Supporting Information

Structure-guided design and in-cell target profiling of a cell-active target engagement probe for PARP inhibitors

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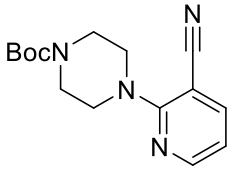
Supplementary Methods

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.[1] The minimal clickable photocrosslinkable group **5** was synthesized as previously reported.[2] Phthalazinone core **8**, **AZ9482** and **AZ0108** were provided by AstraZeneca. Olaparib was purchased from VWR International. NeutrAvidin agarose resin and PreScission™ Protease were purchased from Thermo Fisher Scientific. Streptavidin magnetic beads were purchased from New England BioLabs. Recombinant GST-PARP6 protein and pcDNA3.1™ mammalian expression vector containing FLAG-PARP6 were provided by AstraZeneca. 6-biotin-17-NAD^+ was purchased from Bio-Techne Ltd.

Antibodies: anti-PARP1 (SantaCruz, sc-8007), anti-PARP6 (Sigma, HPA026991), anti-HDLBP (Abcam ab109324), anti-GAPDH (Abcam, ab9485), anti-HSP90 (SantaCruz, sc-69703), FLAG-HRP [M2] (Sigma, A8592), NeutrAvidin-HRP (Invitrogen, A2664), anti-mouse-HRP (Advansta, R-05071-500), anti-rabbit-HRP (Advansta, R-05072-500).

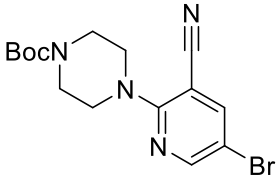
Tert-butyl 4-(3-cyanopyridin-2-yl)piperazine-1-carboxylate (5)



2

2-(Piperazin-1-yl)nicotinonitrile **1** (4.00 g, 21.3 mmol) and di-*tert*-butyl dicarbonate (4.78 g, 21.9 mmol) were dissolved in CH_2Cl_2 (60 mL) and cooled to 0 °C. Et_3N (5.60 mL, 40.8 mmol) was added drop-wise and the solution was stirred for 4 h at rt. The resulting reaction mixture was quenched with H_2O (60 mL) and extracted with CH_2Cl_2 (3 × 60 mL). The combined organic extracts were washed with H_2O (30 mL) and sat. aq. NaCl (30 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography (1:1 EtOAc:*n*-hexane) to afford **2** as a white crystalline solid (2.70 g, 9.38 mmol, 44%). HRMS (ESI^+) found $[M+H]^+$ 289.1661, $C_{15}H_{21}N_4O_2^+$ requires 289.1665; ^1H NMR (400 MHz, Chloroform-*d*) δ ppm 8.35 (dd, *J* = 4.9, 2.0 Hz, 1H), 7.79 (dd, *J* = 7.6, 2.1 Hz, 1H), 6.79 (dd, *J* = 7.5, 4.7 Hz, 1H), 3.69 – 3.65 (m, 4H), 3.61 – 3.57 (m, 4H), 1.48 (s, 9H).

Tert-butyl 4-(5-bromo-3-cyanopyridin-2-yl)piperazine-1-carboxylate (6)

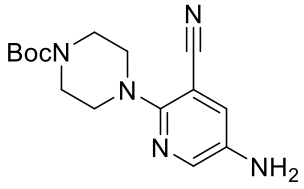


3

Tert-butyl 4-(3-cyanopyridin-2-yl)piperazine-1-carboxylate **2** (2.70 g, 9.38 mmol) was dissolved in DMF (10 mL) and the solution cooled to 0 °C. *N*-bromosuccinimide (2.01 g, 11.3 mmol) was added and the reaction stirred at rt for 3 h. The reaction was quenched with H_2O (5 mL) and concentrated *in vacuo*. The crude residue was dissolved in EtOAc (30 mL) and sat. aq. $NaHCO_3$ (15 mL) and aq. LiCl (5% (w/v), 15 mL) were added. The mixture was extracted with EtOAc (3 × 30 mL) and the combined organic layers were washed successively with sat. aq. $NaHCO_3$ (15 mL), aq. LiCl (5% (w/v), 15 mL) and sat. aq. NaCl (30 mL). The

organics were dried over Na_2SO_4 and concentrated *in vacuo* to yield **3** as a pale yellow solid (3.18 g, 8.66 mmol, 93%) without further purification. HRMS (ESI^+) found $[M+H]^+$ 367.0772, $C_{15}H_{20}N_4O_2{}^{79}Br$ requires 367.0770; ^1H NMR (400 MHz, Chloroform-*d*) δ ppm 8.34 (d, *J* = 2.6 Hz, 1H), 7.84 (d, *J* = 2.5 Hz, 1H), 3.70 – 3.64 (m, 4H), 3.59 – 3.54 (m, 4H), 1.47 (s, 9H).

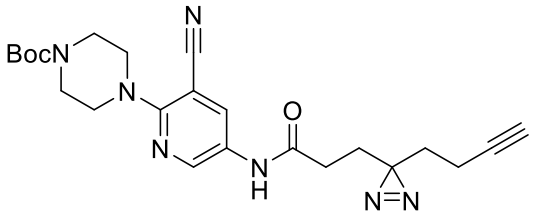
***Tert*-butyl 4-(5-amino-3-cyanopyridin-2-yl)piperazine-1-carboxylate (7)**



4

Tert-butyl 4-(5-bromo-3-cyanopyridin-2-yl)piperazine-1-carboxylate **3** (1.00 g, 2.72 mmol), CuI (103 mg, 0.542 mmol), L-proline (563 mg, 4.08 mmol), K_2CO_3 (563 mg, 4.08 mmol) and a stirrer bar were sealed inside a microwave vial which was flushed with Ar. DMSO (2.5 mL) was added and stirred at rt for 5 min. NH_4OH (30% (w/w), 0.520 mL, 4.08 mmol) was added and the reaction mixture stirred at 90 °C for 12 h. After cooling, the reaction was quenched with H_2O (50 mL) and extracted with Et_2O (3 × 50 mL). The combined organic layers were washed with sat. aq. NaCl (50 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (1:49 MeOH:CH_2Cl_2) to yield **4** as a yellow crystalline solid (426 mg, 1.41 mmol, 52%). HRMS (ESI^+) found $[M+H]^+$ 304.1767, $C_{15}H_{22}N_5O_2{}^+$ requires 304.1774; ^1H NMR (400 MHz, Methanol-d_4) δ ppm 7.93 (d, *J* = 2.7 Hz, 1H), 7.30 (d, *J* = 2.8 Hz, 1H), 3.60 – 3.52 (m, 4H), 3.26 – 3.18 (m, 4H), 1.48 (s, 9H).

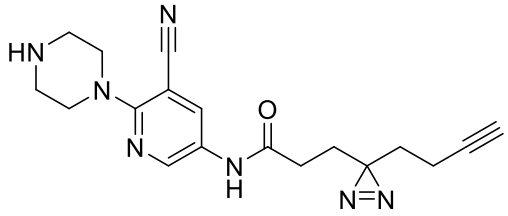
***Tert*-butyl 4-(5-(3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)propanamido)-3-cyanopyridin-2-yl)piperazine-1-carboxylate (9)**



6

DIPEA (110 μL, 0.631 mmol), *tert*-butyl 4-(5-amino-3-cyanopyridin-2-yl)piperazine-1-carboxylate **4** (64 mg, 0.211 mmol), and 3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)propanoic acid **5** (35 mg, 0.211 mmol) were dissolved in DMF (2 mL). Propylphosphonic acid cyclic anhydride (50% (v/v) in EtOAc, 249 μL, 4.19 mmol) was added and the reaction stirred at rt for 1 h. The reaction mixture was diluted with sat. aq. $NaHCO_3$ (5 mL) and extracted with EtOAc (3 × 5 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$ (5 mL) and aq. LiCl (5% (w/v), 2 × 5 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (1:7 acetone:CH_2Cl_2) to yield **6** as a brown oil (219 mg, 0.448 mmol, 75%). HRMS (ESI^+) found $[M+H]^+$ 452.2419, $C_{23}H_{30}N_7O_3{}^+$ requires 452.2410; ^1H NMR (400 MHz, Chloroform-*d*) δ ppm 8.32 (d, *J* = 2.7 Hz, 1H), 8.23 (d, *J* = 2.8 Hz, 1H), 7.85 (s, 1H), 3.56 (s, 8H), 2.11 (t, *J* = 7.4 Hz, 2H), 2.05 – 1.97 (m, 3H), 1.93 (t, *J* = 7.4 Hz, 2H), 1.66 (t, *J* = 7.3 Hz, 2H), 1.47 (s, 9H).

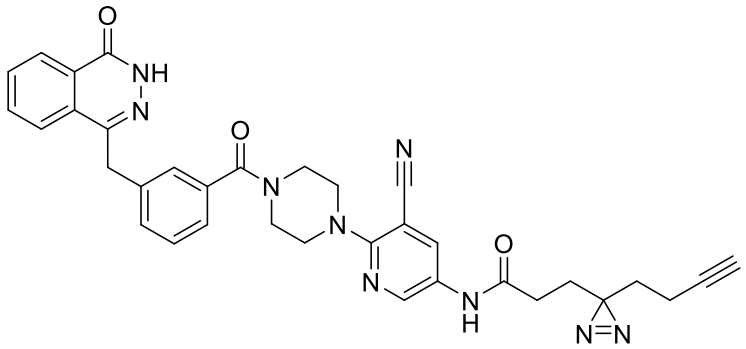
3-(3-(But-3-yn-1-yl)-3*H*-diazirin-3-yl)-*N*-(5-cyano-6-(piperazin-1-yl)pyridin-3-yl)propanamide (10)



7

Tert-butyl 4-(5-(3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)propanamido)-3-cyanopyridin-2-yl)piperazine-1-carboxylate **6** (37 mg, 0.0819 mmol) was dissolved in CH_2Cl_2 (3 mL). *N*-methylmorpholine (0.460 mL, 0.419 mmol) and trimethylsilyl iodide (0.330 mL, 0.231 mmol) were added and stirred under Ar at rt for 24 h. The reaction mixture was quenched with sat. aq. $NaHCO_3$ (5 mL) and the aqueous layer extracted with CH_2Cl_2 (3 × 5 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield **7** as a crude brown oil (28 mg, 0.0798 mmol, 96%) that was taken forward without further purification. HRMS (ESI^+) found $[M+H]^+$ 352.1893, $C_{18}H_{22}N_7O^+$ requires 352.1886; ^1H NMR (400 MHz, Methanol-d_4) δ ppm 8.47 (d, *J* = 2.4 Hz, 1H), 8.25 (d, *J* = 2.7 Hz, 1H), 3.72 – 3.65 (m, 4H), 3.05 – 3.00 (m, 4H), 2.27 (t, *J* = 2.9 Hz, 1H), 2.21 (t, *J* = 7.6 Hz, 2H), 2.04 (td, *J* = 7.5, 2.7 Hz, 2H), 1.84 (t, *J* = 7.6 Hz, 2H), 1.64 (t, *J* = 7.4 Hz, 2H).

3-(3-(But-3-yn-1-yl)-3*H*-diazirin-3-yl)-*N*-(5-cyano-6-(4-(3-((4-oxo-3,4-dihydrophthalazin-1-yl)methyl)benzoyl)piperazin-1-yl)pyridin-3-yl)propanamide (PARPYnD 3)



PARPYnD

DIPEA (51 μL, 0.293 mmol), 3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)-*N*-(5-cyano-6-(piperazin-1-yl)pyridin-3-yl)propanamide **7** (28 mg, 0.0798 mmol), and 3-((4-oxo-3,4-dihydrophthalazin-1-yl)methyl)benzoic acid **8** (22 mg, 0.0798 mmol) were dissolved in DMF (500 μL). Propylphosphonic acid cyclic anhydride (50% (v/v) in EtOAc, 95 μL, 0.293 mmol) was added and the reaction stirred at rt for 30 min. The reaction mixture was diluted with sat. aq. $NaHCO_3$ (5 mL) and extracted with EtOAc (3 × 5 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$ (5 mL) and aq. LiCl (5% (w/v), 2 × 5 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude residue was purified by preparative reverse phase LC-MS (50–98% MeCN in H_2O (0.1% HCO_2H)) to yield **PARPYnD** as a white solid (12 mg, 0.0195 mmol, 24%). MS: ES^+ 614.47; HRMS (ESI^-) found $[M-H]^-$ 612.2465, $C_{34}H_{30}N_9O_3^-$ requires 612.2472; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 12.60 (s, 1H), 10.33 (s, 1H), 8.52 (d, J = 2.7 Hz, 1H), 8.29 (d, J = 2.7 Hz, 1H), 8.26 (d, J = 7.7 Hz, 1H), 7.97 (d, J = 7.9 Hz, 1H), 7.89 (t, J = 7.5 Hz, 1H), 7.82 (t, J = 7.5 Hz, 1H), 7.40 (q, J = 7.0 Hz, 3H), 7.28 (d, J = 7.1 Hz,

1H), 4.36 (s, 2H), 3.80 – 3.66 (m, 2H), 3.60 – 3.42 (m, 6H), 2.84 (t, J = 2.7 Hz, 1H), 2.16 (t, J = 7.6 Hz, 2H), 2.01 (td, J = 7.6, 2.9 Hz, 2H), 1.75 (t, J = 7.5 Hz, 2H), 1.60 (t, J = 7.4 Hz, 2H).

Tissue Culture

All cell culturing was carried out in a sterile tissue culture cabinet sprayed with 70% (v/v) EtOH before and after use. All cell lines were cultured at 37 °C in a 5% CO_2 incubator. MDA-MB-468 cells were cultured in Dubecco's Modified Eagle Medium – low glucose (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS). Cell harvesting was achieved by washing with Dubecco's PBS and treatment with 0.25% (w/v) Trypsin-EDTA. After 5 min incubation at 37 °C, the trypsin was quenched with DMEM to the appropriate volume for passage and aliquoted into the appropriate number of cell culture plates. Mycoplasma tests were carried out monthly. Passage number was limited to 20–25 and stocks of early passages were frozen at –150 °C containing ~10^6 cells in 1 mL FBS containing 10% (v/v) DMSO.

Multipolar Spindle Assay

This was performed as described previously.[3] Briefly, HeLa cells were plated in 96-well plates at 7,000 cells per well and incubated at 37 °C overnight. The cells were treated with compounds in a dose-dependent manner from 0 to 11 μM for 48 h. The cells were fixed by 4% (v/v) formaldehyde at room temperature for 10 min and followed by ice-cold methanol fixation for another 10 min. After washing with PBS four times, the cells were blocked in blocking buffer for 1 h at room temperature. The cells were labelled with primary antibodies, 1:2000 dilution of anti-cyclin B antibody (Thermo Fisher) and 1:4000 dilution of anti-pericentrin antibody (Abcam), for 16 h at 4 °C. After washing with PBS four times, the cells were labelled with secondary antibodies, 1:200 Alexa Fluor 488 anti-rabbit antibody and Alexa Fluor 594 anti-mouse antibody, for 1 h at room temperature. After washing with PBS twice, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. The cells were washed twice with PBS and then applied to image acquisition by ImageXpress Micro High Content Screening System (Molecular Devices). The data were analyzed by MetaXpress and accessed by AcuityXpress (Molecular Devices). The 16 fields in each well were acquired by ImageXpress Micro. The cyclin B was labelled for scoring the mitotic cells and pericentrin was labelled for scoring the centrosome number in each mitotic cell. Value output was taken as % mitotic cells with greater than 2 centrosomes.

***In vitro* PARP fluorescence anisotropy binding assays**

Dilution of various PARP proteins and fluorescence anisotropy probe

Recombinant full length 6HIS-tagged PARP1 protein was diluted to 6 nM with 50 mM Tris pH 8, 0.001% (v/v) Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl and incubated for four hours with an equivalent volume of 2 nM fluorescent probe diluted with 50 mM Tris pH 8, 0.001% (v/v) Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl. The final DMSO concentration of the probe was kept below 1% (v/v).

Recombinant full length PARP2 protein was diluted to 6 nM with 50 mM Tris pH 8, 0.001% (v/v) Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl and incubated for four hours with an equivalent volume of 2 nM fluorescent probe diluted with 50 mM Tris pH 8, 0.001% (v/v) Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl. The final DMSO concentration of the probe was kept below 1% (v/v).

