 7.5 Hz, 4H), 2.69 (t, J = 7.3 Hz, 4H), 2.11 (p, J = 7.5, 7.3 Hz, 4H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 144.12, 138.38, 126.33, 110.86, 33.18, 29.10, 25.63; HRMS found 174.1284 ($C_{12}H_{16}N$, $[M+H]^+$ requires 174.1277).

4-(N-((1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl)sulfamoyl)-N-(2-(3-methyl-3H-diazirin-3-yl)ethyl)-N-(prop-2-yn-1-yl)benzamide (IMP2070)



IMP2070

Triphosgene (45.7 mg, 0.154 mmol) in THF (154 µL) was added to a solution of triethylamine (129 µL, 0.923 mmol) and compound **9** (80 mg, 0.462 mmol) in THF (10 mL). The reaction mixture was refluxed for three hours and the reaction was taken to the next step without any purification. To a solution of molecule **6** (75 mg, 0.234 mmol) in THF (5 mL) was added NaOMe (13 mg, 0.234 mmol) and the reaction was stirred for one hour. The crude isocyanate was added dropwise to the reaction mixture and stirred overnight. The reaction mixture was filtered, and the filtrate dry-loaded onto a C-18 reverse-phased column and purified using automated column chromatography, eluting with a gradient of MeCN /H_2O (50-98%) to afford the title compound **IMP2070** as a white solid (12 mg, 0.023 mmol, 10%). ^{1}H NMR (400 MHz, DMSO-d_6, 100 °C) δ 7.93 (d, J = 8.6 Hz, 2H), 7.58 (d, J = 8.6 Hz, 2H), 7.18 (s, 2H), 6.38 (s, 1H), 4.11 (d, J = 2.6 Hz, 2H), 3.44 (t, J = 7.5 Hz, 2H), 3.13 (t, J = 2.6 Hz, 1H), 2.75 (t, J = 7.4 Hz, 4H), 2.62 (t, J = 7.4 Hz, 4H), 2.00 (p, J = 7.4, 7.4 Hz, 4H), 1.70 (t, J = 7.5 Hz, 2H), 1.00 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6, 100 °C) δ 168.66, 149.16, 144.82, 142.08, 138.90, 138.50, 126.41, 125.40, 124.55, 108.18, 78.67, 74.38, 41.29, 32.11, 31.81, 28.29, 24.34, 23.66, 18.62, one quaternary carbon was not observed; LC-MS 18 minutes, 50-98% MeCN in H_2O, retention time 8.52 minutes, ESI m/z $[M+H]^+$ = 520.30; HRMS found 518.1874 ($C_{27}H_{28}N_5O_4S$, $[M-H]^-$ requires 518.1862); IR vmax/cm−1 1622 (C=O), 1582 (C=O).

Tissue culture

Wild-type (WT) THP1 cells were grown at 37 °C in a 5% CO_2 incubator, and mycoplasma tests performed monthly. WT THP1 cells were cultured in RPMI medium (Sigma Aldrich) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 1 mM sodium pyruvate (Sigma Aldrich) and 10 mM HEPES (Sigma Aldrich). THP1 cells were differentiated into macrophages using 100 ng mL^{-1}PMA for 72 hours prior to treatments. Unless otherwise stated, media was refreshed before treatments began.

Incucyte cell death assay

THP1 cells were seeded in 96 well plates with 1.5×10^5 cells per well 72 hours prior to the assay. Cells were treated in triplicate with 250 ng mL^{-1} LPS and either MCC950 or IMP2070 with a dilution concentration range 24 nM - 50 µM, in media containing 250 nM Sytox Green. After incubation for 3 hours, the cells were treated with 10 µM nigericin and placed in the Incucyte. Green filter and phase readings were taken of the wells after 1 hour.

Cell treatments and Western blots

THP1 cells were seeded in 24 well plates with 7.5×10^5 cells per well 72 hours prior to the assay. Cells were treated for 3 hours with 250 ng mL^{-1} LPS and either MCC950 or IMP2070 with a dilution concentration range 0.5 - 20 µM. Cells were then treated with OptiMEM (Gibco) containing 10 µM nigericin. After 1 hour, supernatants were collected and remaining cells lysed in 1% Triton, 0.1% SDS, 1 × protease inhibitor in 1 × PBS. Supernatant were precipitated with the sequential addition of 1 vol H_2O, 2 vol MeOH and 0.5 vol $CHCl_3$, before centrifugation at 4 °C at 17,000 × g for 10 minutes. The top layer of supernatant was removed by pipetting, without disrupting the pellet. A further 3 vol MeOH was added to wash the pellet, and samples sonicated. Samples were then centrifuged for ten minutes at 4 °C at 17,000 × g before tipping off the supernatant and drying the protein pellets at RT for 30 minutes, or until the pellets were visibly dry, before resuspension in NuPAGE LDS sample buffer with 5% β-mercaptoethanol. Lysates were centrifuged at 4 °C at 17,000 × g for 10 minutes before dilution with the same loading buffer. Proteins were separated by mass by SDS-PAGE before being transferred to nitrocellulose membranes by wet transfer at 100 V for 1 hour. Membranes were blocked in 5% fat free milk in TBST for one hour at RT, before incubation at 4 °C overnight with the desired primary antibodies. Membranes were incubated with secondary antibodies for one hour at RT. Blots were imaged following addition of HRP luminata solution, using an ImageQuant LAS 400 imager.

