Electronic Supplementary Material (ESI) for Chemical Science.

Structure-guided design and characterization of a clickable, covalent PARP16 inhibitor

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURES

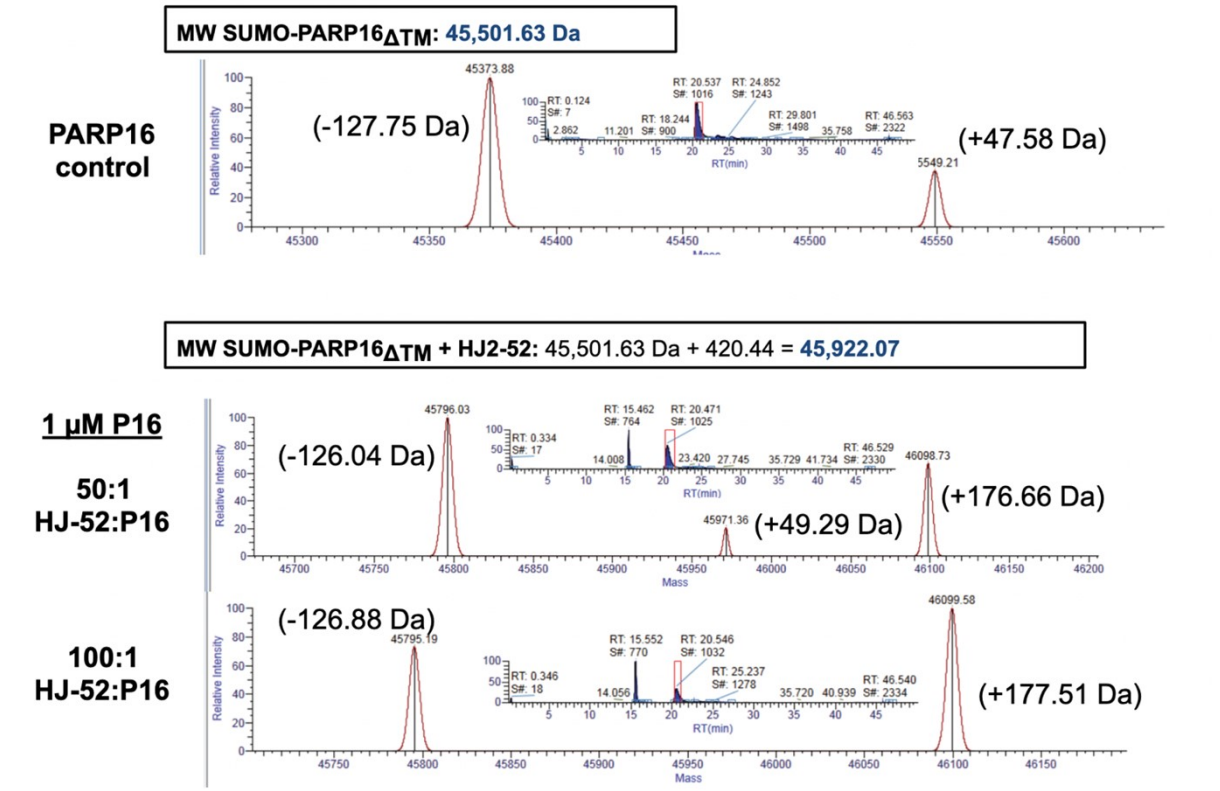


Figure S1. Covalent labeling of purified SUMO-PARP16$_{\Delta TM}$ with 50- or 100-fold excess HJ-52 determined by whole protein electrospray ionization (ESI) mass spectrometry. PARP16 was incubated with HJ-52 for 18 h, then the protein sample was injected onto a protein micro trap cartridge (Optimize Technologies, Oregon City, OR) at a flow rate of 20 µl/min in mobile phase containing 0.1% formic acid. After 5 min the flow diverted to a 1 x 250 mm C4 column (Vydac, SN 214MS51, Grace, Deerfield, IL). Protein was then eluted by increasing the acetonitrile concentration from 2-7.5% over one min, then 7.5-60% over 30 min and data collection on the mass spectrometer started 10 min into the separation. Masses of eluted protein were determined by on-line electrospray ionization using an LTQ Velos linear ion trap (Thermo Scientific, San Jose, CA) fitted with a HESI-II probed fitted with a 34 gauge metal needle, using a 5.8 kV source voltage, 325 °C ion transfer tube temperature and sheath gas setting of 15. The instrument acquired full MS scans in profile mode over a range of m/z = 350-2000 while averaging 10 µscans. Spectra acquired during elution of protein peaks were then further averaged and deconvoluted using the Manual Respect module for isotopically unresolved data in Protein Deconvolution 4.0 software (Thermo Scientific).