Recombinant PARP5a binding domain was diluted to 160 nM with 50 mM Tris pH 8, 0.001% (v/v) Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl and incubated for four hours with an equivalent volume of 6 nM fluorescent probe diluted with 50 mM Tris pH 8, 0.001% (v/v) Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl. The final DMSO concentration of the probe was kept below 1% (v/v).

Recombinant full length GST-tagged PARP6 protein was diluted to 160 nM with 50 mM Tris pH 8, 0.001% (v/v) Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl and incubated for four hours with an equivalent volume of 6 nM fluorescent probe diluted with 50 mM Tris pH 8, 0.001% (v/v) Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl. The final DMSO concentration of the probe was kept below 1% (v/v).

Experimental protocol
Fluorescence anisotropy of the probe when bound to the proteins was measured using a BMG Pherastar FS© in the presence of test compounds or solvent control and the effect on anisotropy determined. % inhibition values for different test compound concentrations were calculated and fitted to a four parameter logistic plot in order to determine the IC_{50} value.

Cell Viability Assay (MTS)
MDA-MB-468 cells were seeded 24 h before treatment in a sterile treated 96-well plate at a density of 8000 cells per well to a final volume of 50 μL in DMEM. PBS (100 μL) was added to the outer wells. DMEM (50 μL) containing 0.2% (v/v) DMSO (positive control, final amount 0.1% (v/v) on plate) or puromycin (negative control, 8 mM, final concentration 4 mM on plate), or different concentrations of compound in DMSO to be tested (final 0.1% (v/v) DMSO, prepared by serial dilution, dilution factor 3, starting from 3 μM, final plate concentration starting from 1.5 μM) were added to the cells in triplicate. 72 h later, a solution of MTS assay powder (3.28 mM in PBS 1×, Promega) and phenazine methosulfate (3 mM in H_2O, Sigma-Aldrich) was prepared (20:1) and 20 μL was added to each well. Absorbance was measured 4 times per well at 490 nm and the average absorbance taken. The average of the negative control was subtracted from every value and viability was calculated as a percentage relative to the positive control. EC_{50} values were calculated by fitting data to the IC_{50} function using GraphPad Prism 5 software.

Probe labelling assays
Probe incubation was carried out in sterile treated 6-well plates (2 mL working volume) or 10 cm dishes (7 mL working volume). All compound treatments were performed with a pre-prepared 1000× stock of the desired concentration in DMSO and added directly to the relevant plate/well with mixing. Irradiation was performed with an in-house designed and built UV LED box (C. Saunders) with a monochromatic wavelength of 365 nm. Optimal probe incubation times were determined previously through irradiation and lysis at various time points and selection of the earliest saturating time point.

For each experiment, the plates/dishes were pre-seeded with MDA-MB-468 cells and the experiment carried out when cells had achieved 90–100% confluency. DMEM was replaced and the plates/dishes were incubated at 37 °C for 15 min. For competition experiments only, the relevant plates/wells were first treated with DMSO vehicle (0.1% (v/v)) or varying concentrations of parent compound in DMSO as indicated and incubated at 37 °C for 1 h before treating with probe. For all probe labelling experiments, competition or otherwise, the plates/dishes were treated with either DMSO vehicle (0.1% (v/v)) or varying concentrations of probe as required by the experiment and incubated at 37 °C for 3 h.

For photocrosslinking, the following was performed 1 plate/3 dishes at a time out of the incubator. Each plate/dish had media replaced and was irradiated with UV light for 30 s (365 nm) and placed on ice while irradiation of other samples was performed.

The cells were relieved of media, washed twice with PBS, then lysed with lysis buffer (70 μL (6-well plates), 300 μL (10 cm dishes); 1% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulfate (SDS), EDTA-free complete protease inhibitor cocktail (1×, Roche) in PBS) on ice for 10 min. The lysates were scraped and transferred to corresponding Lo-Bind Eppendorfs. Each lysate

was probe sonicated (20% amplitude, 20 s (2 s pulse, 3 s rest)) to shear the nuclear DNA. Protein concentration was determined using the DC Protein Assay (Bio-Rad) in a 96-well plate as per manufacturer's instructions.

Click reaction

The desired amount of lysed protein from each sample was made up to 0.5–2 mg mL^{-1} with PBS to a total volume of ≤300 μL. The following "click mixture" was prepared separately, preparing 6 μL for every 100 μL of lysate:

- Click reagent (AzTB or AzRB, 10 mM in DMSO, 1 vol; final concentration in reaction 0.1 mM),
- $CuSO_4$ (50 mM in H_2O, 2 vol; final concentration in reaction 1 mM),
- Tris(2-carboxyethyl)phosphine (TCEP, 50 mM in H_2O, 2 vol; final concentration in reaction 1 mM),
- Tris(benzyltriazolylmethyl)amine (TBTA, 10 mM in DMSO, 1 vol; final concentration in reaction 0.1 mM).

The click mixture was vortexed and incubated at rt for 2 min before 6 μL of the mixture was added to every 100 μL of lysate. The reaction mixtures were shaken at rt for 1 h before being quenched with EDTA (500 mM in H_2O) to a final concentration of 5 mM.

A table-top centrifuge was pre-chilled to 4 °C. Proteins were precipitated by adding H_2O (1 vol), MeOH (2 vol) and $CHCl_3$ (0.5 vol), vortexing briefly then centrifuging at 17,000 × g for 5 min. The $CHCl_3$ and H_2O/MeOH layers were discarded and the middle layer of protein pellet was retained. The pellet was washed with MeOH (300 μL), sonicated to break up the pellet then stored at –80 °C for at least 20 min. The proteins were pelleted by centrifugation at 10,000–17000 × g for 5–10 min or until a compact pellet was formed. The MeOH was decanted and the pellet air-dried for 5 min. The pellet was resuspended by completely dissolving in 1% (w/v) SDS in PBS (to 5 mg mL^{-1} protein) before being made up to 1 mg mL^{-1} protein with PBS.

In-gel fluorescence

10- or 15-well SDS-polyacrylamide gels with a 12% resolving gel and 4% stacking gel were used for all gel electrophoresis experiments and were prepared using the following recipe (makes 2 gels):

Reagent	Resolving gel (12%)	Stacking gel (4%)
H_2O	3.4 mL	3.05 mL
Resolving/Stacking Buffer (National Diagnostics)	2.5 mL	1.25 mL
Protogel 30% (National Diagnostics)	4 mL	0.65 mL
Ammonium Persulfate (10% (w/v) in H_2O)	100 μL	25 μL
N,N,N′,N′ - tetramethylethylene diamine	10 μL	5 μL

All gels were run using a Bio-Rad Mini-PROTEIN® Tetra Cell with a Bio-Rad PowerPac™ Basic power supply. In general, 15 μg of protein was run per well in a volume of 15 μL.

Samples were prepared by adding 5 μL of 4× loading buffer (1:4 β-mercaptoethanol:5× NuPAGE LDS sample buffer) to 15 μL of sample and boiling at 95 °C for 10 min. The samples were briefly centrifuged then 15 μL of each sample was added to a well of the gel, with at least one well also containing Precision Plus Protein™ All Blue Prestained Protein Standard (2 μL, Bio-Rad). The gels were run in running buffer (25 mM trizma base, 194 mM glycine, 1% (w/v)

SDS) for 10 min at 85 V then up to 1 h at 180 V. The fluorescence on the gel was detected using a Typhoon™ FLA 9500 biomolecular imager (GE Healthcare Life Sciences) detecting TAMRA fluorescence, and the contrast normalized using Fiji (ImageJ) software. The protein loading was verified by staining with Coomassie Brilliant Blue. Coomassie stained gels were imaged using the digitization method (trans-illumination) on an ImageQuant LAS-4000 Imaging System (Fujifilm) and the contrast normalized in Fiji.

Pull down

50 μL of Pierce™ NeutrAvidin™ Agarose beads (proteomics) or 300 μL of streptavidin coated magnetic beads (western blot) were used per 1 mg of total protein per sample to an absolute minimum of 20 μL of agarose beads or 15 μL magnetic beads. All bead washes were performed by moderate shaking for 1 min then either briefly pelleting by table-top centrifuge then vacuum aspirating the supernatant with fine-end pipette tips (agarose) or by partitioning the beads using a magnet (magnetic). The beads were pre-washed three times with 0.2% (w/v) SDS in PBS, then protein samples (1 mg mL^{-1}) were added over the beads and incubated with moderate shaking at rt for 2 h.

For western blot analysis, the beads were washed three times with 300 μL 0.2% (w/v) SDS in PBS and captured proteins were released from the beads by boiling in 14 μL 2× sample loading buffer (95 °C, 10 min), briefly centrifuging and the supernatant loaded straight on to an SDS-PAGE gel. For proteomic analysis (agarose beads), the beads were treated as described below.

Western Blot

SDS-PAGE gels intended for western blot were prepared and run as outlined above. Proteins were wet transferred to nitrocellulose membrane (GE healthcare) in transfer buffer (120 mM tris, 40 mM glycine, 20% (v/v) MeOH) at 100 V for 1 h. Successful transfer was confirmed by staining with Ponceau S. Membranes were blocked (5% (w/v) non-fat dried skimmed milk powder in TBS-T (1× tris-buffered saline, 0.1% (v/v) Tween-20) or 3% (w/v) bovine serum albumin (BSA) in TBS-T) for 1 h at rt. Staining with primary antibody was performed either in milk TBS-T or 0.3% (w/v) BSA in TBS-T for 1 h at rt or overnight at 4 °C. The blot was washed with TBS-T (3 × 5 min) and, if necessary, stained with secondary antibody in milk/BSA TBS-T for 1 h at rt. The blot was washed with TBS-T (3 × 5 min) and visualized using Immobilon Crescendo Western HRP substrate, imaging using the chemiluminescence method on an ImageQuant LAS-4000 Imaging System.

Chemical Proteomics

Lysates for all proteomics experiments were derived from cells cultured in 10 cm dishes in triplicate for each experimental condition. 600 μg of labelled protein clicked to AzRB were enriched on NeutrAvidin agarose, all as described above. All buffers were prepared fresh and filtered (0.2 μm) and the work surface cleaned with 70% (v/v) EtOH.

The beads were washed twice with 300 μL 1% (w/v) SDS in 50 mM HEPES (pH 8). Proteins were reduced and alkylated with 5 mM TCEP and 10 mM chloroacetamide in 60 μL 50 mM HEPES with moderate shaking for 30 min at rt. The beads were washed 3 times with 300 μL 50 mM HEPES. Beads were resuspended in 30 μL 50 mM HEPES and proteins were digested on-bead by treatment with 1 μL trypsin (Promega, 20 μg dissolved in 100 μL 50 mM HEPES) with vigorous shaking at 37 °C overnight. The samples were briefly centrifuged and 10 μL of the supernatant from each sample was TMT-labelled by combining with 10 μL of the appropriate TMT10plex™ Isobaric Mass Tag Labelling Reagent (Thermo Scientific) dissolved in acetonitrile (8 mg mL^{-1}) with moderate shaking for 2 h at rt (see **Extended Data 2** for TMT label used for each sample). TMT-labelling was quenched by the addition of 1.1 μL of 5% (w/v)

hydroxylamine and the samples from each TMT set were combined into one “plex” solution. These samples were evaporated to dryness.

6-layer fractionation

Each stage tip was prepared by cutting 3× polystyrene-divinylbenzene copolymer modified with sulfonic acid (SCX) disks (3M) and using these to plug a p200 pipette tip. The tip was activated with 150 μL MeCN by centrifugation (3000 × g, 3 min) then equilibrated with 150 μL H_2O by centrifugation (3000 × g, 3 min). Samples were dissolved in 1% (v/v) aqueous trifluoroacetic acid and each sample transferred to a stage tip. Peptides were loaded onto the SCX column by centrifugation as above. Peptides were desalted by centrifugation with 3× 60 μL of 0.2% (v/v) trifluoroacetic acid. Peptides were then liberated from the column sequentially by centrifugation with 60 μL of each of the following buffers into separate Lo-Bind Eppendorfs:

Fraction	Buffer composition
1	75 mM ammonium acetate, 20% (v/v) MeCN, 0.5% (v/v) Formic Acid
2	125 mM ammonium acetate, 20% (v/v) MeCN, 0.5% (v/v) Formic Acid
3	200 mM ammonium acetate, 20% (v/v) MeCN, 0.5% (v/v) Formic Acid
4	300 mM ammonium acetate, 20% (v/v) MeCN, 0.5% (v/v) Formic Acid
5	400 mM ammonium acetate, 20% (v/v) MeCN, 0.5% (v/v) Formic Acid
6	5% (v/v) ammonium hydroxide, 80% (v/v) MeCN

All fractions of each sample were evaporated to dryness and stored at –80 °C.

LC-MS/MS methodology

Samples were rehydrated in 0.5% (v/v) formic acid, 2% (v/v) UPLC grade MeCN in Optima™ LC/MS H_2O (Fisher Scientific) and dissolved completely by vortexing and sonication. Samples were filtered through 3x Durapore® membrane filters (Millipore) plugged into a p20 pipette tip by centrifuging the samples through the filters (4000 × g, 5 min) into a mass spectrometry vial. Samples were stored at 4 °C until ready for analysis.

Peptides were separated on an EASY-Spray™ Acclaim PepMap C_{18} column (50 cm × 75 μm inner diameter, Thermo Fisher Scientific) using a 3-hour linear gradient separation of 0–100% solvent B (80% MeCN supplemented with 0.1% formic acid): solvent A (2% MeCN supplemented with 0.1% formic acid) at a flow rate of 250 nL min^{-1}. The liquid chromatography was coupled to a QExactive mass spectrometer via an easy-spray source (Thermo Fisher Scientific) which operated in data-dependent mode with survey scans acquired at a resolution of 70,000 at *m/z* 200. Scans were acquired from 350 to 1800 *m/z*. Up to 10 of the most abundant isotope patterns with charge +2 or higher from the survey scan were selected with an isolation window of 1.6 *m/z* and fragmented by HCD with normalized collision energy of 25. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 35,000 at *m/z* 200) were 20 and 120 ms, respectively. The ion target value for MS was set to 10^6 and for MS/MS to 10^5, and the intensity threshold was set to 8.3×10^2.

Data analysis

Peptide searches were performed in MaxQuant version 1.6.0.2. Under group-specific parameters and type, reporter ion MS2 was selected, and the appropriate TMT10plex™ isobaric labels selected for both lysines and N-termini. For all experiments, oxidation (M) and acetyl (protein N-term) were set as variable modifications, carbamidomethyl (C) was set as a fixed modification, trypsin/P was set as the digestion mode, re-quantify and match between runs were selected, and up to date UniProt FASTA files for the human proteome and contaminants databases were used.

Data analysis was performed in Perseus version 1.6.7.0. Reporter intensity corrected values were loaded into the matrix. Data was filtered by removing rows based on “only identified by site”, “reverse”, and “potential contaminant” columns. Data were log_2 transformed and filtered by row, retaining those that had 2 valid values in each triplicate condition. TMT data were normalized by subtracting the mean of each row within each TMT “plex” (if appropriate) and subtracting the median of each column. Volcano plots were generated using a pairwise Student’s T-Test and the cut-offs generated using the false discovery rate (FDR) and S_0 values indicated.

PARP6 auto-ADP ribosylation assay

This protocol was adapted from Hutin, Grimaldi and Matthews.[4] Briefly, reaction tubes containing 1× assay buffer (20×: 1 M Tris-HCl, pH 8.0, 4 mM DTT, 80 mM $MgCl_2$), 25 μM NAD^+-biotin (added last), 0.3 μM GST-PARP6, appropriate concentration of inhibitor (from 10× stock (1% (v/v) DMSO)) were made up to final volume with H_2O, substituting for appropriate controls. The reactions were shaken on ice for 30 min, quenched with 4× sample loading buffer and boiled at 95 °C for 5 min. The samples were separated on to two SDS-PAGE gels and transferred to nitrocellulose. Each membrane was blotted separately using NeutrAvidin-HRP and anti-PARP6 (total protein).

Transfection

Plasmid production

All microbiology work was carried out in a work area sterilized with 70% (v/v) EtOH and in the presence of an open flame. All equipment and media was either bought sterile or sterilized by autoclave. DNA concentration was measured using a NanoDrop OneC (Thermo Scientific). Sequencing was performed by Genewiz.