Antibody	Source	Buffer	Dilution
α-Caspase-1	Adipogen, AG-20B-0048-C100	5% milk in TBST	1 in 1000
α-IL-1β	R&D Systems, MAB201	5% milk in TBST	1 in 1000
α-NLRP3	Adipogen, AG-20B-0014-C100	5% BSA in TBST	1 in 1000
α-GAPDH	Abcam, ab9485	5% milk in TBST	1 in 1000
α-HSP90	SantaCruz, sc-69703	5% milk in TBST	1 in 1000
α-rabbit-HRP	Advansta, R-05072-500	5% milk in TBST	1 in 10,000
α-mouse-HRP	Advansta, R-05071-500	5% milk in TBST	1 in 10,000

Pull-down Experiments

THP1 cells were seeded in 6 well plates with 3×10^6 cells per well 72 hours prior to the assay. Cells were treated with 250 ng mL^{-1} LPS and IMP2070 with or without MCC950. After incubation for 3 hours, cells were UV irradiated with 365 nm radiation for 60 seconds, using a UV lightbox. Cells were washed with PBS and then lysed in 1% Triton, 0.1% SDS, 1 × protease inhibitor in 1 × PBS, and protein concentrations measured using Detergent Compatible Protein Assay.

Stock reagents of AzTB, copper (II) sulphate ($CuSO_4$), Tris(2-carboxyethyl)phosphine hydroxide (TCEP) and Tris(benzyltriazolylmethyl)amine (TBTA) were prepared previously and frozen in aliquots as described below.

Reagent	Stock concentration	Stock solvent	Concentration
AzTB	10 mM	DMSO	100 ×
$CuSO_4$	50 mM	H_2O	50 ×
TCEP	50 mM	H_2O	50 ×
TBTA	10 mM	DMSO	100 ×

A 'click mix' was prepared with sequential addition of 1:2:2:1 ratios of AzTB:CuSO4:TCEP:TBTA and mixed. For each 100 µL of protein in a CuAAC reaction, 6 µL of 'click mix' was be added to give final reaction concentrations of 6 µM AzTB, 120 µM copper(II) sulphate ($CuSO_4$), 120 µM Tris(2-carboxyethyl)phosphine hydroxide (TCEP) and 6 µM Tris(benzyltriazolylmethyl)amine (TBTA). For pull-down experiments, 250 µg CuAAC reactions at 2 mg mL^{-1} were agitated with click reagents at RT for 1 hour, before quenching with the addition of 5 mM ethylenediaminetetraacetic acid (EDTA) final concentration. Proteins were precipitated with MeOH and $CHCl_3$, and washed with MeOH, as described above. Protein pellets were resuspended in 50 µL 10 mM DTT, 0.5% SDS in PBS and sonicated to dissolve, before dilution with 75 µL of PBS, resulting in a final concentration of 0.2% SDS. 10 µL of each sample was taken for 'input' samples, and kept on ice for future comparisons. For each sample, 35 µL of Neutravidin-agarose beads were washed with 0.2% SDS in PBS (3 × 500 µL). Samples were added to the prepared Neutravidin-agarose beads and agitated gently at RT for 1 hour. The supernatant was discarded, and beads washed with 0.2% SDS in PBS (3 × 500 µL), before the addition of 16 µL 4 × NuPAGE LDS sample buffer containing 5% β-mercaptoethanol. The beads were boiled for 10 minutes at 95 °C, before collecting the supernatants as the 'pull-down' samples. Input samples were also diluted with 1 × loading buffer. Labelled and enriched proteins were separated by mass by SDS-PAGE on 12% gels. The SDS-PAGE gels of fluorescently labelled proteins were imaged using a Typhoon FLA 9500 (GE Healthcare) using 532/575 nm excitation/emission wavelengths at 750 V. Proteins were then transferred to membranes and analysed by western blotting as described above.

Photoaffinity Chemical Proteomics

THP1 cells were seeded in 6 well plates 72 hours prior to the experiment. Media was removed and replaced with fresh RPMI media containing 250 ng mL^{-1}LPS and chosen concentrations of **IMP2070** and MCC950 for 3 hours. Each condition was performed in triplicate, with 2 wells of cells for each replicate. Cells were UV irradiated, washed and lysed as described above. CuAAC reactions were set up at 600 µg (2 mg mL^{-1}) with AzRB as described above and agitated at RT for one hour. Reactions were quenched with 5 mM EDTA, and proteins precipitated as above before pellets were left to dry at RT until visibly dry. Protein pellets were resuspended in 120 µL 10 mM DTT, 0.5% SDS in PBS and sonicated to dissolve. Tubes were centrifuged to check the pellet was fully dissolved, before dilution with further 180 µL of PBS, resulting in a final concentration of 0.2% SDS. For each sample, 80 µL of Neutravidin-agarose bead slurry was washed with of 0.2% SDS in PBS (3 × 1000 µL). Proteins were added to the equilibriated beads and agitated gently at RT for 2 hours. The supernatant was discarded, and beads washed with 1% SDS in 50 mM HEPES pH 8.0 (3 × 1000 µL).

5 mM TCEP and 10 mM CAA in 50 mM HEPES pH 8.0 was added to the beads and the samples shaken at RT for 30 minutes. Supernatants were removed and the beads washed with 50 mM HEPES (3 × 300 µL), before resuspending in 28 µL 50 mM HEPES. To each sample 0.2 µg Trypsin was added and agitated overnight at 37 °C.

0.08 mg TMT10plex™ Isobaric Mass Tag Labelling Reagent (Thermo Scientific) were dissolved in 30 µL proteomics-grade MeCN. The supernatant containing the peptides were collected from the trypsinised samples, and added to the corresponding TMT reagent. TMT reactions were shaken at RT for two hours before quenching with 1 µL 5% hydroxylamine (vol/vol) in H_2O. Samples were combined within their 10-plex and evaporated to dryness using a SpeedVac concentrator (ThermoFisher).