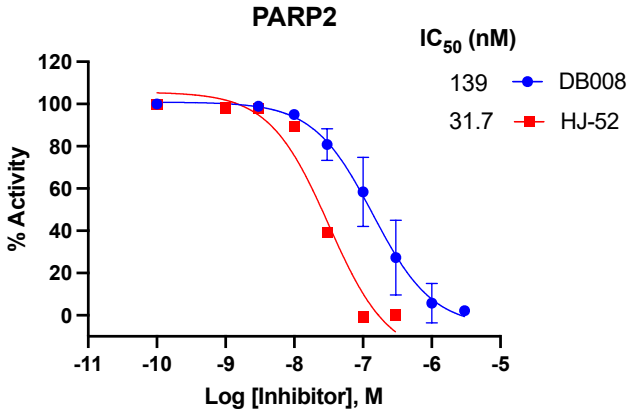


Figure S2. Inhibition of PARP2 is decreased with DB008 compared to HJ-52. PARP2 inhibtion was assayed using our PASTA assay. The addition of the ethynyl group decreases the potency for DB008 against PARP2 by ~4-fold, compared to HJ-52.

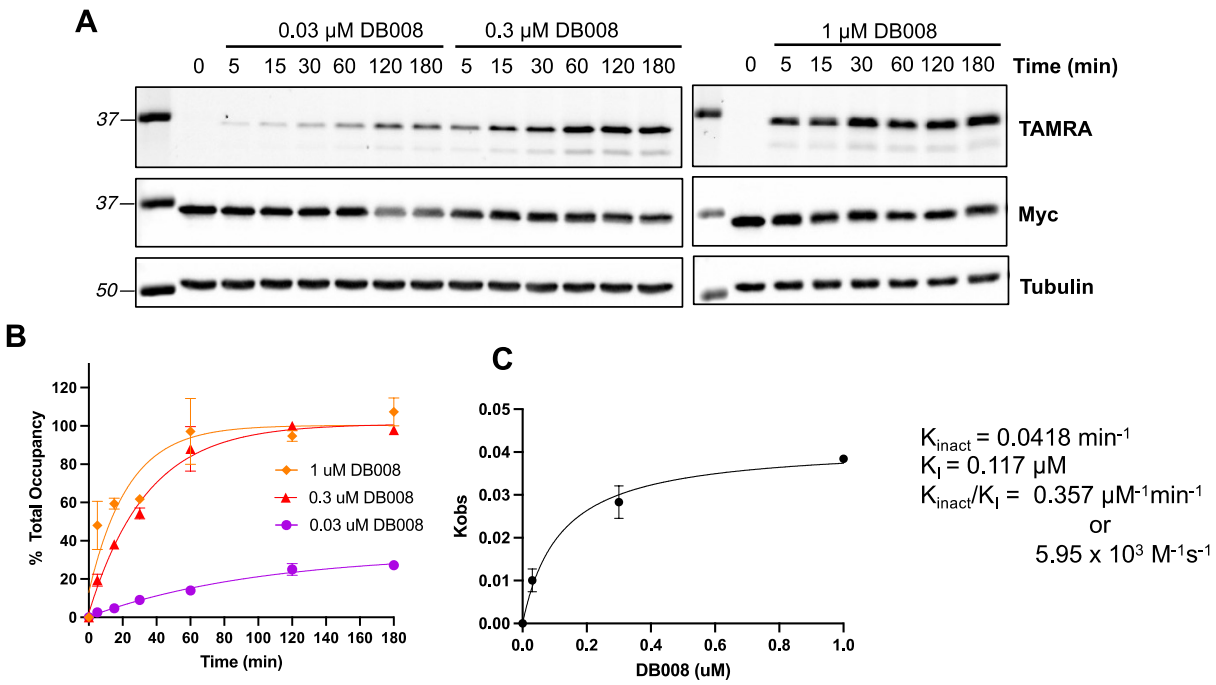


Figure S3. Determining efficiency of covalent bond formation (K_{inact}/K_I) for DB008. (**A**) HEK 293T cells transiently expressing Myc2x-PARP16 were dosed with DB008 at inidated concentrations and time points, followed by lysis and clicking to TAMRA-azide for in-gel fluorescence detection of PARP16 labeling. (**B**) Quantification of labeling in (**A**), n = 2 biological replicates. (**C**) Calculation of K_{inact}/K_I using K_{obs} calculated from (**B**).