1 μL of pcDNA™ 3.1 plasmid (70 ng μL^{-1}) containing FLAG-PARP6 was transformed into 50 μL competent DH5α *E. coli* cells (Invitrogen) by heat shock treatment (30 min on ice, 42 °C for 45 s, 2 min on ice). Cells were cultured in Super Optimal broth with Catabolite repression (S. O. C.) medium (1 h, 900 rpm, 37 °C) then 50 μL spread on lysogeny broth (LB) agar supplemented with 100 μg μL^{-1} ampicillin which was grown overnight at 37 °C. Individual colonies were then cultured overnight (37 °C, 170 rpm) in 15 mL LB medium supplemented with 100 μg μL^{-1} ampicillin. 5 mL of this culture was used to inoculate a further 150 mL LB medium supplemented with 100 μg μL^{-1} ampicillin and grown overnight (37 °C, 170 rpm). Plasmids were purified from the culture using the QIAGEN® Maxiprep kit and sequence identity confirmed by Sanger sequencing.

Mammalian cell uptake

Transfections were performed with Lipofectamine® LTX with Plus™ Reagent according to standard manufacturer’s protocol. Briefly, MDA-MB-468 cells were seeded in sterile treated 6-well plates and grown to confluency. Transfections were performed at 1:3 DNA:Lipofectamine with 2 μg DNA, incubating for 24 h. Cells were then treated and irradiated as described above and analyzed by in-gel fluorescence and western blot.

Lysate-based photocrosslinking assay

Native MDA-MB-468 lysates were generated by trypsination of one 75 cm^2 flask of cells as described above. Trypsin was quenched and removed by aspiration after centrifugation (200 × *g*, 5 min). The cells were washed similarly in PBS and resuspended in cold PBS (250 μL). Cells were lysed on ice by probe sonication (20% amplitude, 18 s (3 s pulse, 3 s rest)) and centrifuged to remove cell debris. Protein concentration was determined as above and the lysate snap frozen in liquid N_2 and stored at –80 °C. Lysates were thawed on ice before use.

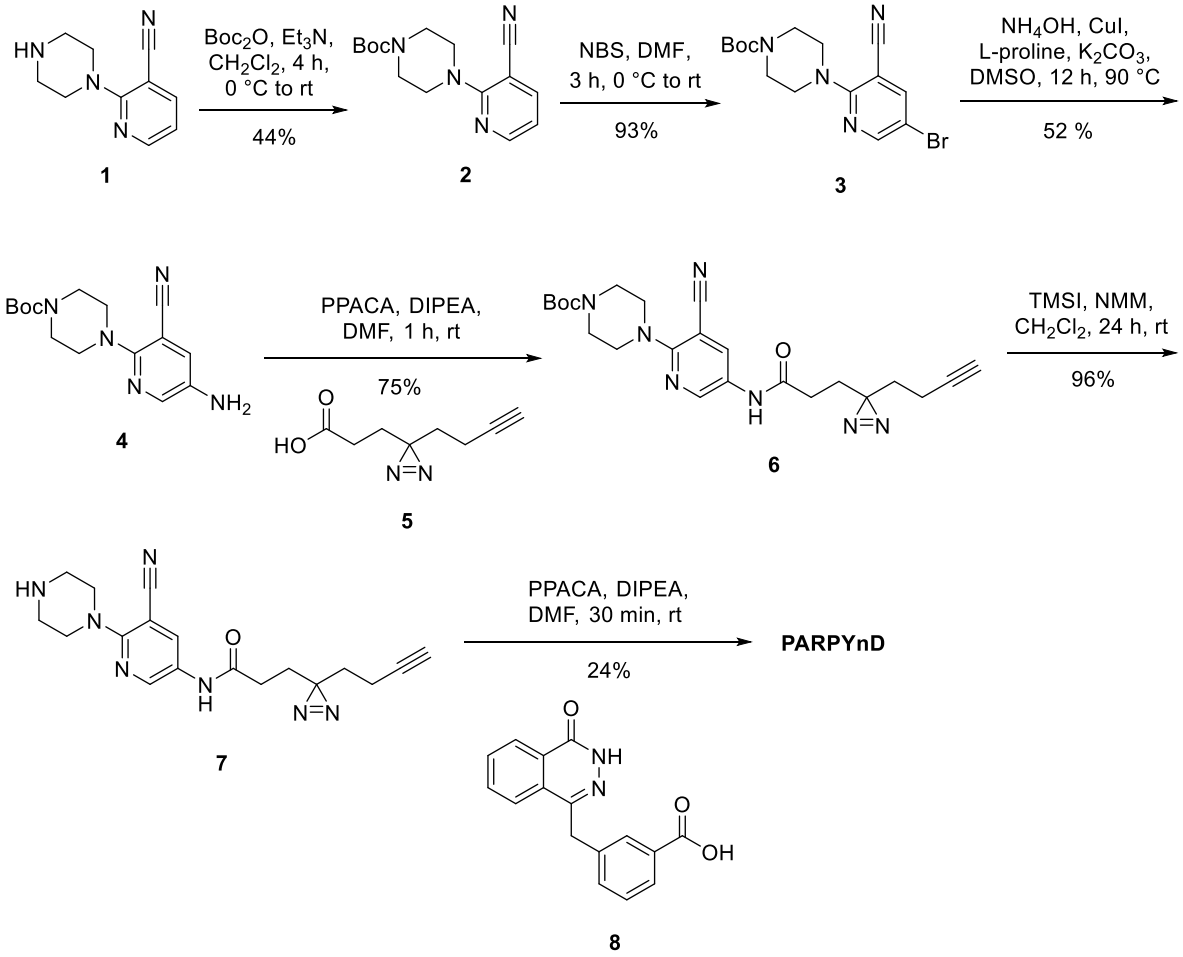
In a clear 96-well plate, wells containing 2 mg mL^{-1} native MDA-MB-468 lysate, 0.1 μM GST-PARP6 (or blank storage buffer), appropriate concentration of **PARPYnD**/**AZ0108** (from 100× stock (10% (v/v) DMSO) – added last) were made up to final volume with PBS and incubated on ice for 30 min in the dark. The plate was irradiated with UV light for 5 min (365 nm monochromatic) and each sample transferred to Eppendorfs. The proteins were precipitated as described above to remove Tris (in GST-PARP6 storage buffer) which would otherwise inhibit the click reaction. Proteins were clicked to AzTB and enriched as described above, and the results analyzed by gel and western blot.

Variation with Prescission™ Protease

When also treating the samples with PreScission™ Preotease, samples were made up with the cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0) rather than PBS, and GST-PARP6 was added to a concentration of 0.3 μM. Cleavage was performed using 1 U of enzyme overnight with moderate shaking at 4 °C. Samples were irradiated and prepared for gel-based analysis as described above.

Supporting Schemes

Scheme S1 – Synthesis of **PARPYnD.**



Supporting Figures

Figure S1. PARP *in vitro* binding assays.
Dose-response curves for **PARPYnD** tested in fluorescence anisotropy competition assay with various recombinant PARP enzymes. N, number of biological replicates – data displayed ±SEM of these replicates, fit to 4 parameter dose-response function. PARP1 IC_{50} = 0.038 μM, PARP2 IC_{50} = 0.006 μM, PARP6 IC_{50} = 0.230 μM. Raw data can be found in **Extended Data 1**.

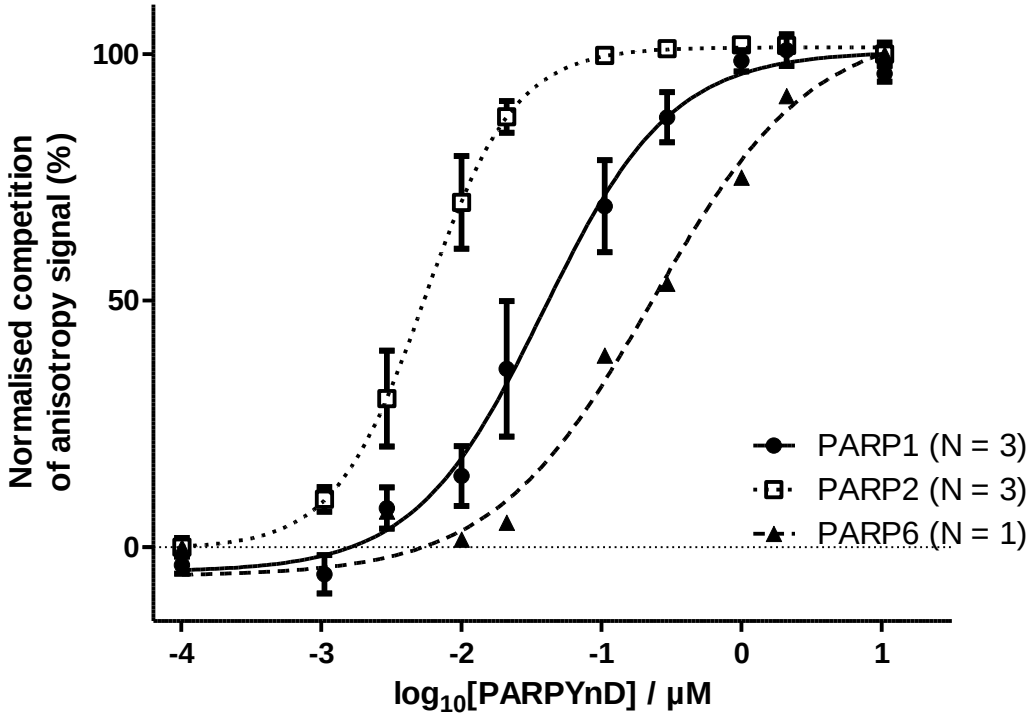
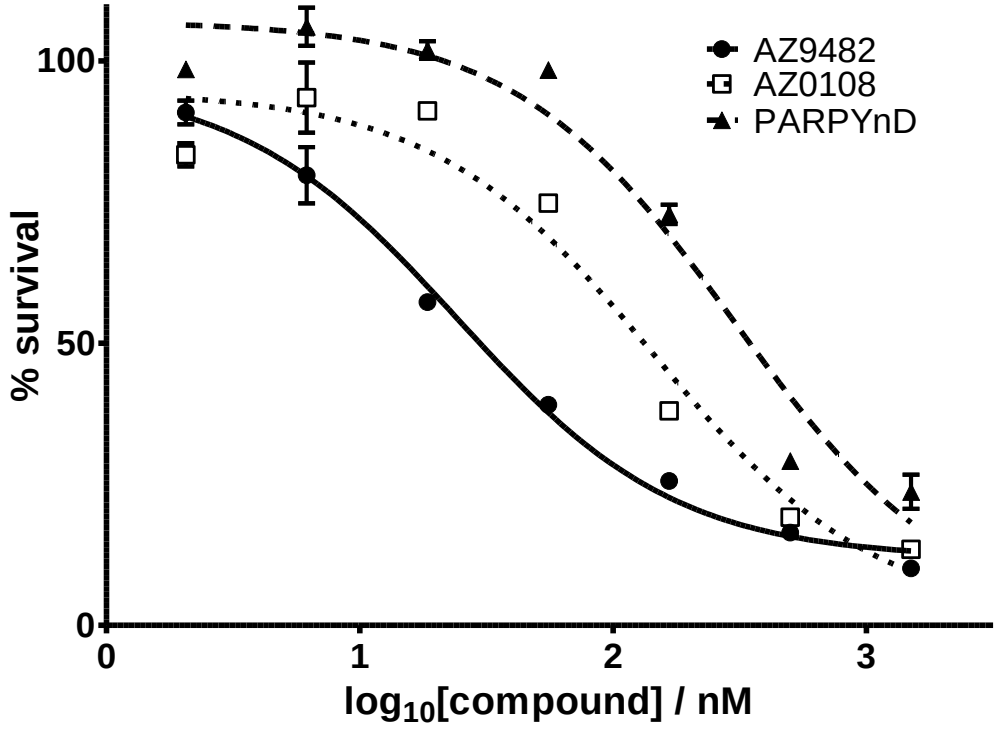
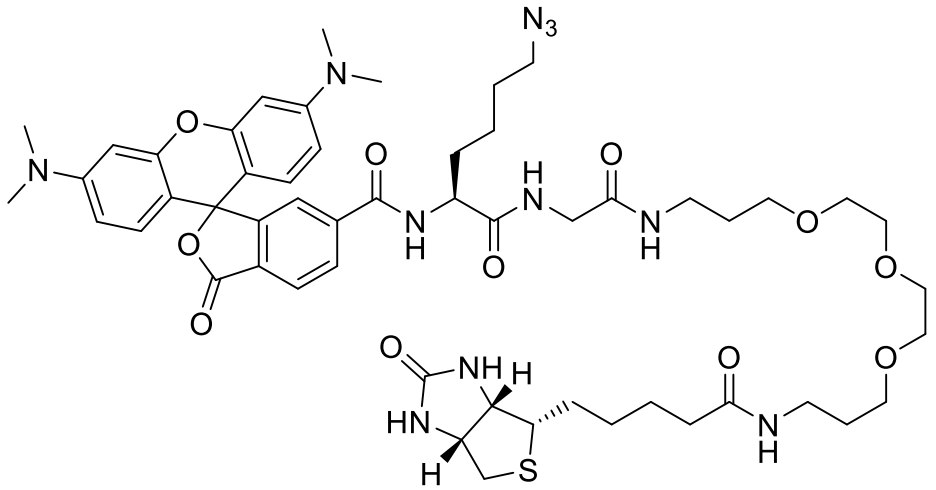


Figure S2. Cell viability assay.
Dose-response curves for MDA-MB-468 cells treated with varying concentrations of each compound indicated for 3 days. Cell viability was measured using the MTS assay, % survival was measured relative to 0.1% (v/v) DMSO control. Raw survival values are displayed in the table below. Data points are average of three technical replicates (±SEM), data fit to 3 parameter dose-response function. EC_{50} values: 24 nM (**AZ9482**), 140 nM (**AZ0108**), 301 nM (**PARPYnD**).

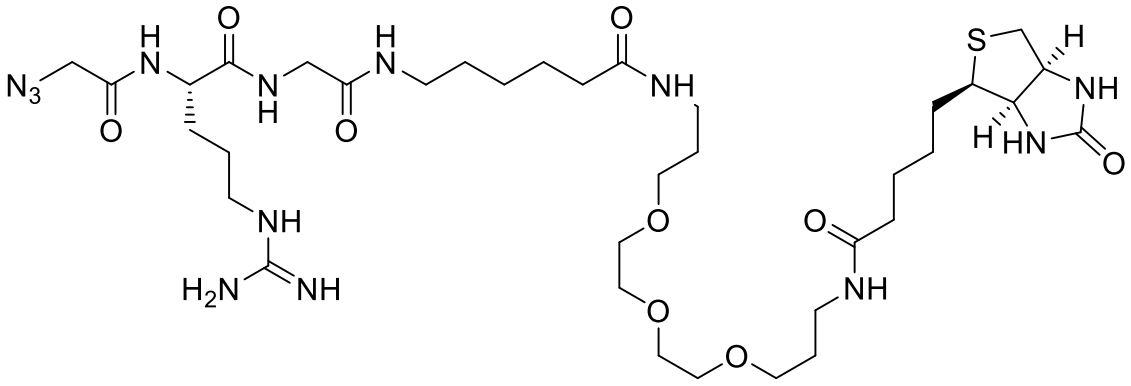


log_{10}[compound] / nM	**AZ9482**			**AZ0108**			**PARPYnD**		
0.31	92.9	86.7	92.9	79.7	83.7	86.7	97.2	98.6	99.6
0.79	88.2	70.9	80.1	86.9	87.7	105.9	105.4	100.5	112.1
1.27	57.2	58.7	55.9	90.9	91.5	91.1	102.9	103.9	98.9
1.74	37.9	38.5	40.8	74.3	76.8	73.4	96.0	98.8	100.3
2.22	25.8	26.4	24.5	35.6	39.1	39.3	71.4	76.2	70.7
2.70	17.8	15.6	15.9	20.0	17.3	20.2	30.0	28.0	29.3
3.18	7.7	10.9	11.7	13.2	13.0	14.3	28.3	24.6	18.0

Figure S3. Structure of the clickable capture reagents.
(**A**) AzTB – Azide-TAMRA-Biotin and (**B**) AzRB – Azide-Arginine-Biotin.[1]



AzTB



AzRB

Figure S4. Photocrosslinking dose-response assay up to 10 μM.
MDA-MB-468 cells were treated with up to 10 μM **PARPYnD** for 3 h, irradiated (365 nm), lysed and ligated to AzTB (**Figure S3**). Samples were enriched on magnetic streptavidin beads and both lysate (input) and pull down were analyzed by in-gel fluorescence and immunoblot. PARP6 cannot be detected in the enriched fraction over any concentration of **PARPYnD** up to 10 μM, over 10 times the reported IC_{50}.