Samples were further fractionated into 6 layers on stage-tip membranes, which were prepared by the insertion of 3 layers of sulfonic acid (SCX) membrane into 200 µL pipette tips as previously reported.[1] Tips were placed in 2 mL tubes with perforated lids, before activation of the membranes with the addition of 150 µL MeCN followed by centrifuging at 1,100 × g for two minutes, then equilibration with the addition of 150 µL H_2O followed by centrifuging at 1,100 × g for two minutes. Buffers were prepared as described below:

Buffer	Composition
Fraction 1	75 mM ammonium formate, 20% (v/v) MeCN, 0.5% (v/v) formic acid
Fraction 2	125 mM ammonium formate, 20% (v/v) MeCN, 0.5% (v/v) formic acid
Fraction 3	200 mM ammonium formate, 20% (v/v) MeCN, 0.5% (v/v) formic acid
Fraction 4	300 mM ammonium formate, 20% (v/v) MeCN, 0.5% (v/v) formic acid
Fraction 5	400 mM ammonium formate, 20% (v/v) MeCN, 0.5% (v/v) formic acid
Fraction 6	5% ammonium hydroxide, 80% (v/v) formic acid

The dried peptides were resuspended in 150 µL 1% TFA in H_2O, and centrifuged for 5 minutes at maximum speed at RT. 135 µL of the samples were added to the stage tip, before tip centrifugation at 1,100 × g for 2 minutes, or until all the sample had passed through the membrane. Samples were desalted with three washes of 60 µL of 0.2% TFA in H_2O and centrifugation at 1,100 × g for 2 minutes, or until all the solution had passed through the membrane. The stage tip was transferred to a new, labelled collection tube and the first fraction eluted with 60 µL Fraction 1 buffer and centrifugation at 1,100 × g for 2 minutes, or until all the solution had passed through the membrane. For each following fraction, the stage tip was then transferred to a new, labelled collection tube and the peptides eluted as described with 60 µL of the corresponding elution buffer. Samples were evaporated to dryness before storing at -80 °C.

Stage-tip filters were prepared by the insertion of 3 layers of PVDF Durapore Filter into 10 µL pipette tips as described above. The stage tips were inserted into the caps of labelled LC-MS vials. Samples were resuspended in 10 µL of 2% MeCN, 0.5%TFA in LC-MS grade H_2O, and added to the stage tips before centrifugation at 4000 × g for 3 minutes, or until the sample had passed through the membrane. Peptides were separated on an EASY-SpraTM Acclaim PepMap C18 column (Thermo Fisher Scientific) using a 3-hour gradient of 0–100% (solvent A 2% MeCN with 0.1% formic acid; solvent B 80% MeCN with 0.1% formic acid) at a flow rate of 250 nL/min. This coupled to a QExactive mass spectrometer (Thermo Fisher Scientific). Survey scans were acquired from 350 to 1800 m/z, with the 10 most abundant isotope patterns with charge +2 or higher from each scan selected and fragmented further in MS/MS scans. The following settings were used:

- maximum ion injection time: survey scan 20 ms; MS/MS scans 120 ms
- ion target value: survey scan 10^6; MS/MS 10^5
- intensity threshold: 8.3×10^2

Peptide searches were performed in MaxQuant (version 1.6.0.7) using default parameters, except for the ones listed hereafter. Under group-specific parameters and type, reporter ion MS2 was selected, and the appropriate TMT10plex labels selected for lysines and N-termini, with their corresponding correction factors. Oxidation (M) and acetyl (protein N-term) were set as variable modifications and carbamidomethyl (C) was set as a fixed modification. Trypsin/P

was set as the digestion mode and re-quantify and match between runs were enabled. Searches were run in the built-in Andromeda search engine using the human Swissprot curated database with isoforms. Data analysis was performed in Perseus (version 1.6.2.3). Reporter intensity corrected values were loaded, and data was filtered against 'only identified by site', 'reverse', and 'potential contaminants'. Data was log2 transformed and filtered by valid values, retaining those that had 2 valid values in each triplicate condition. TMT data was normalized by subtracting the mean of each row within each TMT plex, before median subtraction across samples (columns). Volcano plots were generated using a pairwise Student's T-Test.

Thermal Protein Profiling

THP1 cells were seeded in 6-well plates 72 hours prior to the assay. Cells were treated with 250 ng mL^{-1} LPS and 0.1, 1.0, 5.0 or 10.0 µM MCC950 or DMSO for 3 hours. The media was removed and the cells washed with PBS, before the addition of non-enzymatic cell dissociation buffer. The plates were incubated on ice for ten minutes while the cells dissociated. Cells were then washed with PBS, and pelleted by centrifugation at 200 RCF for 5 minutes. The cells were resuspended in 400 µL PBS containing protease inhibitor and each condition aliquoted into 4 PCR tubes of 90 µL. Samples were then heated for 3 minutes in a thermal cycler at 50.5, 54, 57 and 60 °C for each concentration. The cells were then held at RT for 3 minutes before lysis by repeated freeze-thaw cycles, and centrifugation at 17,000 × g for 20 minutes at 4 °C. Soluble proteins (supernatant) were transferred onto a new tube and 70 µL of each sample was precipitated and reduced and alkylated as previously described, before addition of 0.3 µg trypsin and overnight incubation at 37 °C. TMT10plex™ Isobaric Mass Tag Labelling Reagents were prepared and incubated with the trypsinised peptides as described above. 50.5 and 60 °C samples of each concentration were in pooled into one 10-plex TMT set, and the 54 and 56 °C into a second 10-plex TMT set. Samples were subjected to 6-layer fractionation and stage tipped as described above. Mass spectrometry analysis and peptide search using MaxQuant (version 1.6.0.1) were performed as above. ProteinGroups output file was loaded into Perseus (version 1.6.0.2) was filtered against 'only identified by site', 'reverse', and 'potential contaminants'. Data was log2 transformed and filtered by valid values, retaining only those with 100% valid values.*
For pseudo melt curves (Figure S2B), from *, a two-sample test was performed between the TMT runs, and biased proteins removed. Data was then normalised within each concentration by the subtraction of the 50.5 °C value for that concentration. Proteins were identified as potential binders of MCC950 if there was an observable dose-dependent shift in the pseudo melt curve of the protein towards a higher temperature in at least two concentrations of MCC950 when compared to DMSO treated samples.
For analysis within temperature sets (Figure 3D), from *, TMT data was normalized by subtracting the mean of each row within each TMT plex, before median subtraction across samples (columns). Proteins were identified as potential binders of MCC950 if there was an observable dose-dependent increase in the quantity of soluble protein present at two or more temperatures.