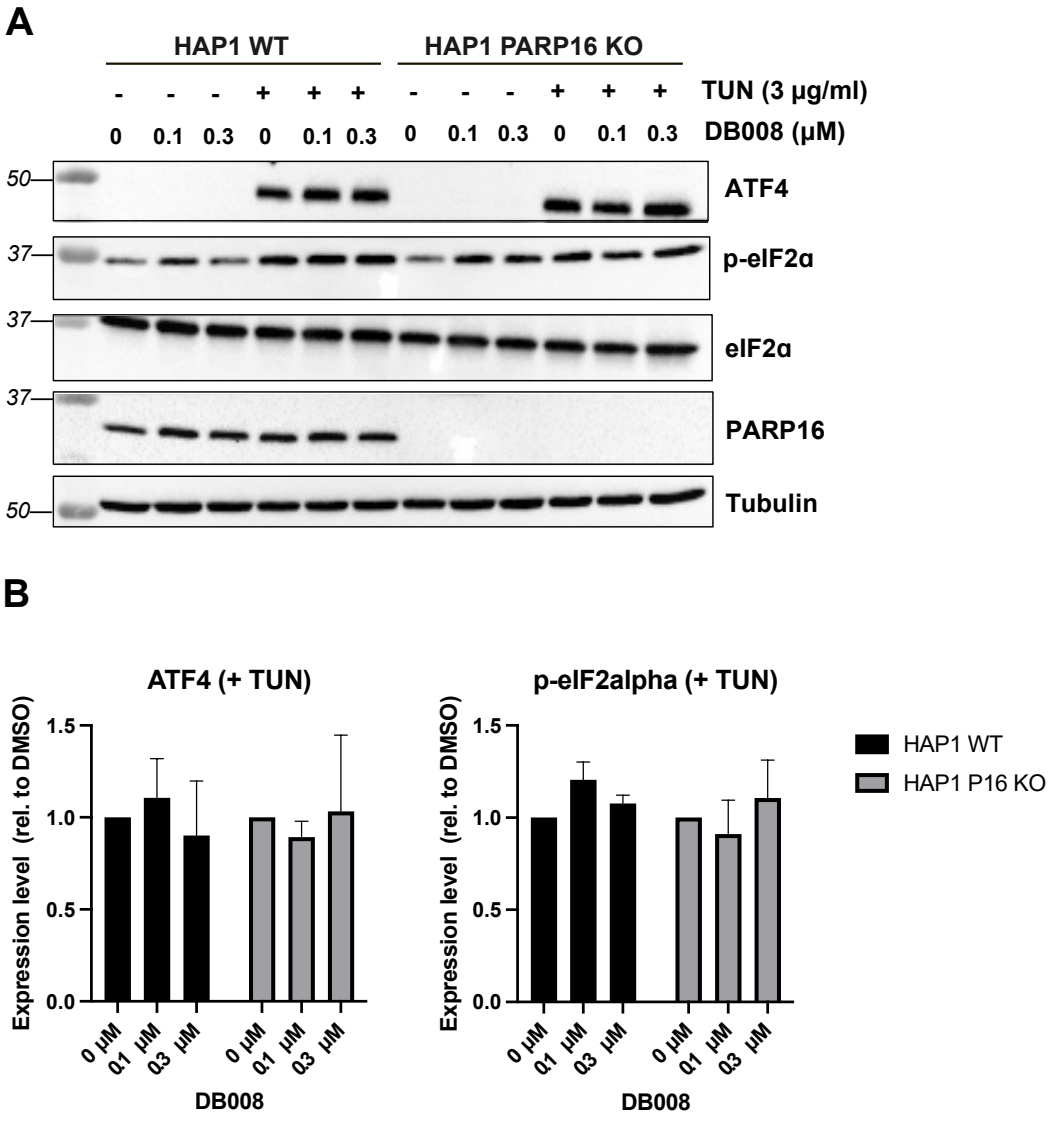


Figure S4. Inhibtion or KO of PARP16 does not impact tunicamycin-induced unfolded protein response in HAP1 cells. (**A**) HAP1 WT and HAP1 PARP16 KO cells were co-treated with tunicamycin (3 µg/ml) and a dose-response of DB008 for 4 hours. (**B**) Quantification of ATF4 and phosphorylated eIF2α levels from n = 3 biological replicates.