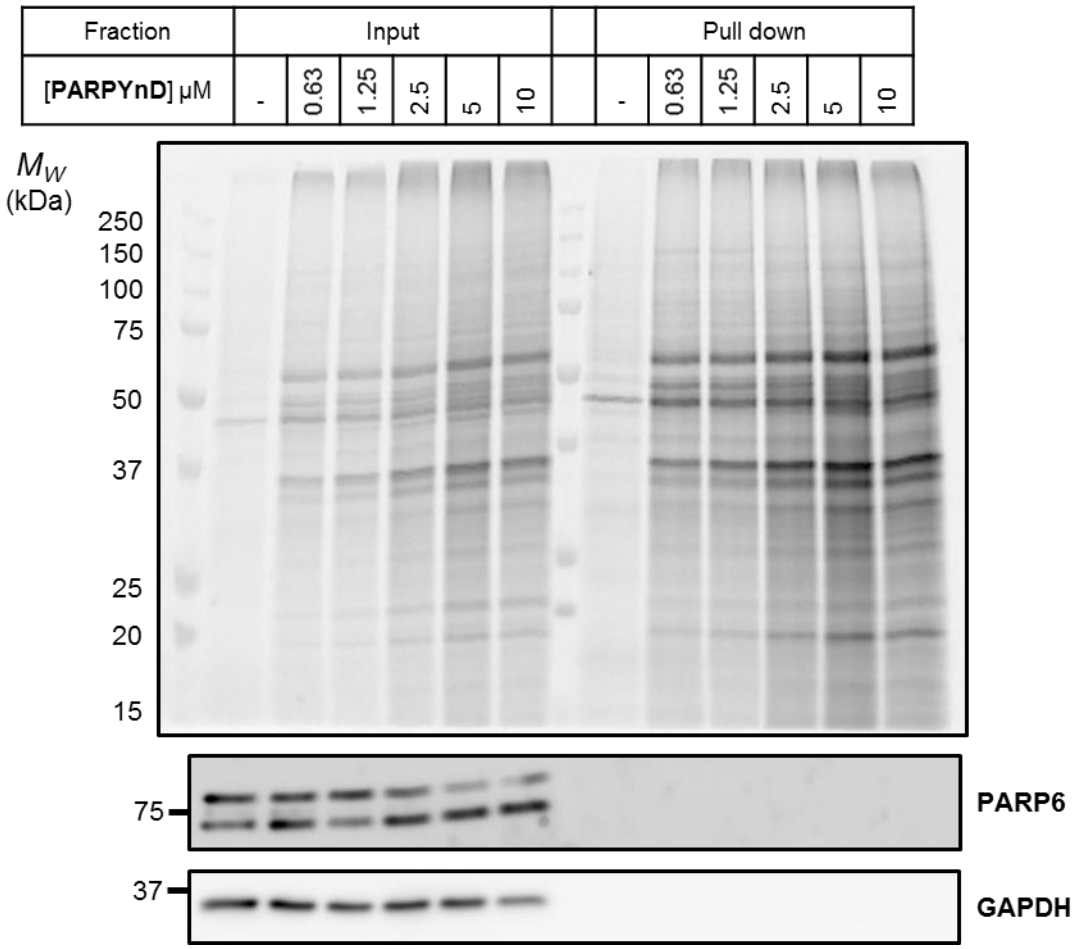
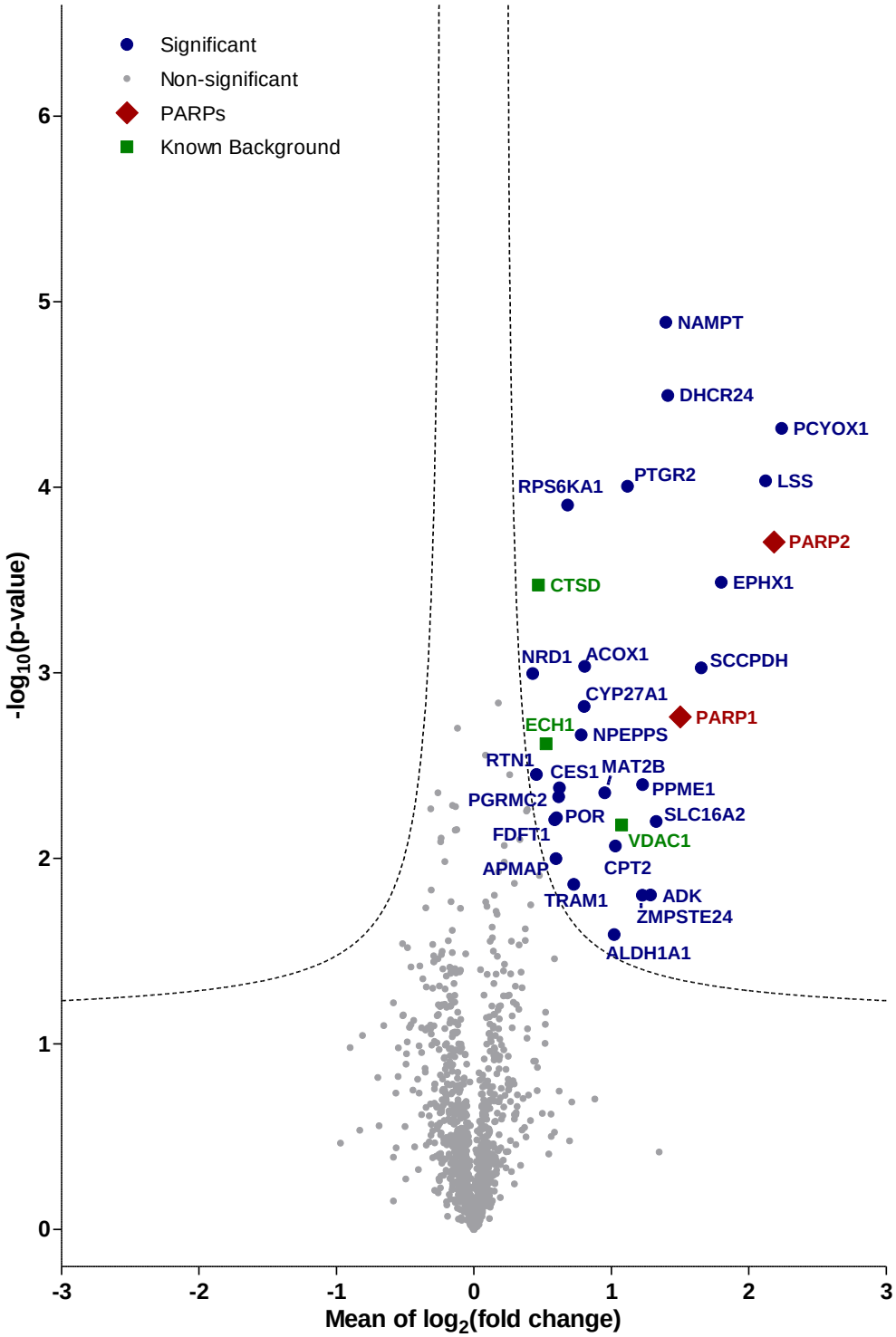
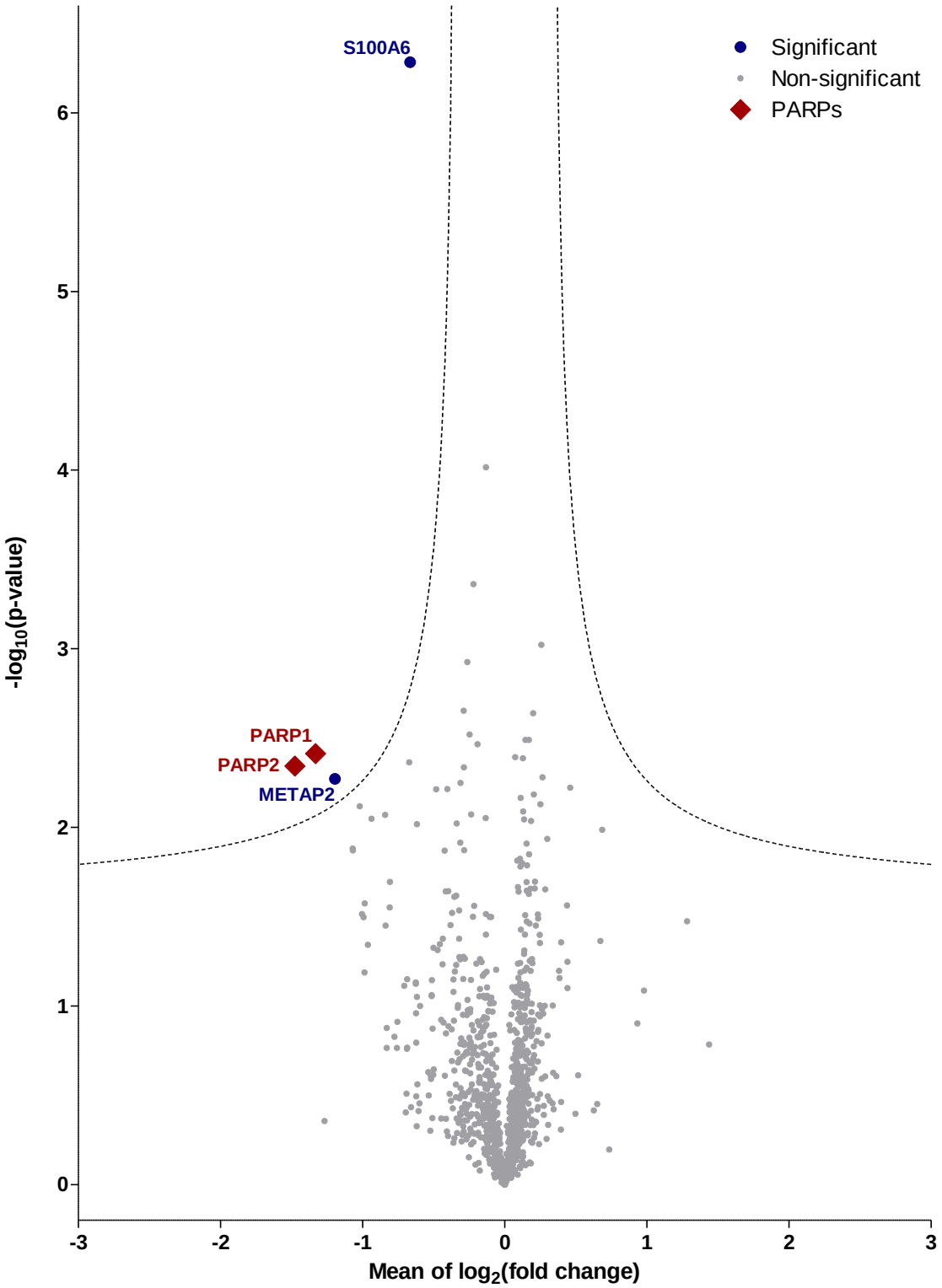


Figure S5. Supplementary volcano and profile plots for **PARPYnD**.
A-**D**: Complete annotated volcano plots for **Figure 3**. Volcano plots demonstrate enrichment (x axis) of one sample versus another and the associated significance (y axis), determined by pairwise Student's T-test.

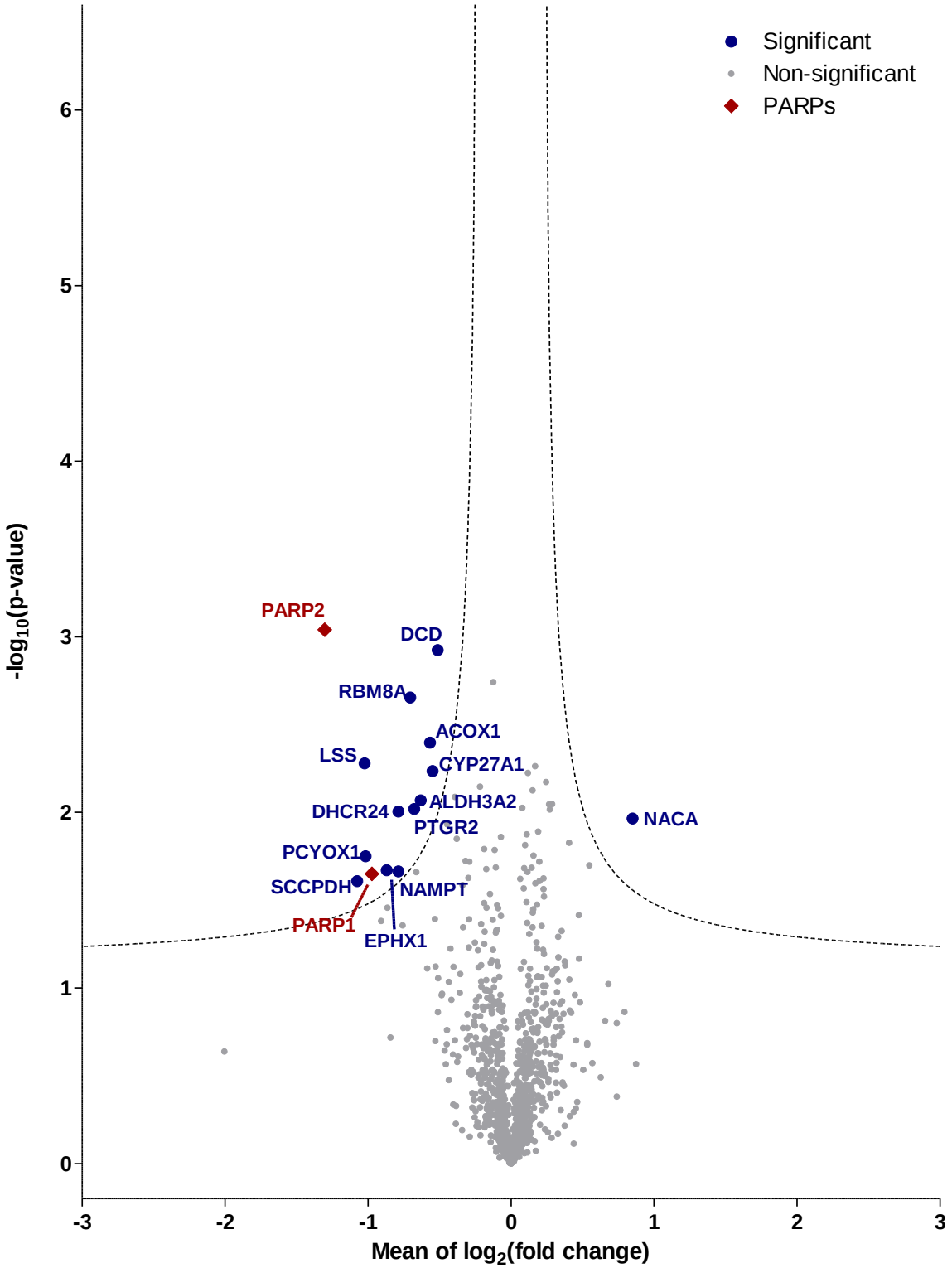
(**A**) **PARPYnD** (1 μM) vs. DMSO (S_0 = 0.1, FDR = 0.05).