CA2 activity assay

Recombinant CA2 (Sigma-Aldrich, C6165) was dissolved in 12.5 mM Tris, 75 mM NaCl pH 7.5. Esterase activity was assessed with the following final concentrations: 5 µg mL^{-1} CA2, 1 mM *para*-nitrophenol acetate (*p*-NPA) substrate, 0.19 – 100 µM MC950, in 100 µL of assay buffer (12.5 mM Tris, 75 mM NaCl pH 7.5). The reactions were initiated with the addition of *p*-NPA and were measured by monitoring the formation of 4-nitrophenol at 405 nm every 90 seconds at 37 °C. Initial reaction rates were calculated by taking away background *p*-NPA hydrolysis and plotting absorbance against time. For Line-Weaver Burk analysis, the assay was performed with 2.5 µg mL^{-1} CA2, 2.56 – 100 µM MCC950, and 125 µM – 1 mM *p*-NPA,

and reaction rates calculated as described. Each experiment was set up as technical triplicates and the means of those plotted and used for statistical analyses, and concentrations of p-NP product calculated using the reported extinction coefficient of 18,000 $M^{-1}cm^{-1}$. MCC950 purity was confirmed by LC-MS and NMR to affirm that inhibition was not due the presence of sulfonamide impurities: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.53 (s, 1H), 7.36 (d, *J* = 1.1 Hz, 1H), 6.76 (s, 1H), 6.55 (d, *J* = 1.1 Hz, 1H), 4.91 (s, 1H), 2.75 (t, *J* = 7.4 Hz, 4H), 2.66 (t, *J* = 7.1 Hz, 4H), 1.34 (s, 6H). LC-MS 18 minutes, 2-98% MeCN in H_2O, retention time 12.98 minutes, ESI m/z $[M+H]^+$ = 427.20.

Molecular Docking

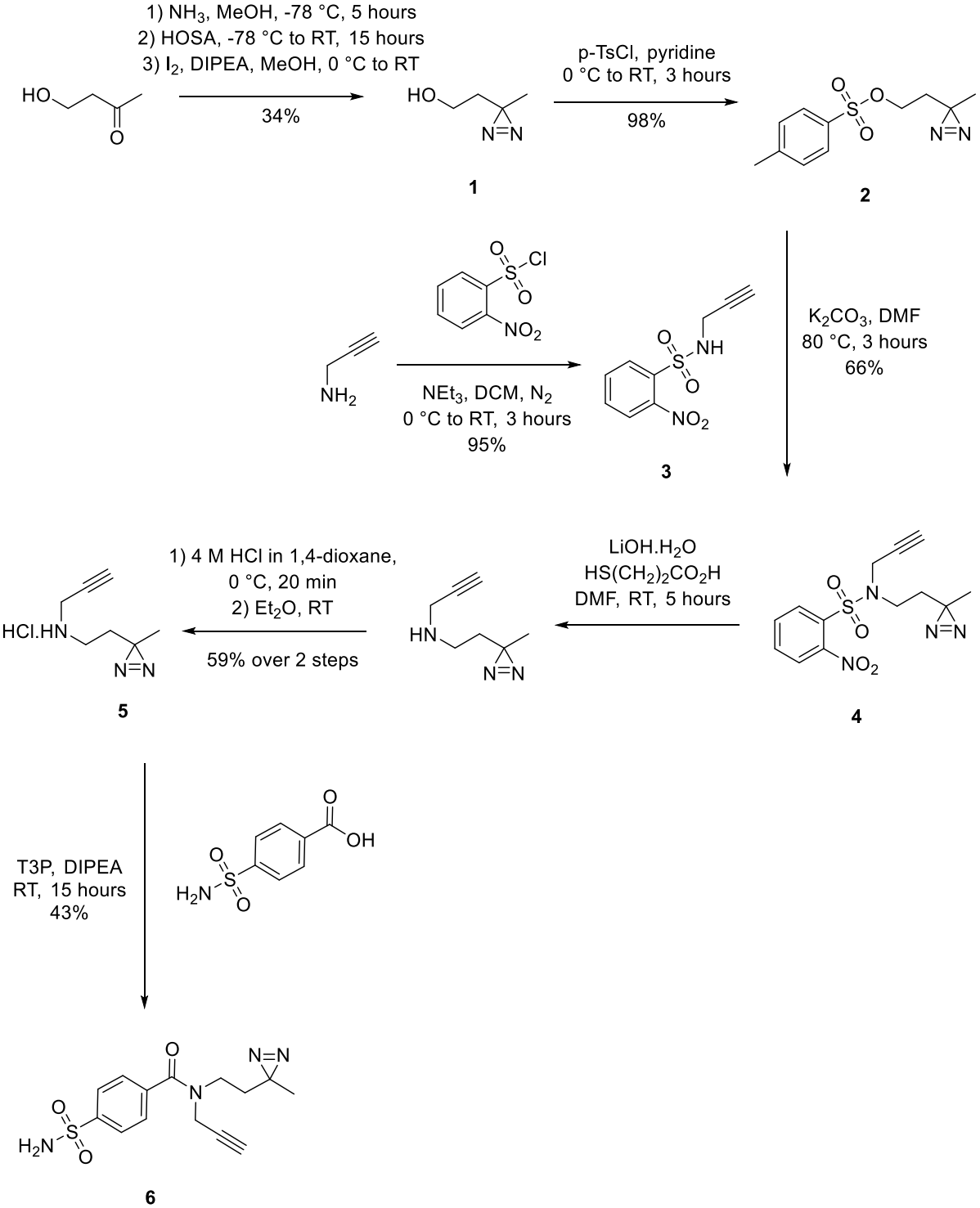
Molecular docking studies were conducted using MOE (purchased from the CCG) using version MOE2019.0102. Amber10:EHT forcefield was selected and docking studies were performed using the in-built 'dock' function. For placement method, the triangle matcher was set to generate 50 poses using London dG scoring. Refinement was then carried out using the rigid receptor method, based on the GBVI/WSA dG scoring functionality, to give 10 final poses.