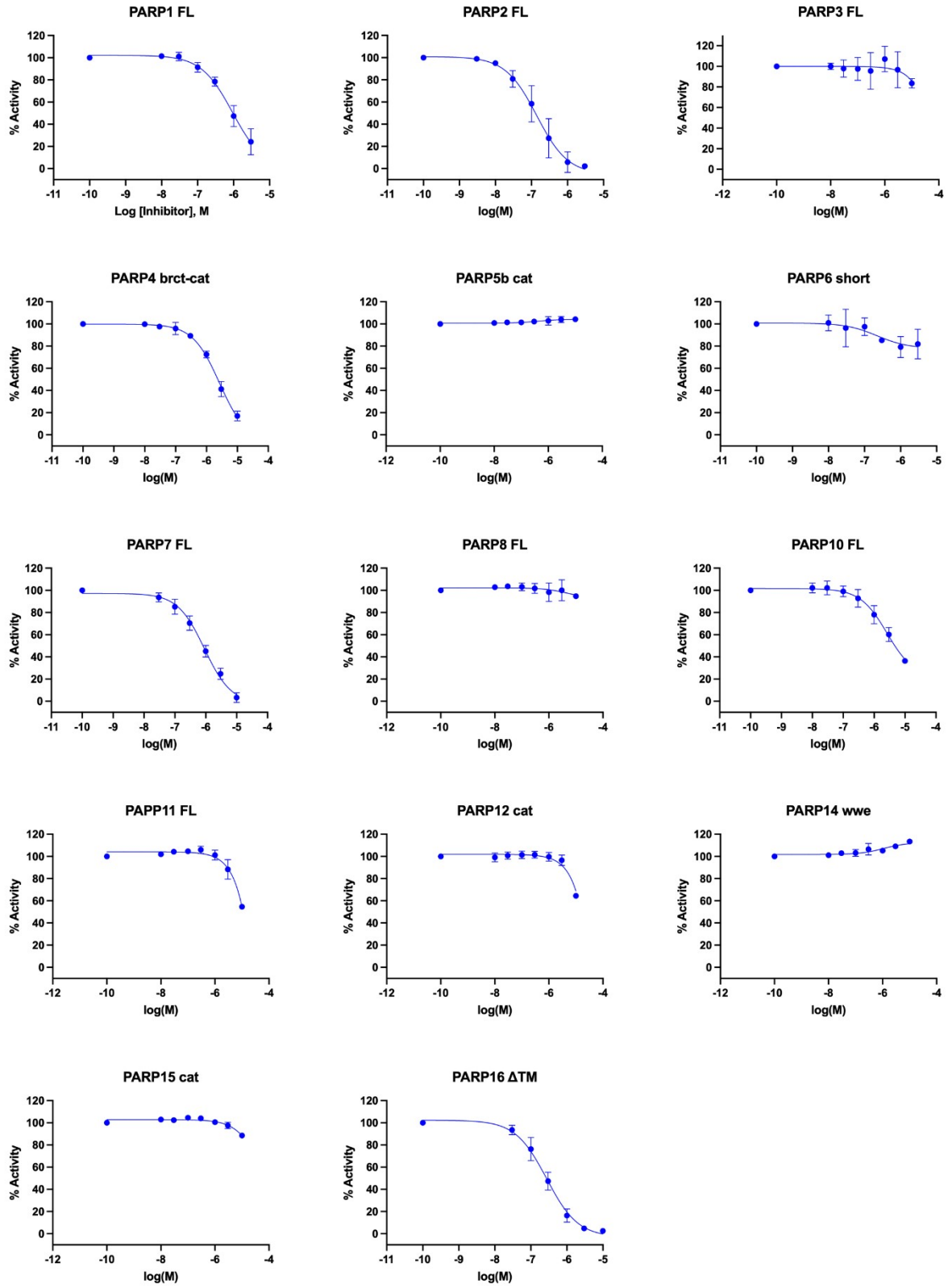


Figure S5. DB008 PASTA screen against PARP family members. IC_{50} values for each curve is reported in **Fig 2A**.

SUPPLEMENTARY METHODS

General synthetic methods

Glassware was oven dried prior to use for air and water sensitive reactions. Reactions were performed under argon. Tetrahydrofuran (THF), N,N-dimethylformamide (DMF), and dichloromethane (CH_2Cl_2) were dried using a solvent purification system manufactured by Glass Contour, Inc. (Laguna Beach, CA). All other solvents were of ACS chemical grade (Fisher Scientific) and used without further purification unless otherwise stated. Commercially available starting reagents were used without further purification. Thin-layer chromatography was carried out with 250 μm glass-backed silica (XHL) plates. Detection of compounds was achieved by UV absorption (254 nm) and by staining with cerium-ammonium-molybdate (CAM) or ninhydrin solution. Flash column chromatography was conducted using self-packed columns containing 200-400 mesh silica gel (SiliCycle) on a Teledyne ISCO Combiflash Rf 150. Microwave reactions were performed using a Biotage Initiator+ SP Wave Microwave Reactor.

NMR spectra were recorded on a Bruker DPX spectrometer at 400 MHz. Chemical shifts (δ) are reported as parts per million (ppm) downfield from an internal tetramethylsilane standard or solvent references. Multiplicities are abbreviated as: s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet.

High-resolution (35,000) mass spectrometry was acquired on a vanquish UHPLC/HPLC system coupled to a Q-Exactive MS equipped with an electrospray ionization source (ESI) at the BioAnalytical Mass Spectra Facility of Portland State University.

Cell culture

All cells were cultured at 37°C and 5% CO_2. HEK 293T cells were cultured in DMEM (Gibco, 11965118) with 10% FBS and 1X GlutaMAX (Gibco), and HAP1 cells were cultured in IMDM (Gibco,12440053) with 10% FBS.

Antibodies and compounds

The following antibodies were used from Cell Signaling Technology: Poly/Mono-ADP Ribose rabbit monoclonal antibody (E6F6A, 1:1000), PARP1 (46D11, 1:1000), Tubulin (DM1A, 1:2000), Myc-Tag (9B11, 1:1000), phosph-eIF2α (1:1000, D9G8), eIF2α (1:1000, D7D3), ATF4 (1:1000, D4B8), p62 (1:1000, #5114), and ubiquitin (1:1000, P4D1). The remaining antibodies used are PARP16 (1:8000, GeneTex, GTX123450) and puromycin (1:2000, Millipore Sigma, MABE343).