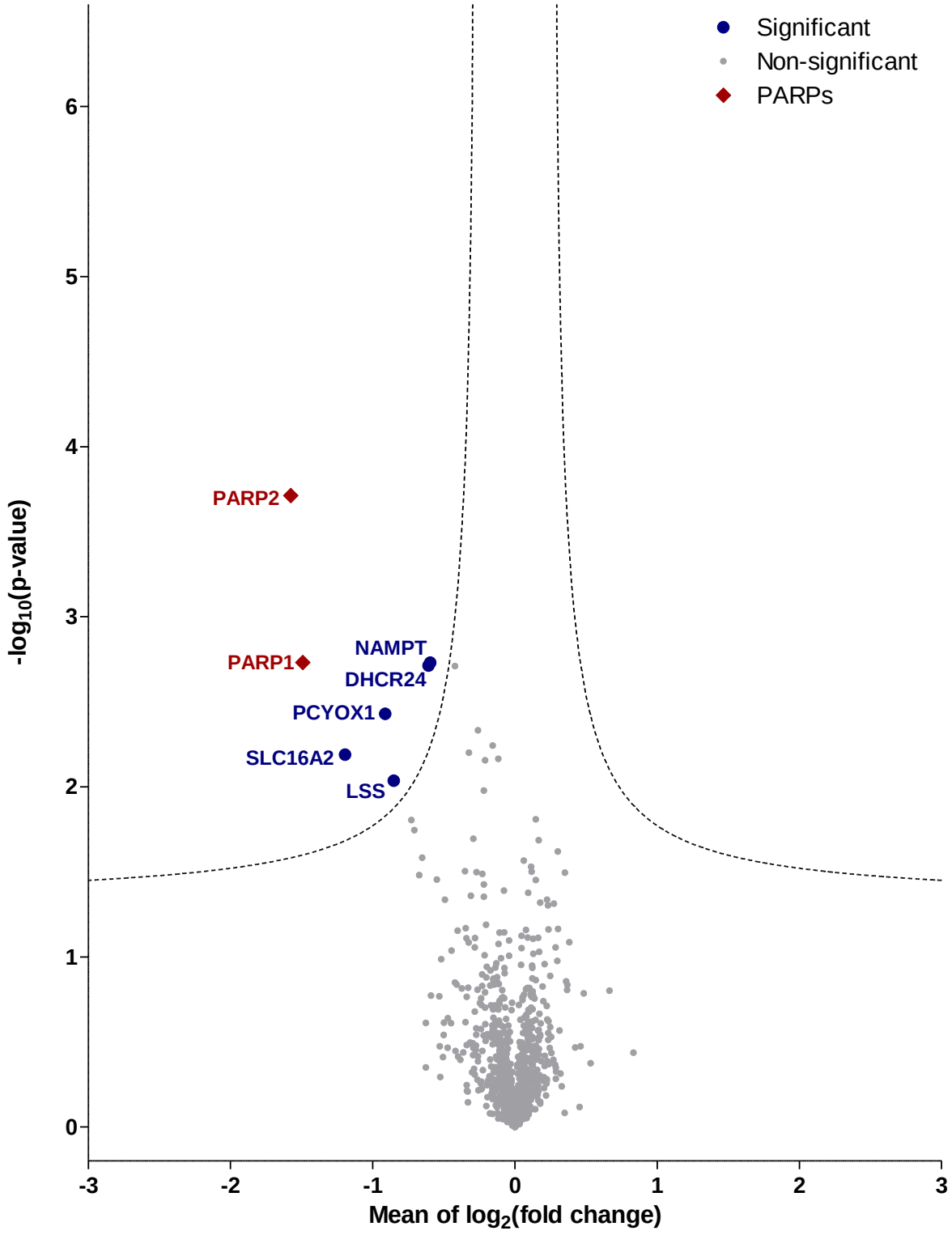
(**B**) **PARPYnD** (1 μM) *and* **AZ9482** (5 μM) vs. **PARPYnD** (1 μM) *only* (S_0 = 0.1, FDR = 0.15).



(**C**) **PARPYnD** (1 μM) *and* **AZ0108** (5 μM) vs. **PARPYnD** (1 μM) *only* (S_0 = 0.1, FDR = 0.15).

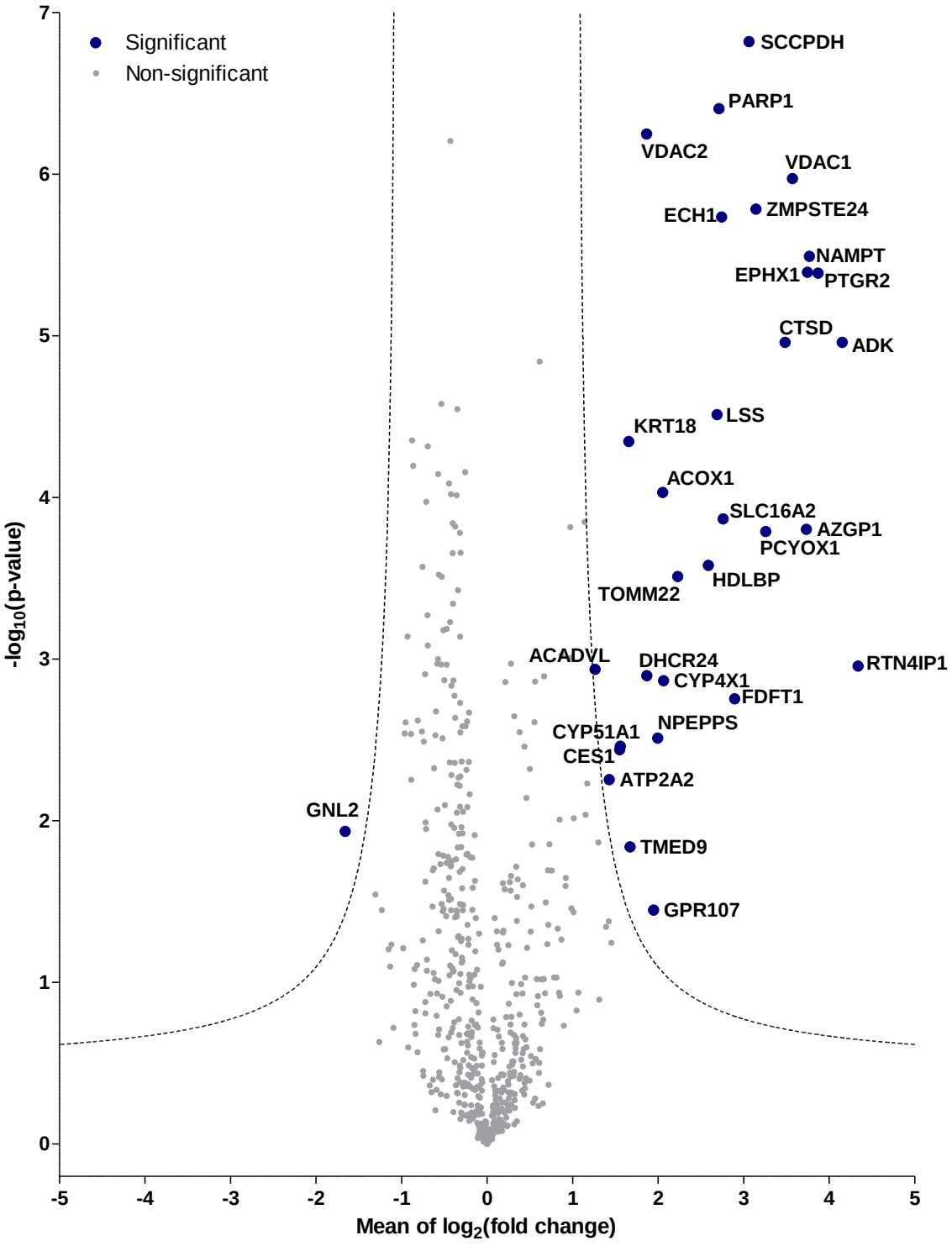


(**D**) **PARPYnD** (1 μM) *and* olaparib (5 μM) vs. **PARPYnD** (1 μM) *only* (S_0 = 0.1, FDR = 0.15).

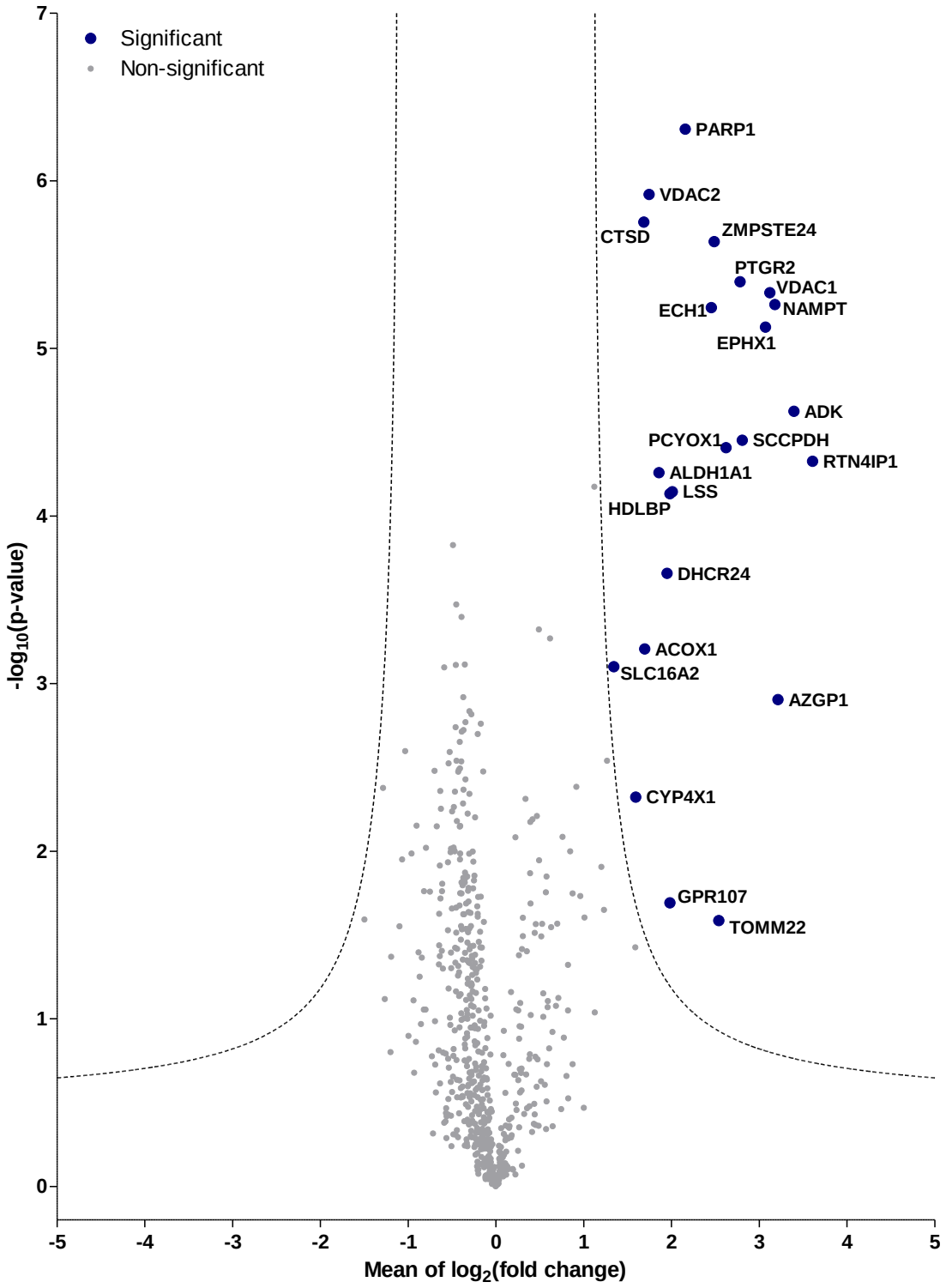


E-**G**: Complete annotated volcano plots for a separate proteomics experiment demonstrating the UV-dependence of target labelling. MDA-MB-468 cells were treated with **PARPYnD** (1 μM) or DMSO for 3 hours and either irradiated with 365 nm UV light or kept in the dark. Labelled proteins were enriched, identified and compared pairwise as previously described (**Figure 3**).

(**E**) **PARPYnD** (1 μM) +UV vs. DMSO +UV (S_0 = 1, FDR = 0.01).



(**F**) **PARPYnD** (1 μM) +UV vs. **PARPYnD** (1 μM) no UV (S_0 = 1, FDR = 0.01)



(**G**) **PARPYnD** (1 μM) no UV vs. DMSO (S_0 = 1, FDR = 0.01)

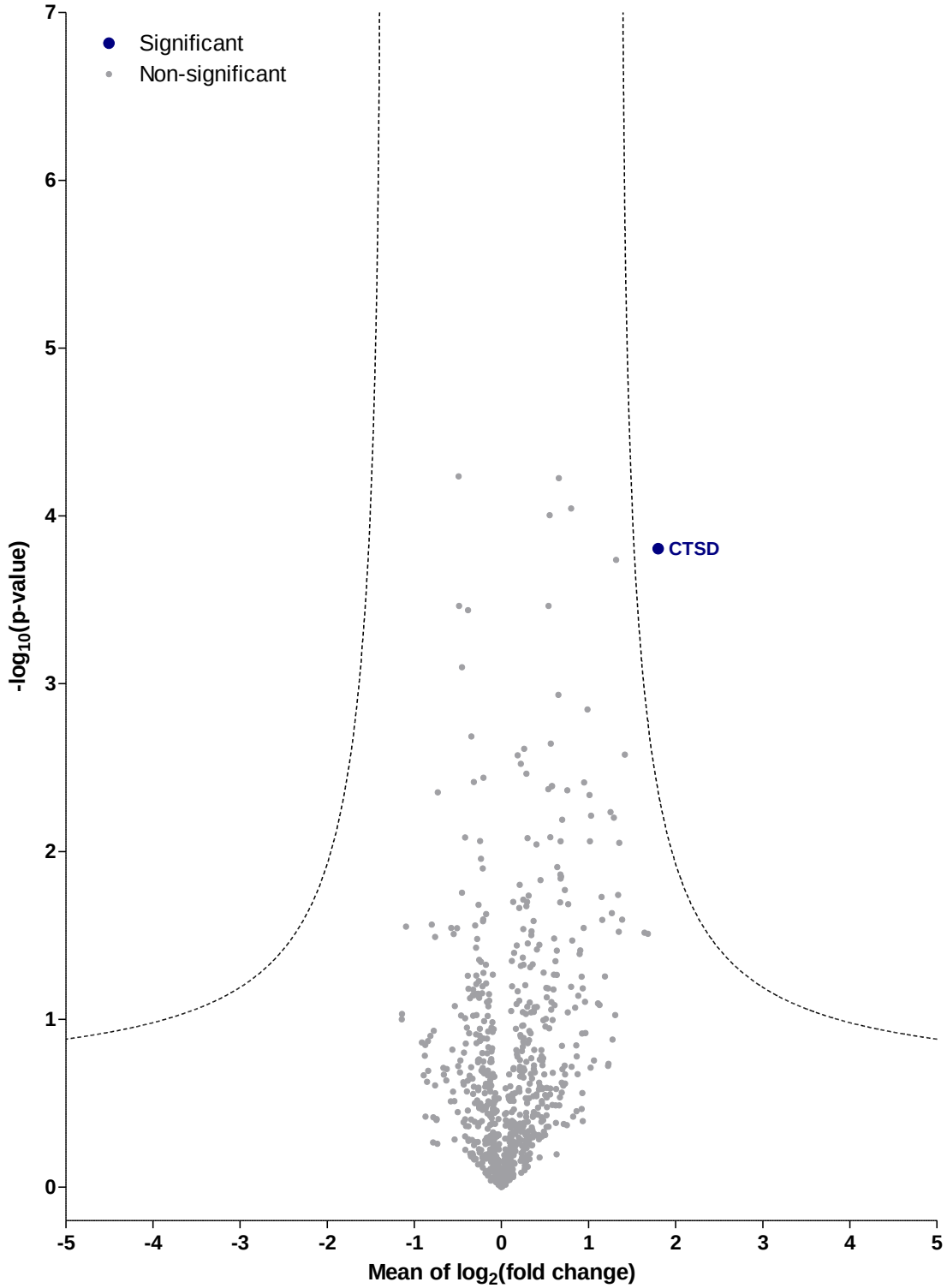


Figure S6. Crosslinking with cleavage experiment.
In-gel fluorescence of **PARPYnD** labelled lysate with PARP6 ±cleavage with PreScission™ Protease. GST tag was found to have no effect on binding and/or labelling. Cleaving the tag off after labelling (lanes 4–6) shows that the 71 kDa PARP6 portion of the protein is labelled (*). Cleaving the tag off before labelling (lanes 7–9) shows that the GST tag does not bias the recombinant protein towards compound binding.