Carbonic anhydrase co-crystal structure (PDB ID 3HS4) was imported into MOE and the in-built 'QuickPrep' function was used. The same was performed for PDB ID 3QYK. MCC950 was imported separately into MOE and an energy minimization was performed *via* the 'Minimize' function. A pharmacophore, based on the native ligand in the co-crystal structure of 3HS4, was created whereby the five-membered aryl ring and one oxygen atom of the sulfonamide moiety were selected as features. These two features were then constrained such that both conditions were satisfied. The MCC950 structure was then docked into the co-crystal structure of 3QYK, with the ligand site defined by the native ligand, using the previously created pharmacophore. The best pose identified by the docking was then taken forward for further molecular analysis.

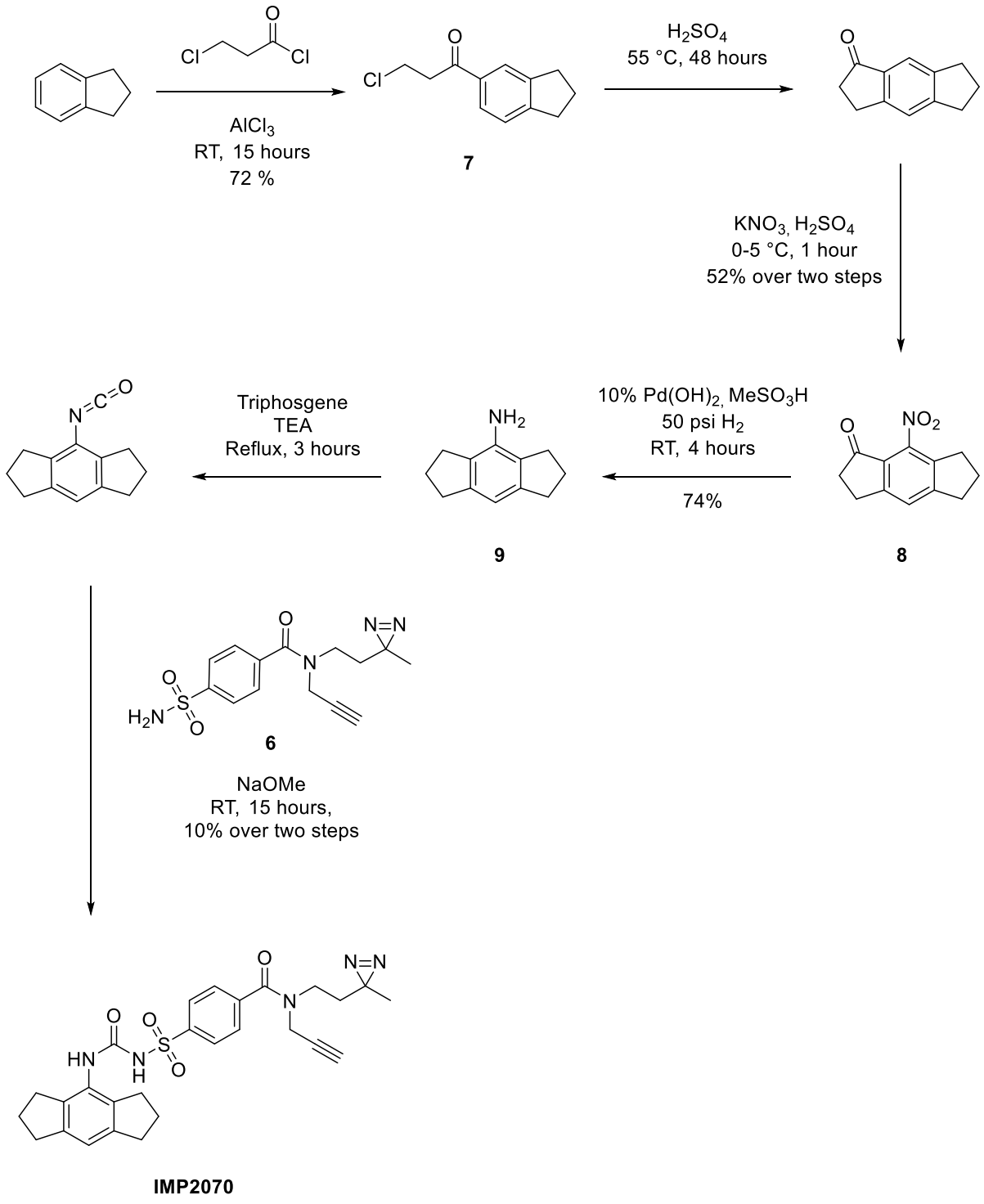
(1) Rappsilber, J.; Ishihama, Y.; Mann, M. Stop And Go Extraction Tips for Matrix-Assisted Laser Desorption/Ionization, Nanoelectrospray, and LC/MS Sample Pretreatment in Proteomics. *Anal. Chem.* **2003**, *75* (3), 663–670. https://doi.org/10.1021/ac026117i.