Compounds used in this paper include olaparib (Selleck Chemical), PDD00017273 (Sigma-Aldrich), puromycin dihydrochloride (Sigma-Aldrich), talazoparib (Cayman), EGCG (Thermo Scientific), MG132 (Selleck Chemical), bafilomycin-A1 (Cayman), and chloroquine (MP biomedicals).

PARP activity screening and inhibitor testing assay (PASTA)

PARPs and SRPK2, containing an N-terminal His-tag and a SUMO-tag were expressed and purified as previously reported[1]. Quantification of protein concentration was done using an in-gel

standard curve of Bovine Serum Albumin (Bio-Rad) and image processing using ImageLab (Bio-Rad).

PARPs 1, 2, $5b_{cat}$, 6, 8, 10, 11, 12_{cat}, 14_{wwe}, and 15_{cat} were assayed in a trans-modification format as previously described[2]. Briefly, 50 µL of 1 µM SPRK2 solution in PARP Buffer for Reaction (PBR) (50 mM HEPES pH 7.5, 100 mM NaCl, 4 mM MgCl2, 0.2 mM TCEP) was added to a nickel-NTA coated 96-well plate (Pierce) for 1 hr at room temperature (RT). The plate was washed with PBST (PBS, 0.1% Tween-20) (3 x 5 min), PBS (1 x 5 min), and PBR (1 x 5 min). Inhibitors at varying concentrations were pre-incubated with 20 µM (2X) 6-alkyne-NAD^+ and then added to plate, followed by equal volume addition of 2X PARP to initiate ADP-ribosylation (50 µL final volume, final concentration; PARP1,2 = 10 nM, PARP5b = 300 nM, PARP6 = 200 nM, PARP8 = 200 nM, PARP10 = 1 nM, PARP11 = 10 nM, $PARP12_{cat}$ = 150 nM, $PARP14_{wwe}$ = 10 nM, $PARP15_{cat}$ = 25 nM). For PARP1 and PARP2, 0.1 mg/mL of activated DNA (Sigma) was supplemented to PBR. The reaction mixture was incubated at 30ºC for 1 hr, then the plate was washed with PBST (3 x 5 min), and PBS (1 x 5 min). A click reaction was performed next by incubation with 50 µL of click buffer containing 100 µM biotin-PEG4-azide (Click Chemistry Tools), 1 mM $CuSO_4$, 100 µM TBTA, and 2 mM TCEP, all in 1X PBS. The plate was then washed with PBST (3 x 5 min), blocked with 1% milk (Carnation) in PBST for 30 min at RT, followed by washing with PBST (3 x 5 min) and PBS (1 x 5 min) before incubation with Strep-HRP (0.05 ng/µL Strep-HRP (Fisher Scientific), 300ng/µL BSA, 1X PBS) for 30 min at RT. The plate was washed with PBST (3 x 5 min), PBS (1 x 5 min), and then developed with QuantaRed Enhanced Chemifluorescent HRP Substrate (Thermo Scientific) according to manufacture protocols. Fluorescence was immediately measured at excitation/emission of 570/600 nm using a Spectra Max i3 (Molecular Devices) and inhibitor dose-response curves were fitted using three parameter nonlinear regression in Prism9 (GraphPad) to calculate IC_{50} values.

PARPs 3, $4_{brct-cat}$, 7, and $16_{\Delta TM}$ were assayed as described above with the following modifications. PARP3, 4, and 16 in PBR (final concentration; PARP3 = 1000 nM, $PARP4_{brct-cat}$ = 600 nM, $PARP16_{\Delta TM}$ = 1000 nM) were directly added to a nickel-NTA plate (50 µL final volume) and incubated for 1 hr at RT. GST-PARP7, expressed and purified as previously described[3], was diluted in PBR (200 nM final concentration) and 50 µL was directly added to a glutathione coated 96-well plate (Pierce) and incubated for 1 hr at RT. The plate was washed with PBST (3 x 5 min), PBS (1 x 5 min), and PBR (1 x 5 min), and then incubated with varying concentrations of inhibitor and native NAD^+(MilliporeSigma) at a concentration of 100 µM for PARPs 3, $4_{brct-cat}$, 7 or 400 µM for $PARP16_{\Delta TM}$. For PARP16, DB008 was pre-incubated at 30ºC for 30 min before addition of NAD^+. The reaction mixture was incubated at 30ºC for 1 hr, then washed with PBST (3 x 5 min) and blocked with 5% Milk (Carnation) in PBST for 30 min at RT. After washing with PBST (3 x 5 min), the plate was incubated with a Poly/Mono-ADP Ribose rabbit monoclonal antibody (Cell Signaling Technology) diluted 1:25,000 (for PARP7 and $PARP16_{\Delta TM}$) or 1:5000 (for PARP3 and $PARP4_{brct-cat}$) in PBST containing 2% BSA and 0.05% NaN_3 for 30 min at RT. The plate was washed with PBST (3 x 5 min) before incubation with Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch) at 1:25,000 (in 5% milk in PBST) for 30 min at RT. Finally, the plate was washed with PBST (3 x 5 min), PBS (1 x 5 min), and developed as described above.