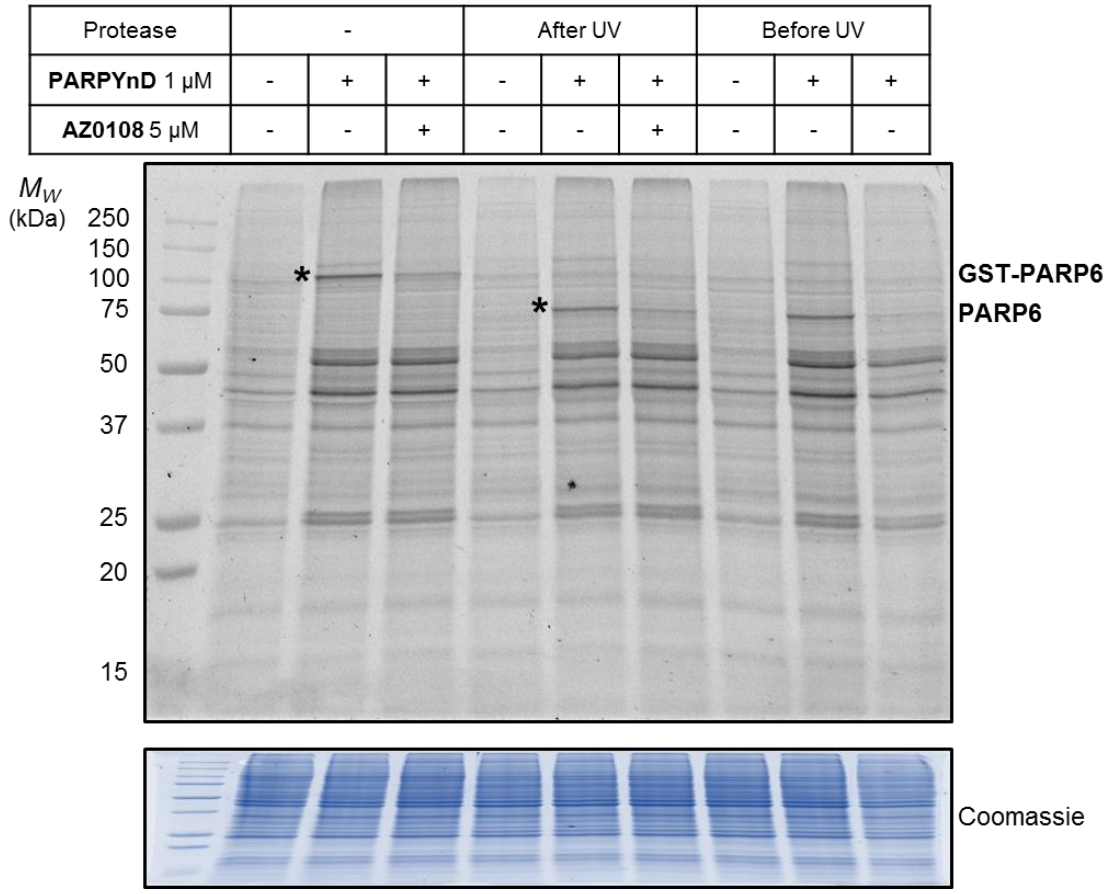


Figure S7. Uncropped gels and immunoblots.
Black boxes indicate portions of the blots taken for figures. Where there is no black box, the whole signal was cropped out and used; I = Input, P = Pull-down fraction, NA = NeutrAvidin.

Figure S2C

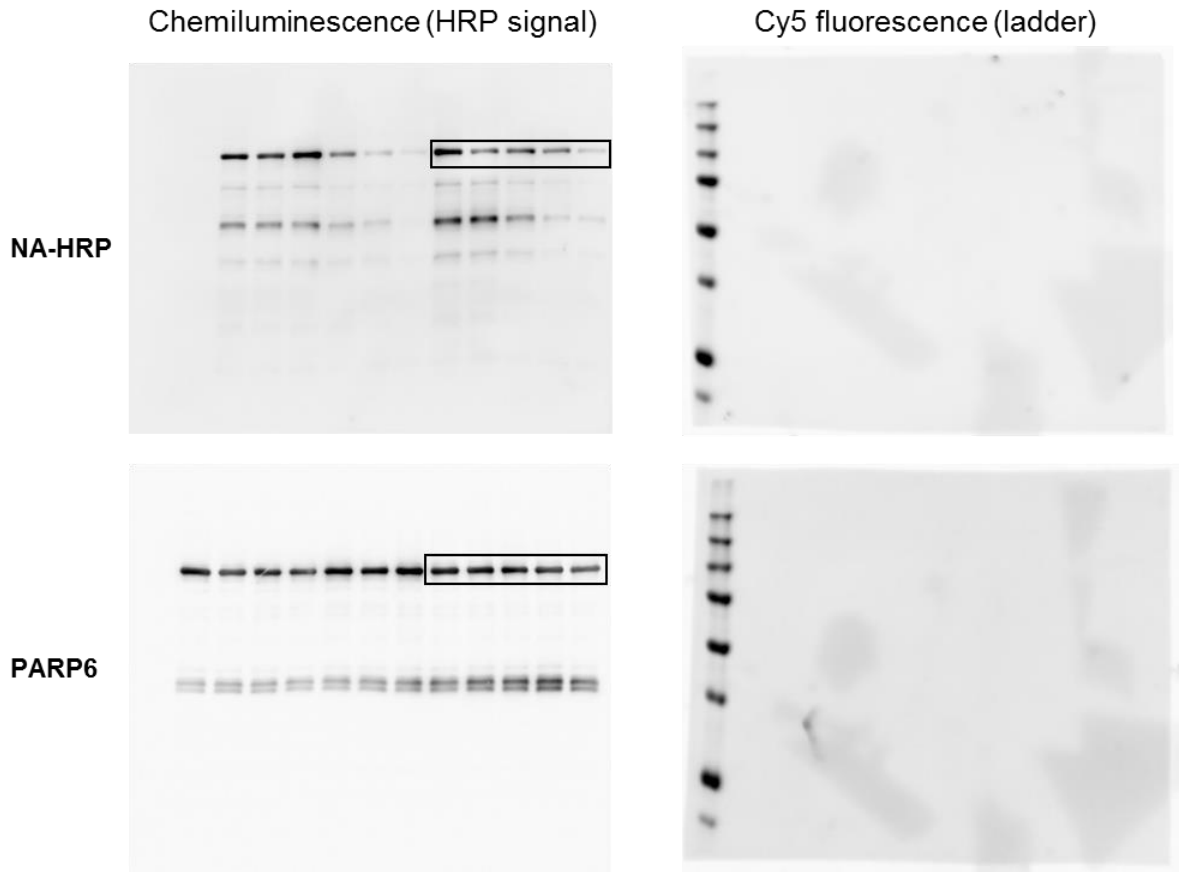
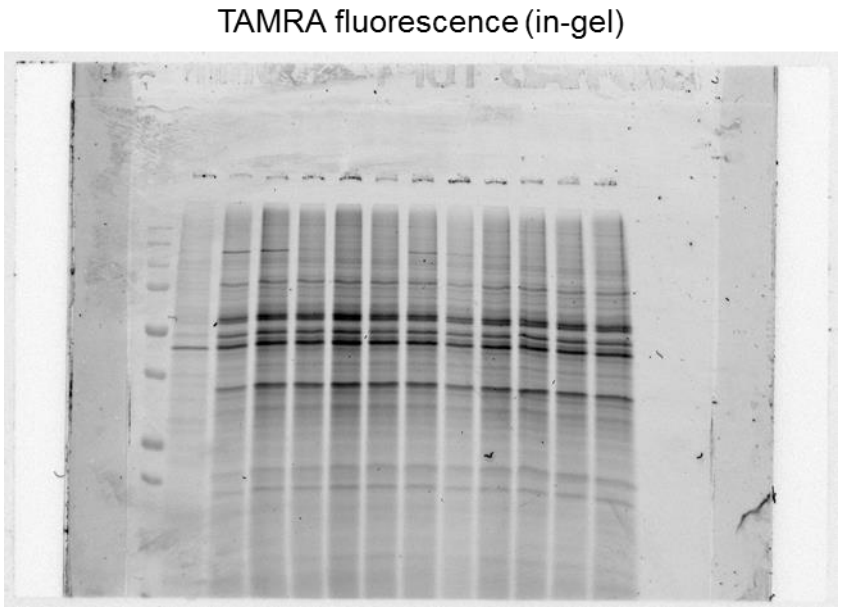


Figure S2D



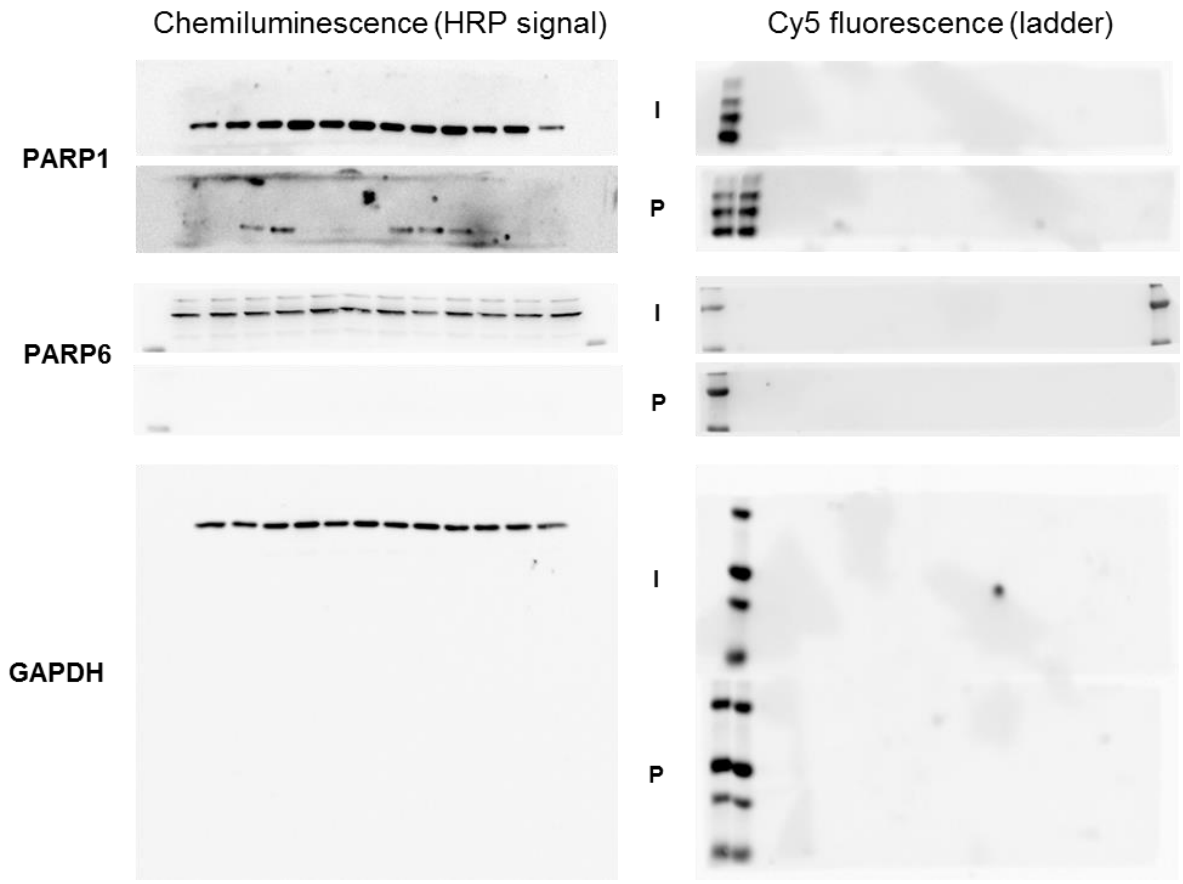

Chemiluminescence (HRP signal)
Cy5 fluorescence (ladder)
PARP1
I
P
PARP6
I
P
GAPDH
I
P

Figure 3E

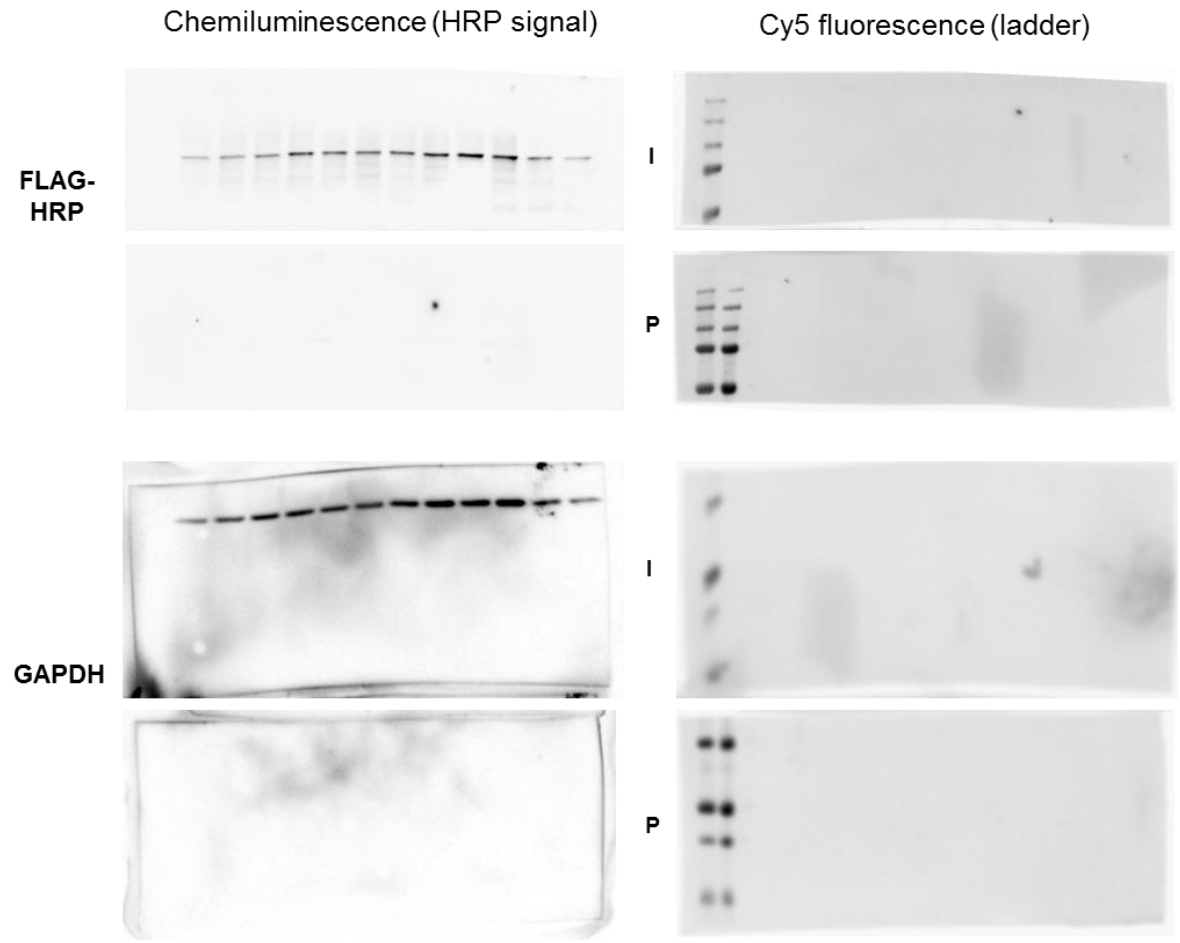
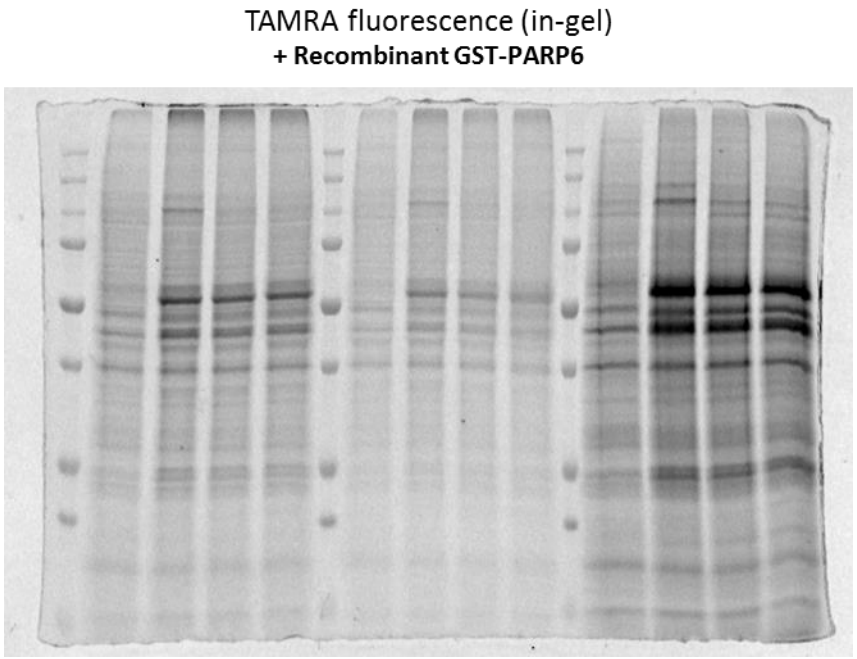
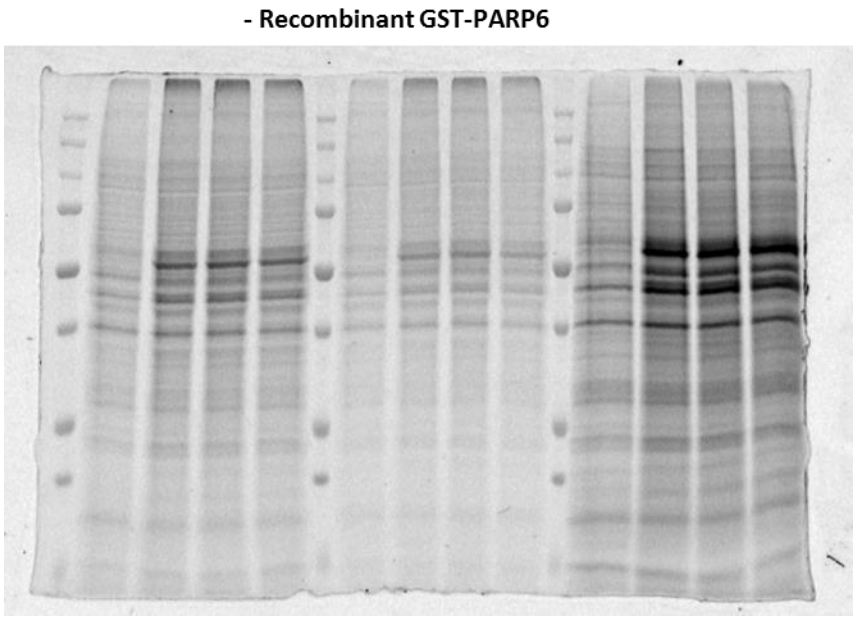