Synthesis schemes

Scheme 1



Scheme 2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72 %
7
H2SO4
55 °C, 48 hours
KNO3, H2SO4
0-5 °C, 1 hour
52% over two steps
10% Pd(OH)2, MeSO3H
50 psi H2
RT, 4 hours
74%
9
8
Triphosgene
TEA
Reflux, 3 hours
6
NaOMe
RT, 15 hours,
10% over two steps
IMP2070

Supporting figures

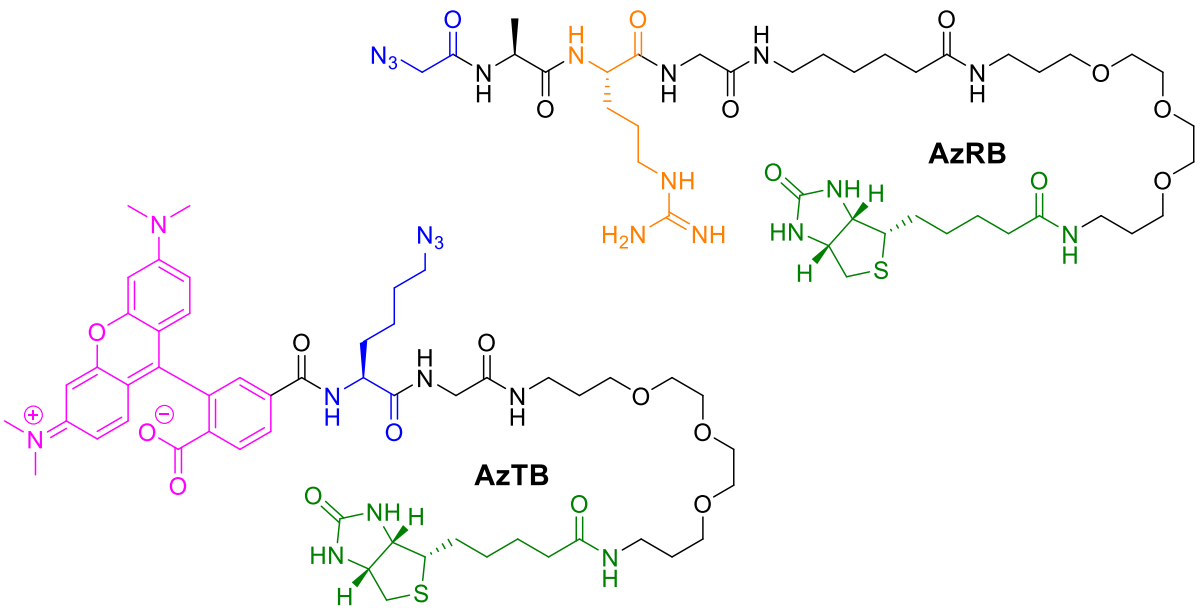

Figure S1: Capture Reagent Structures

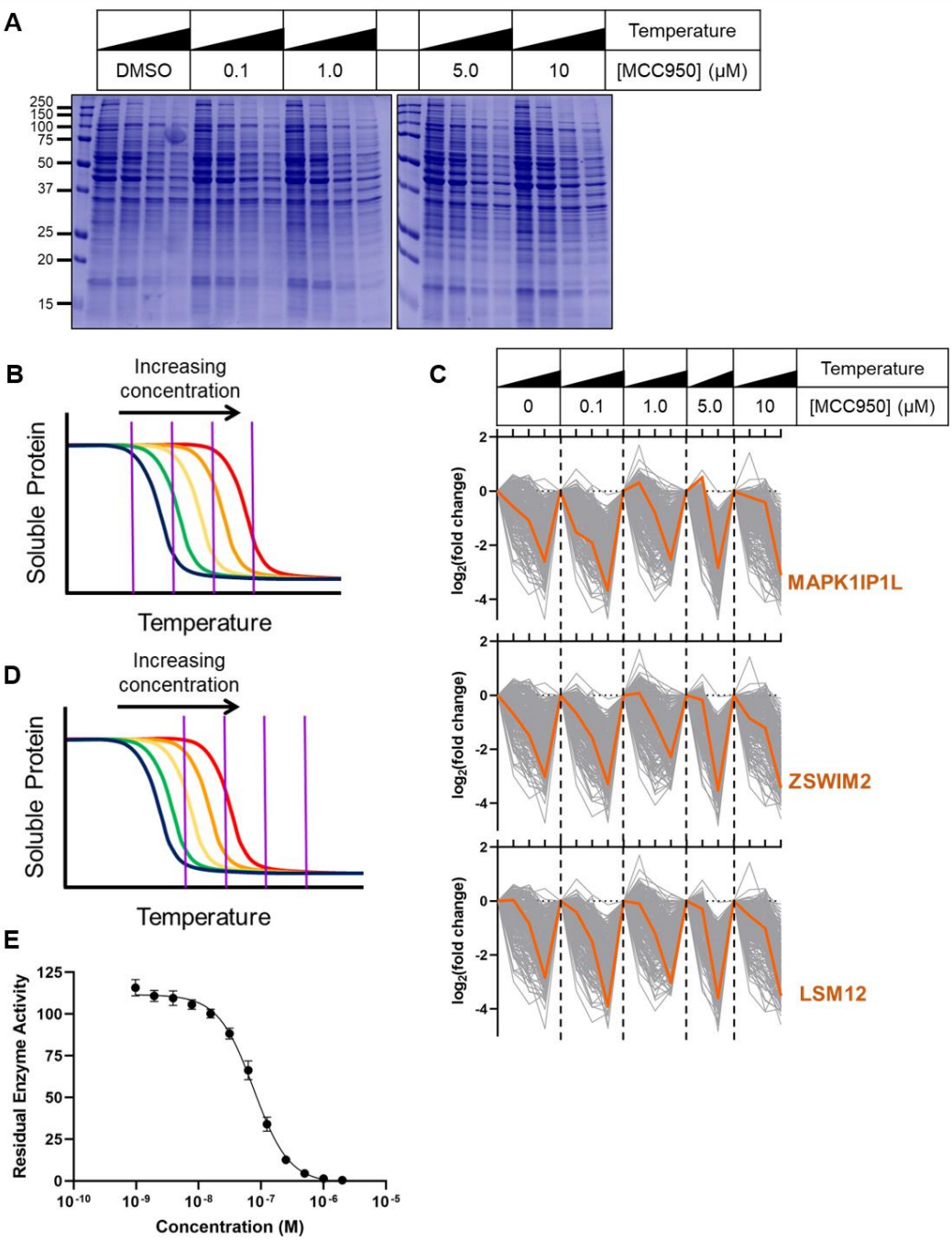

Figure S2: Proteomics Supplementary Figure

a) SDS-PAGE and coomassie analysis of soluble protein fractions used for proteomics TPP, showing uniform proteome degradation. b) Ideal coverage of 2D thermal protein profiling experiments, with increasing concentration of MCC950 stabilizing target proteins across the chosen temperature range. c) 'Pseudo melt curves' for TPP experiments. TMT intensities were normalized to the lowest temperature for each concentration of MCC950. The 5.0 µM, 57 °C data point was removed to remove anomalies. MAPK1IPL1 levels are shown as an example of a protein stabilized as the concentration of MCC950 increases; while ZSWIM2 shows weaker stabilization. LSM12 is given as a protein that is not stabilized by the presence of MCC950. d) Proteins with lower-end melt temperatures are unlikely to be sufficiently stabilized by MCC950 at higher temperatures for stabilization to be observed across the full temperature range chosen. e) Residual esterase enzyme activity of CA2 when treated with AZA. CA2

(5 µg mL^{-1}) was treated with inhibitor in the presence of 1 mM *p*-NPA substrate, and absorbance at 405 nm measured over 1 hour. Error bars represent SEM, N=3. IC_{50} = 0.78 µM; slope = -1.53, R^2 = 0.99.