Cellular PARylation assay

HEK 293T cells at 75% confluency were treated with a dose-response of DB008, olaparib (Selleck Chemicals), or HJ-52 for 30 minutes, followed by treatment with 1 µM PARG inhibitor

PDD00017273 (Sigma-Aldrich) for 15 minutes. Cells were washed once with cold PBS and the plate was frozen in -80ºC prior to lysis. Cells were lysed in cytosolic lysis buffer (CLB: 50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100) containing freshly added 0.1 mM TCEP, 1X cOmplete EDTA-free Protease Inhibitor Cocktail (Roche), 30 µM Phthal 01 (pan-PARP inhibitor)[4], and 10 µM PDD00017273. Lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4ºC and the supernatant was transferred into a fresh tube. Total protein was quantified with a Bradford assay (Bio-Rad), normalized, and sample buffer was added to 1X (10% glycerol, 50mM Tris-Cl (pH 6.8), 2% SDS, 1% β-mercaptoethanol, 0.02% bromophenol blue). Samples were boiled at 95ºC for 5 min and resolved in a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-Rad), blocked in 5% milk (Carnation) in PBST, and probed overnight at 4ºC with primary antibodies. The blots were washed with PBST (3 x 10 min), incubated a goat anti-rabbit (1:10000, Jackson Immuno Reseach Labs) or goat anti-mouse (1:5000, Invitrogen) HRP-conjugated secondary antibody in 5% milk in PBST for 1 hour at room temperature, and washed with PBST (3 x 5 min) before being developed with SuperSignal™ West Pico or Femto chemiluminescent substrate (Thermo Scientific) and imaged on a ChemiDoc Gel Imaging System (Bio-Rad). The washout condition in **Fig. 2D** was performed as follows. Cells were dosed with DB008 (1 µM) for 30 min, followed by removal of media and addition of fresh media lacking inhibitors. The plate was placed in the incubator for 10 minutes. This process was repeated twice more before dosing with 1 µM PDD00017273 for 15 minutes, followed by western blotting as previously described.

Click chemistry for in-gel detection of PARP16 labeling by DB008

HEK 293T cells at 75% confluency were transiently transfected with Myc2x-PARP16 (2 µg DNA/well of a 6-well plate) using Calphos mammalian transfection kit (Clontech Labs). The cells were washed once with media 5 hr post transfection. After 24 hr post transfection, cells were treated with inhibitors, followed by lysis in CLB (lacking PARG inhibitor) as described above. Clarified lysates were diluted to 2 mg/mL and click buffer was added to 1X (100 µM TBTA, 1 mM $CuSO_4$, 100 µM TAMRA-azide (Click Chemistry Tools), 1 mM TCEP, 1% SDS, all in 1X PBS). The click reaction was incubated at room temperate for 1 hr (protected from light) and quenched with sample buffer. Samples were boiled at 95ºC for 5 min, and total protein was resolved in a 12% SDS-PAGE gel. The gel was rinsed with water and imaged using the ChemiDoc Gel Imaging System (rhodamine setting). Western blots were prepared as previously described.

HAP1 WT and PARP16 KO cells (Horizon Discovery) at 70-80% confluency were treated with a dose-response of DB008 for 2 hr, followed by lysis in CLB, clicking to TAMRA-azide, and western blotting as described above.

Modeling of covalent complex

Residues 418-450 of PARP2 (PDB: 4TVJ) were aligned to residues 142-174 of PARP16 (PDB: 6HXS) to place olaparib within the PARP16 binding pocket. Atoms CAJ and CAK of olaparib were deleted and the complex underwent one cycle of relaxation via RosettaRelax[5]. We then used RosettaLoopModel to generate 1,000 conformations of loop 166-181, and collected models in which the CAI atom of DB008 was placed within 4A of the Cys169 thiol. Of the models that passed this filter, we used DOCKovalent[6] to covalently dock DB008 against the 5 top-scoring models. Each docking run generated 100 poses. We manually reviewed the top 10% of solutions and selected a top scoring pose that recapitulated the polar interactions of

olaparib. Lastly, we performed another cycle of RosettaRelax with AtomPair constraints to enforce the retention of the covalent bond.

Nutrient starvation

HAP1 cells at 85% confluency were washed twice with starvation media (1X Hanks' Balanced Salt Solution (HBSS), Gibco), and incubated with 1X HBSS for 16 hours in the presence of inhibitors. For the puromycin incorporation assay, cells were treated with 5 µg/ml puromycin for 5 minutes prior to wash and lysis. Cells were washed with cold PBS and lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with fresh 0.1 mM TCEP, 1X cOmplete EDTA-free Protease Inhibitor Cocktail, 30 µM Phthal 01, and 10 µM PDD00017273. Samples were briefly vortexed (5 seconds), clarified by centrifugation at 14,000 rpm for 20 min at 4ºC, and the RIPA-soluble fraction (supernatant) was transferred into a new tube and prepared for western blotting as described previously, except samples were not boiled prior to SDS-PAGE.