Figure 4




- Recombinant GST-PARP6

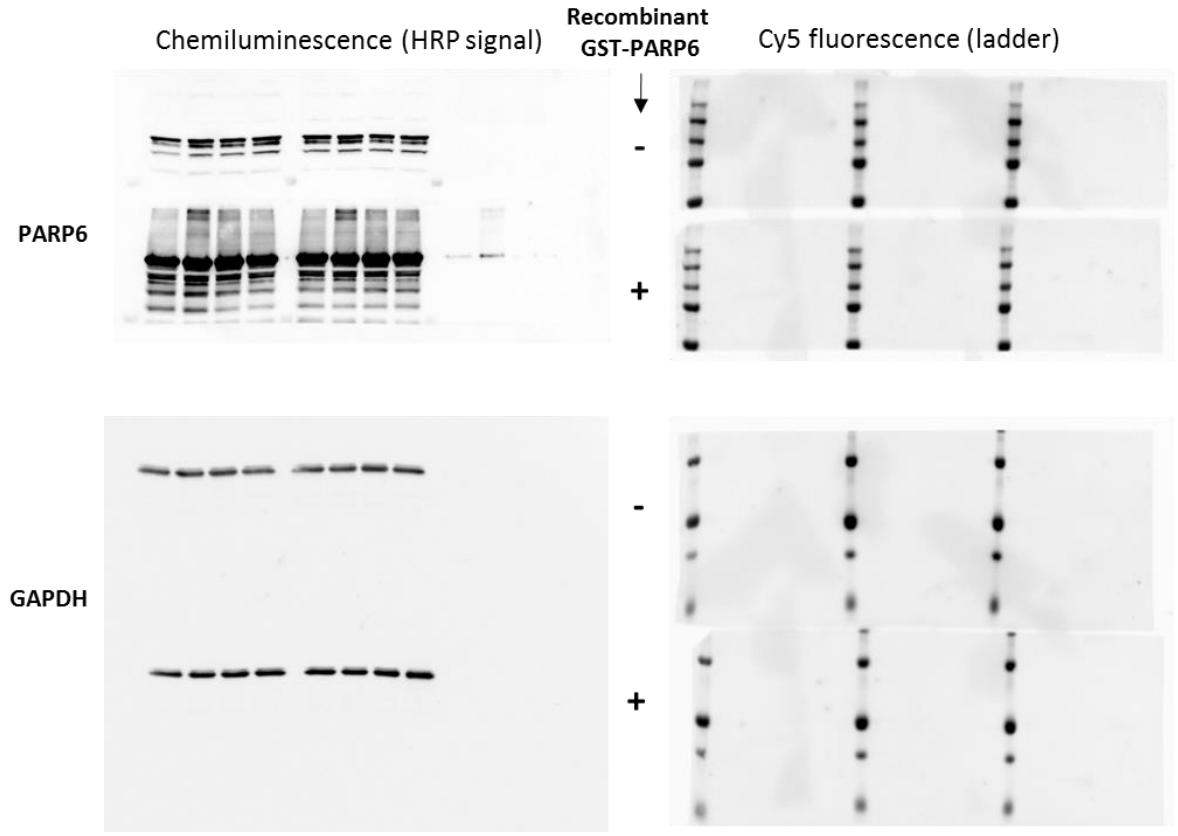

Chemiluminescence (HRP signal)
Recombinant GST-PARP6
Cy5 fluorescence (ladder)
PARP6
GAPDH
-
+
-
+

Figure S4

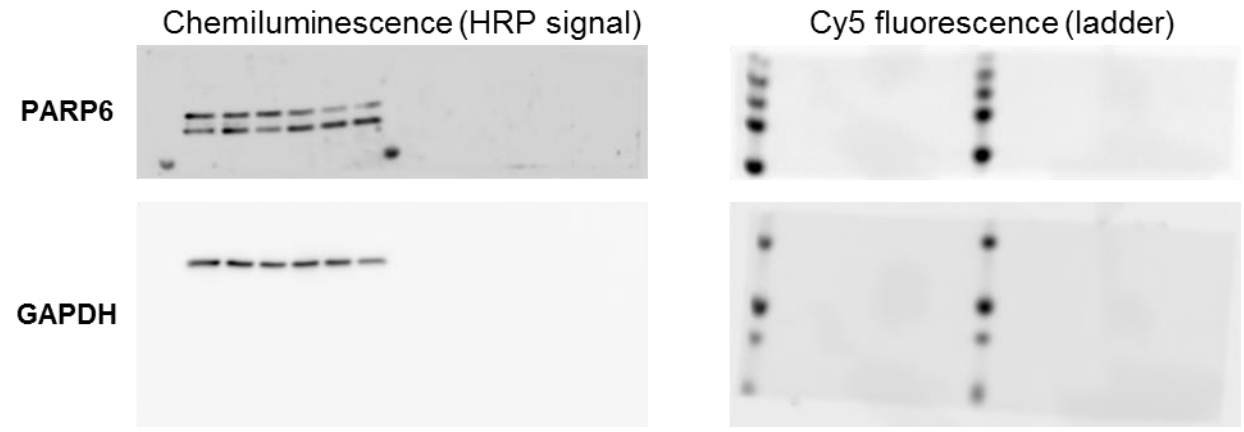
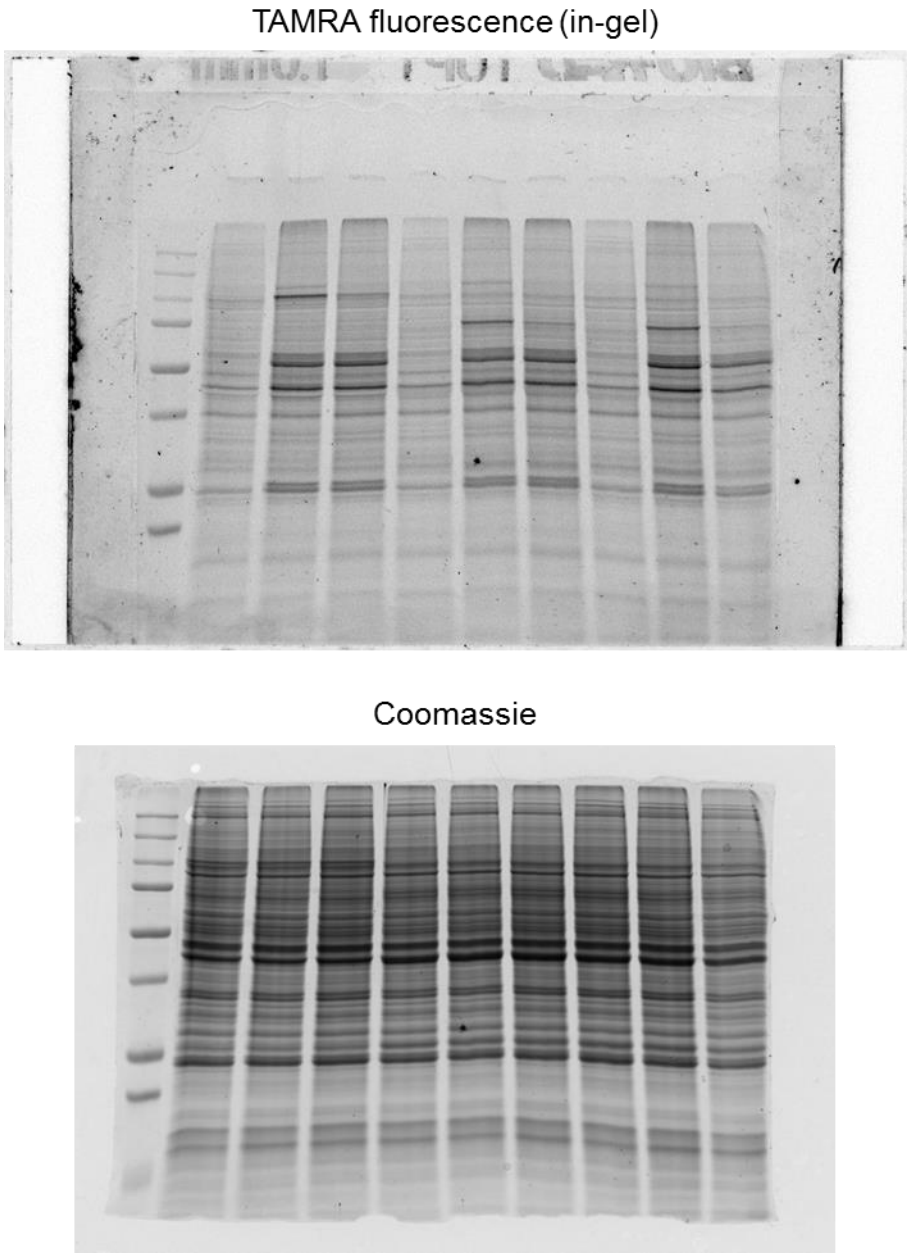


Figure S6



Supporting references

(1) Broncel, M., Serwa, R. A., Ciepla, P., Krause, E., Dallman, M. J., Magee, A. I., and Tate, E. W. (2015) Multifunctional reagents for quantitative proteome-wide analysis of protein modification in human cells and dynamic profiling of protein lipidation during vertebrate development. *Angew. Chem. Int. Ed. Engl. 54*, 5948–51.

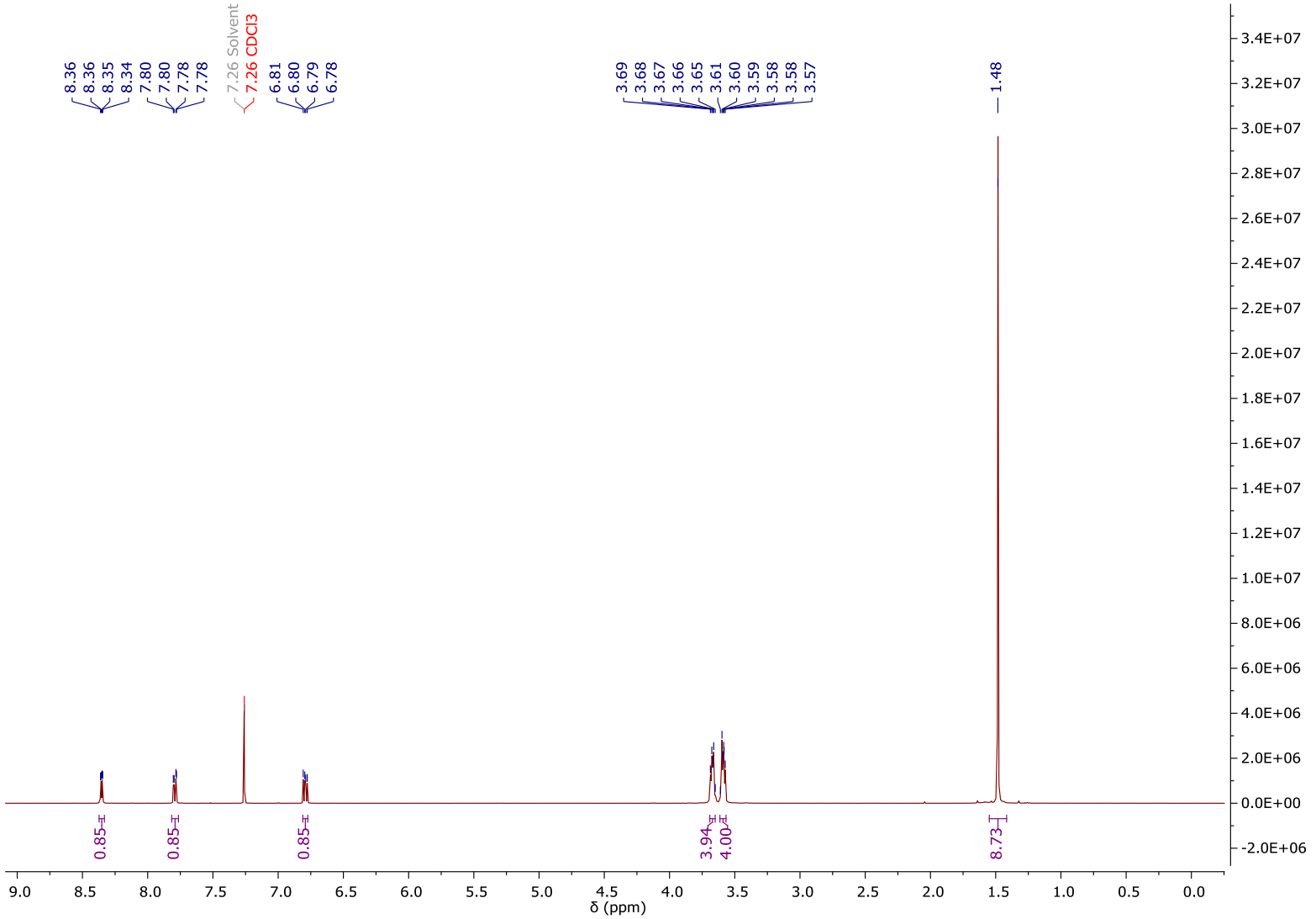
(2) Parker, C. G., Galmozzi, A., Wang, Y., Correia, B. E., Sasaki, K., Joslyn, C. M., Kim, A. S., Cavallaro, C. L., Lawrence, R. M., Johnson, S. R., Narvaiza, I., Saez, E., and Cravatt, B. F. (2017) Ligand and Target Discovery by Fragment-Based Screening in Human Cells. *Cell 168*, 527-541.e29.

(3) Wang, Z., Grosskurth, S. E., Cheung, T., Petteruti, P., Zhang, J., Wang, X., Wang, W., Gharahdaghi, F., Wu, J., Su, N., Howard, R. T., Mayo, M., Widzowski, D., Scott, D. A., Johannes, J. W., Lamb, M. L., Lawson, D., Dry, J. R., Lyne, P. D., Tate, E. W., Zinda, M., Mikule, K., Fawell, S. E., Reimer, C., and Chen, H. (2018) Pharmacological Inhibition of PARP6 Triggers Multipolar Spindle Formation and Elicits Therapeutic Effects in Breast Cancer. *Cancer Res. 78*, 6691–6702.