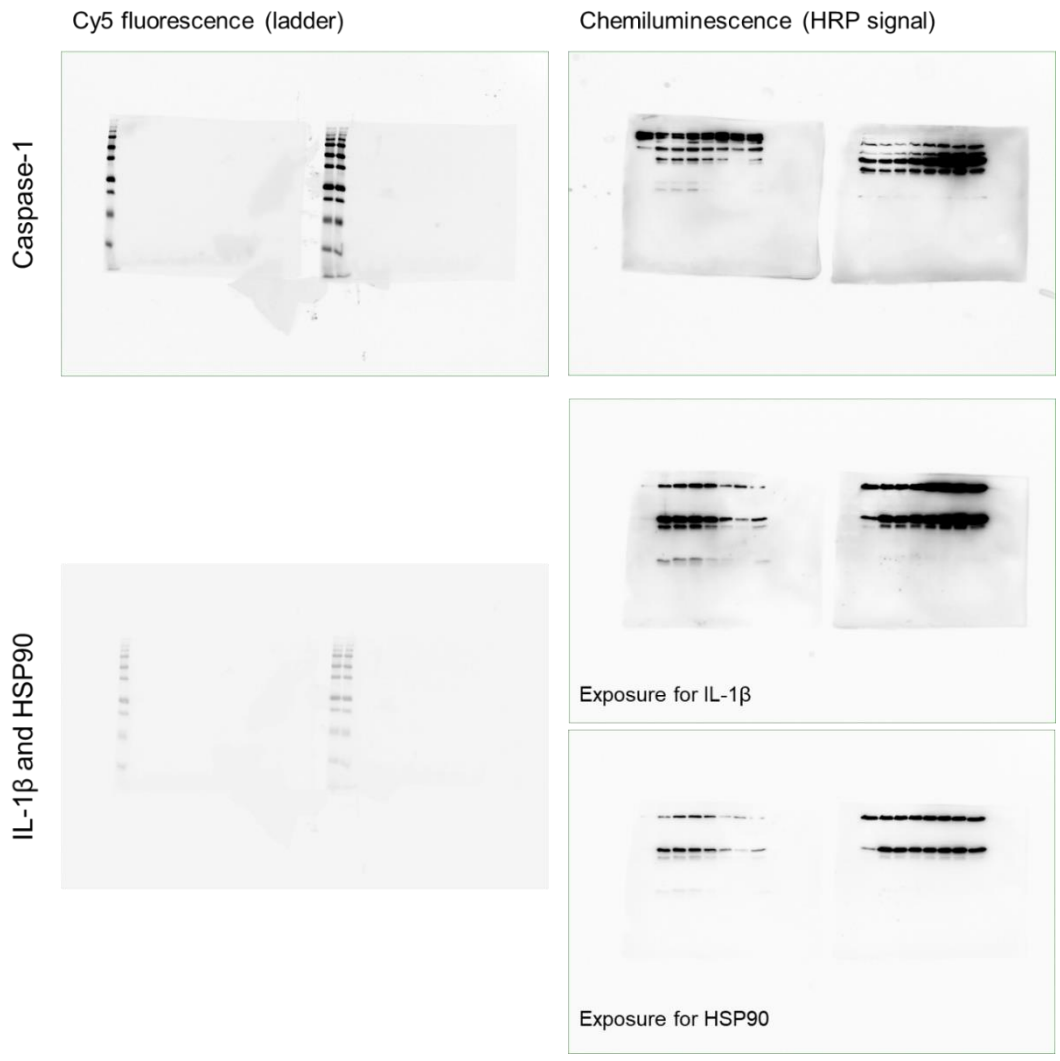

Figure S3: Uncropped blots and gels for Figure 2A

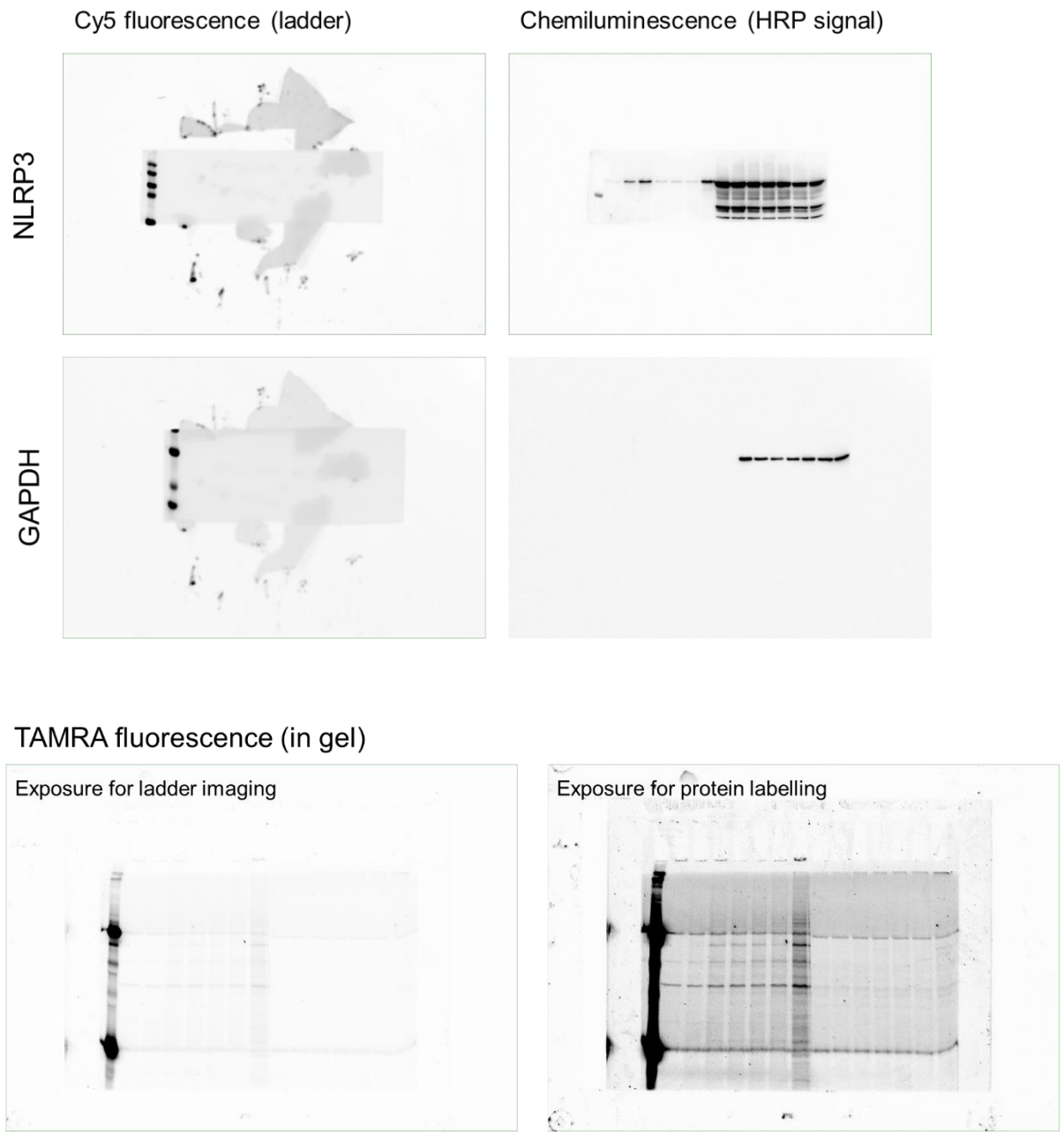

Figure S4: Uncropped blots and gels for Figure 2C

Supporting Tables

Table S1 Proteins identified by proteomics TPP as potential MCC950 binders

Gene Name	Protein
CA2	Carbonic anhydrase 2
CD99	CD99 antigen
CLTB	Clathrin light chain B
DBI	Acyl-CoA-binding protein
EEA1	Early endosome antigen 1
ENO2	Gamma-enolase
HDDC2	HD domain-containing protein 2
HIST1H1D	Histone H1.3
MAPK1IP1L	MAPK-interacting and spindle-stabilizing protein-like
MRPS18C	28S ribosomal protein S18c, mitochondrial
MYCBP	C-Myc-binding protein
NUP37	Nucleoporin Nup37
PEA15	Astrocytic phosphoprotein PEA-15
PEX19	Peroxisomal biogenesis factor 19
PEX7	Peroxisomal targeting signal 2 receptor
PSAP	Prosaposin
RIN2	Ras and Rab interactor 2
RPL10	60S ribosomal protein L10
RPL10A	60S ribosomal protein L10a
RPL15	60S ribosomal protein L15;Ribosomal protein L15
RPL18	60S ribosomal protein L18
RPL23	60S ribosomal protein L23
RPL23A	60S ribosomal protein L23a
RPL24	60S ribosomal protein L24
RPL27A	60S ribosomal protein L27a
ZSWIM2	E3 ubiquitin-protein ligase ZSWIM2