Isolation of RIPA-insoluble (pellet) fraction

After clarification of lysates, the pellet was washed with RIPA buffer (1 x 5 min), centrifuged at 14,000 rpm for 5 min at 4ºC, and washed again with CLB. The pellet was resuspended in CLB containing 25 U benzonase nuclease (Millipore Sigma) per mL of CLB, and incubated at room temperature for 1 hr. Samples were sonicated using a bath sonicator (Fisher Scientific, FS20D) for 5-10 seconds to fully solubilize the pellet, and quantification was done using a Bradford assay. Sample buffer was added to 1X and samples were prepared for western blotting as previously described, except samples were not boiled prior to SDS-PAGE.

<u>Detailed synthetic methods</u>

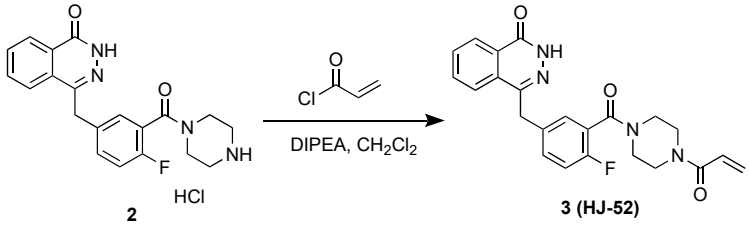
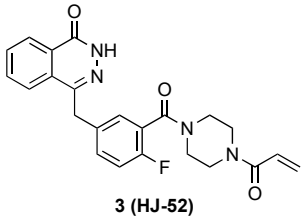
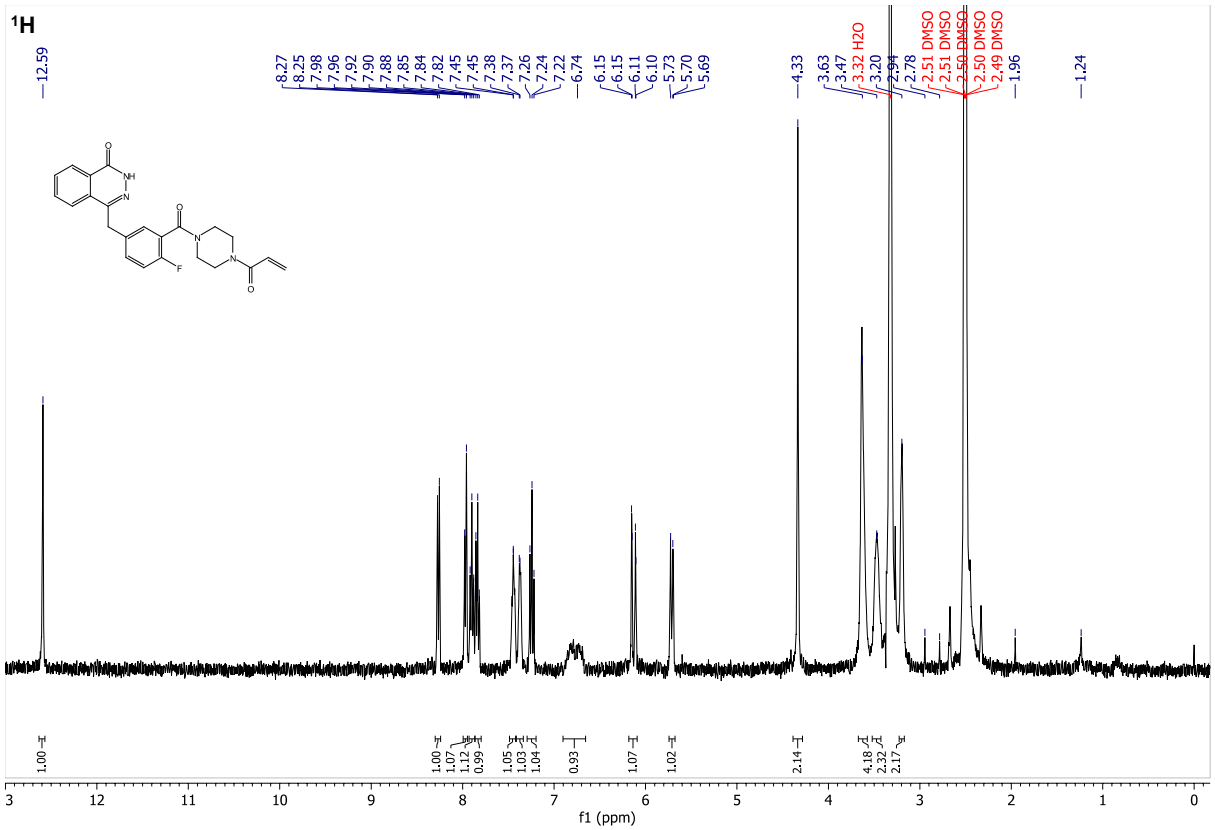


Figure S6. Scheme for HJ-52 synthesis.