(4) Hutin, D., Grimaldi, G., and Matthews, J. (2018) Methods to Study TCDD-Inducible Poly-ADP-Ribose Polymerase (TIPARP) Mono-ADP-Ribosyltransferase Activity, in *ADP-ribosylation and NAD+ Utilising Enzymes*, pp 109–124. Humana, New York, NY.

NMR Spectra

Tert-butyl 4-(3-cyanopyridin-2-yl)piperazine-1-carboxylate **2**



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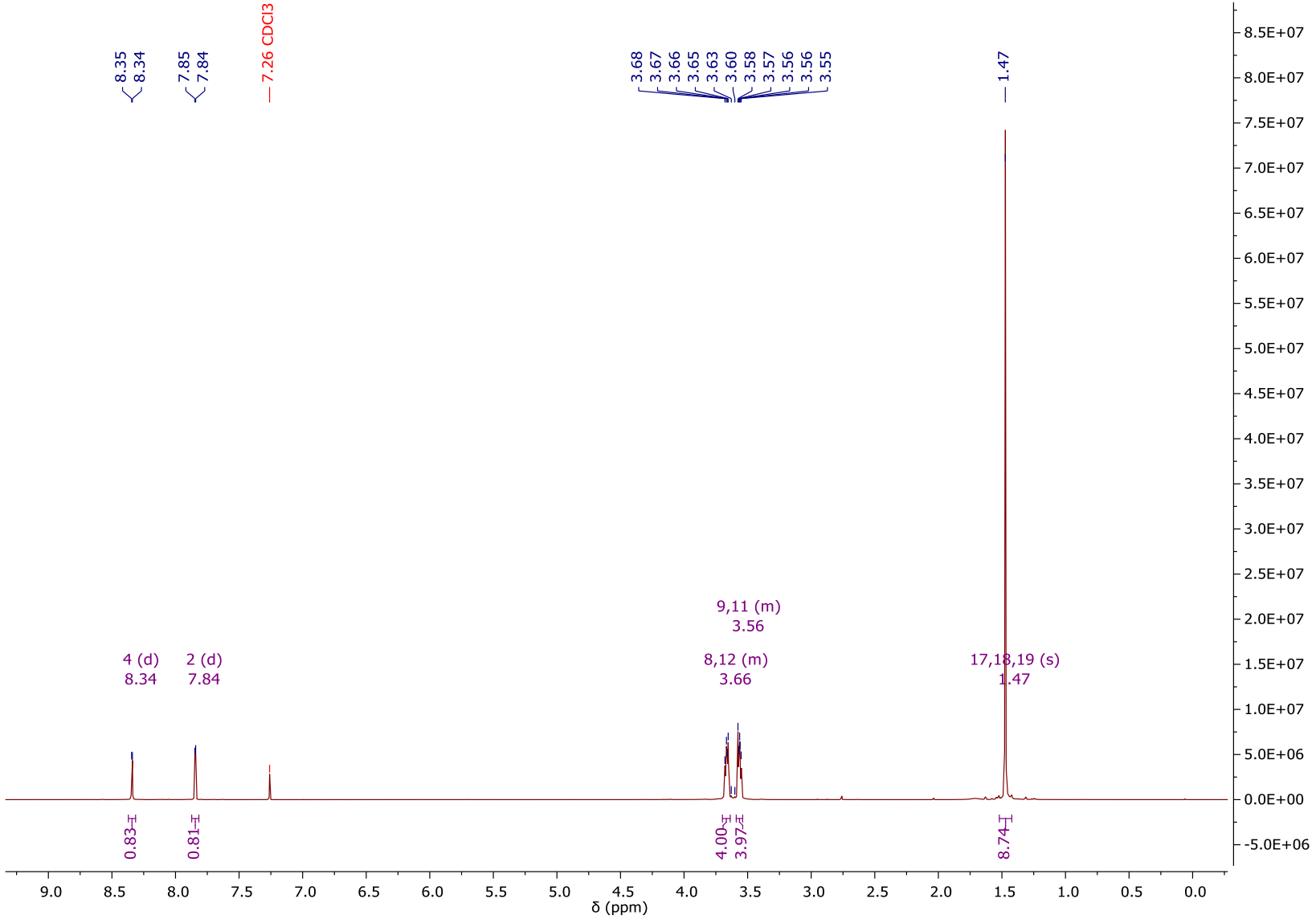

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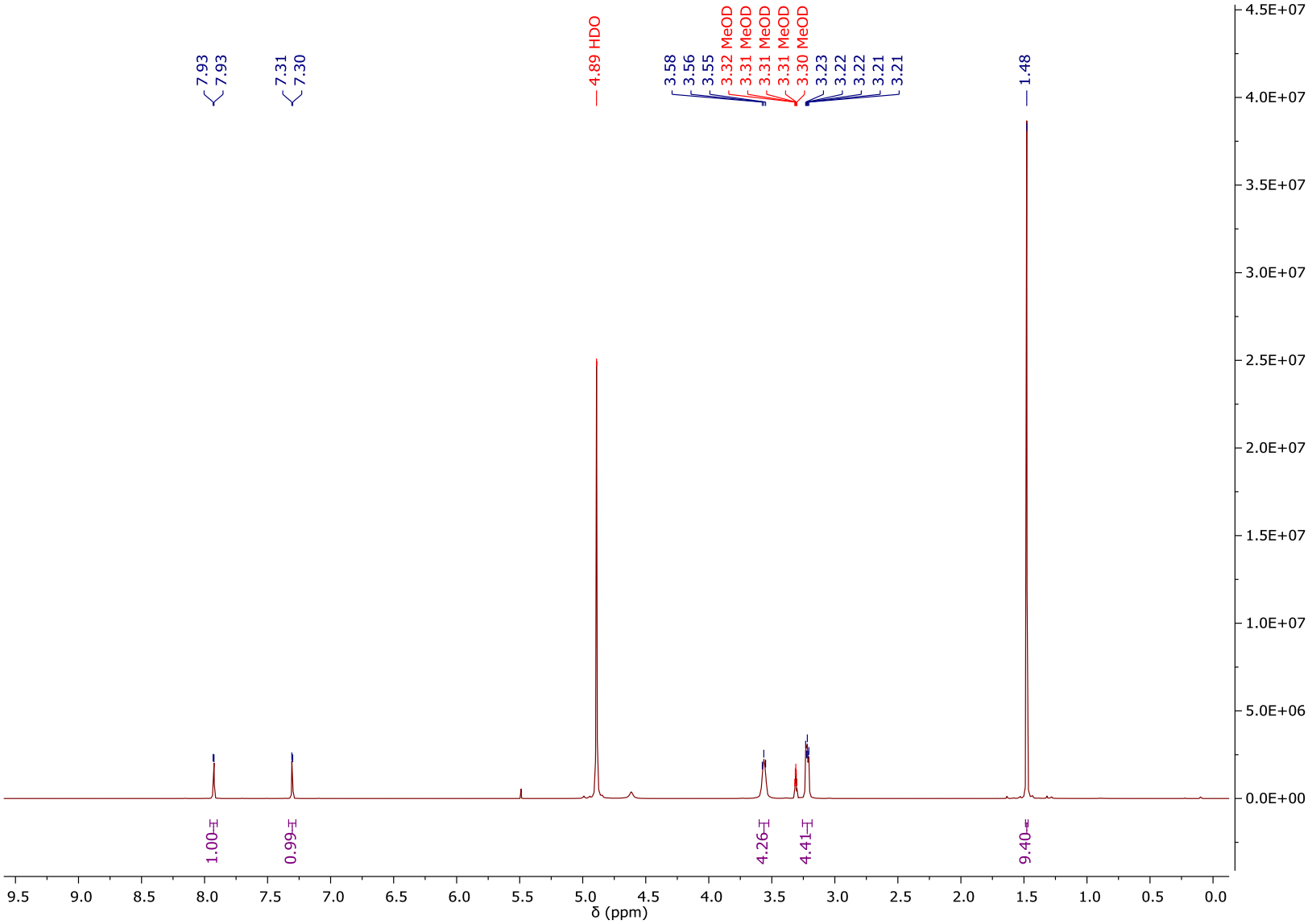
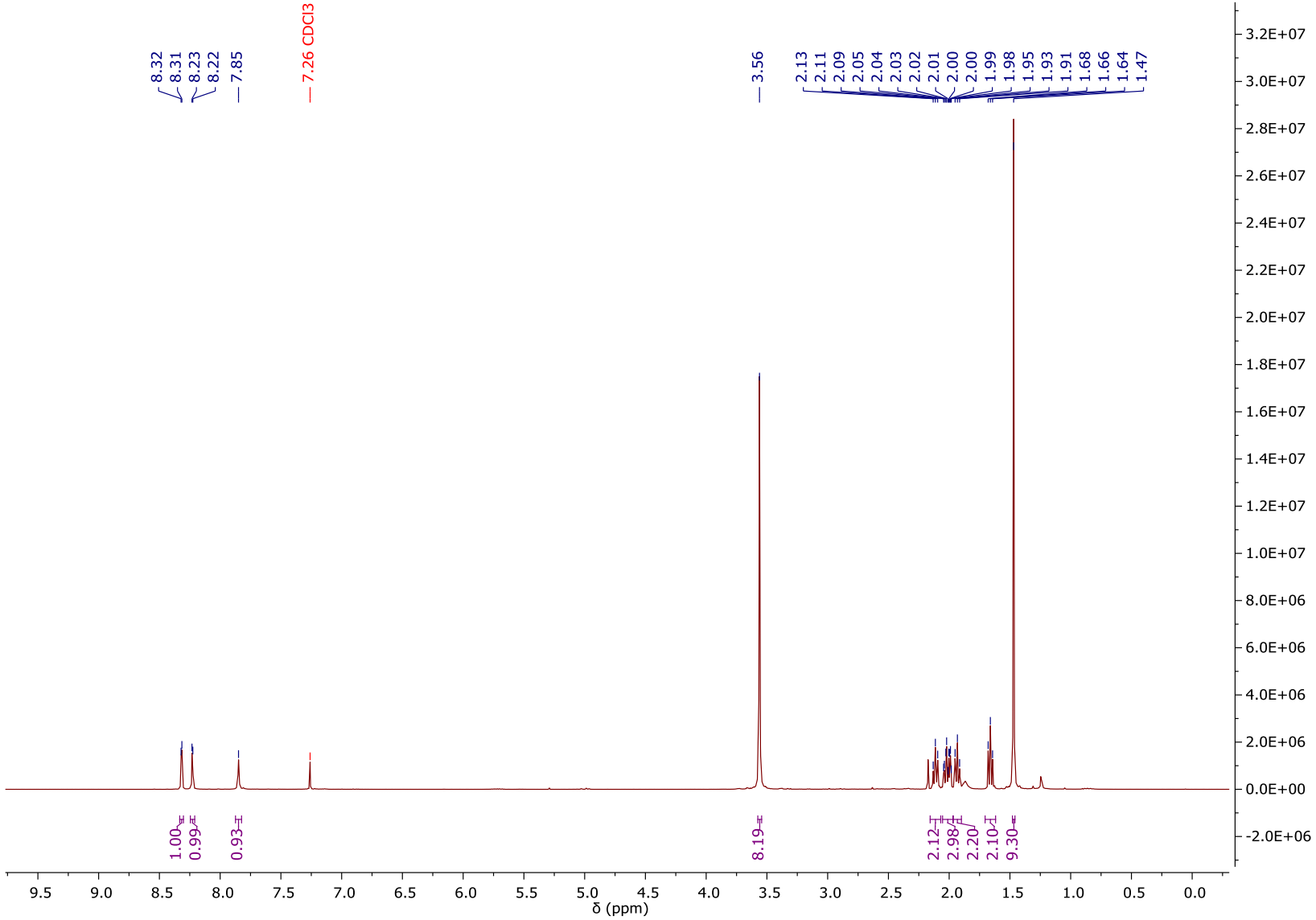
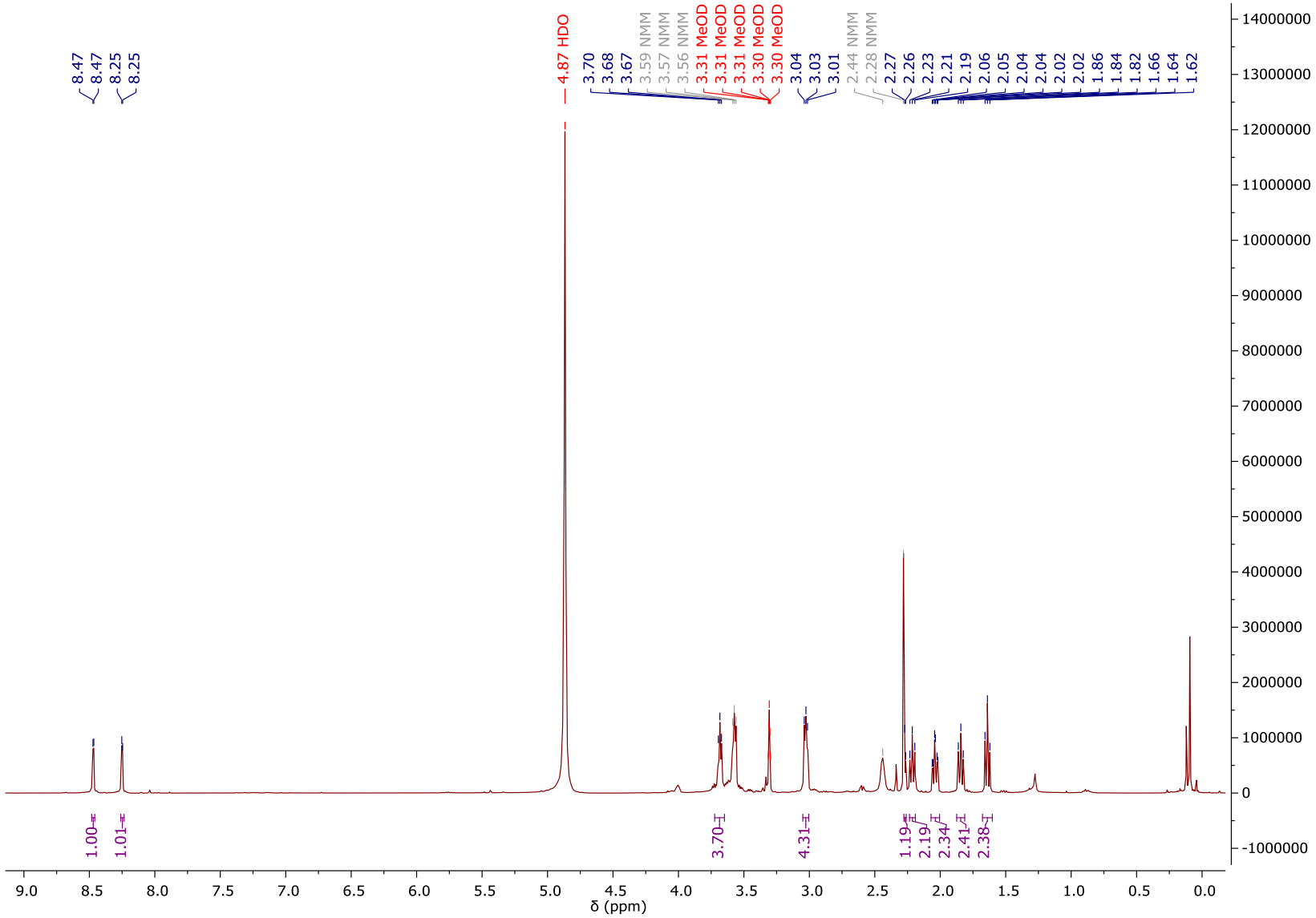


Tert-butyl 4-(5-(3-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)propanamido)-3-cyanopyridin-2-yl)piperazine-1-carboxylate **6**



3-(3-(But-3-yn-1-yl)-3*H*-diazirin-3-yl)-*N*-(5-cyano-6-(piperazin-1-yl)pyridin-3-yl)propanamide **7**



3-(3-(But-3-yn-1-yl)-3*H*-diazirin-3-yl)-*N*-(5-cyano-6-(4-(3-((4-oxo-3,4-dihydrophthalazin-1-yl)methyl)benzoyl)piperazin-1-yl)pyridin-3-yl)propanamide (**PARPYnD**)