Synthesis of 4-(3-(4-acryloylpiperazine-1-carbonyl)-4-fluorobenzyl)phthalazin-1(2H)-one (**3, HJ-52**).



Compound **2** (72 mg, 0.2 mmol), synthesized as previously reported[7], was added to a flame dried flask and dissolved in anhydrous CH_2Cl_2 (5 mL) followed by addition of DIPEA (48.8 mg, 0.38 mmol) and acryloyl chloride (0.025 mL, 0.3 mmol). Once the stating material was consumed by TLC (10% CH_2Cl_2 in MeOH) the reaction was purified on a Teledyne ISCO CombiFlash (0-15% MeOH in CH_2Cl_2) to afford a white solid (20 mg, 24% yield). **^{1}H NMR (400 MHz, DMSO-d6)** δ 12.59 (s, 1H), 8.26 (d, *J* = 7.8 Hz, 1H), 7.97 (d, *J* = 8.0 Hz, 1H), 7.90 (t, *J* = 7.6 Hz, 1H), 7.84 (t, *J* = 7.6 Hz, 1H), 7.50 – 7.41 (m, 1H), 7.40 – 7.34 (m, 1H), 7.24 (t, *J* = 9.0 Hz, 1H), 6.74 (s, 1H), 6.13 (dd, *J* = 16.7, 2.3 Hz, 1H), 5.75 – 5.67 (m, 1H), 4.33 (s, 2H), 3.63 (s, 4H), 3.47 (s, 2H), 3.20 (s, 2H). **LR-MS (ESI)** m/z $[M-H]^-$ for $C_{23}H_{21}FN_4O_3$: 418.7



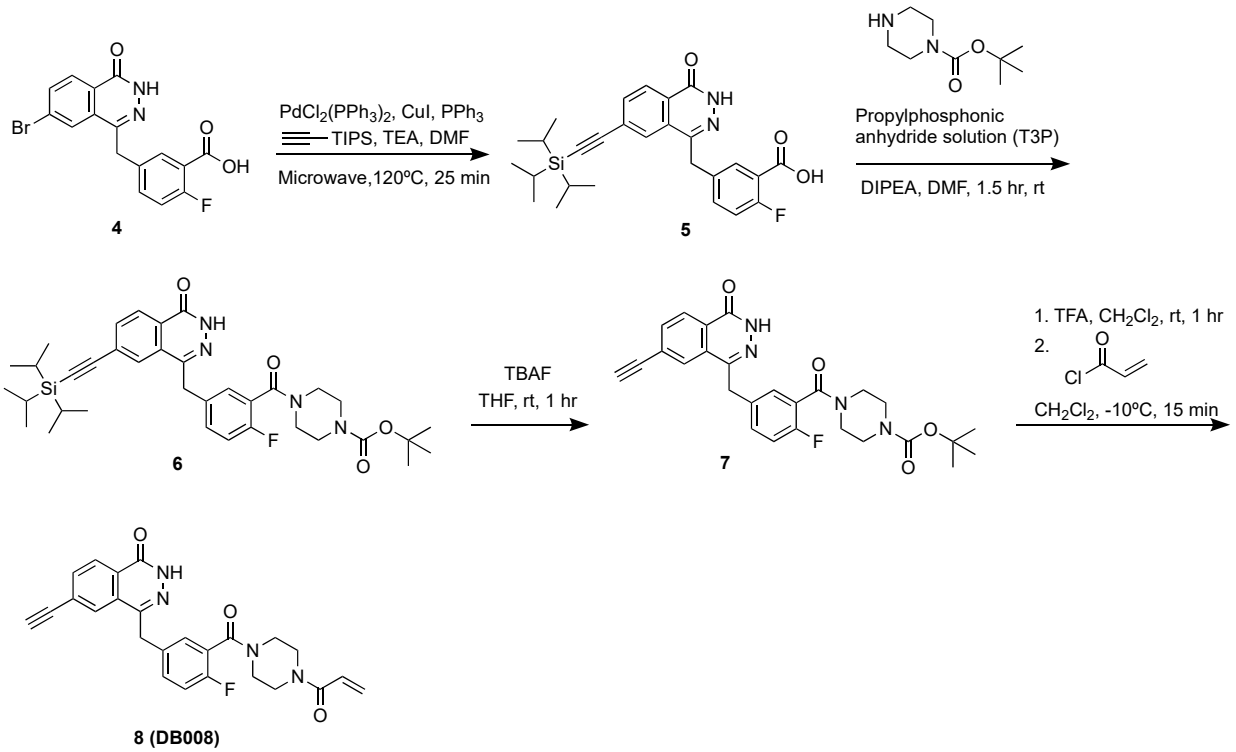
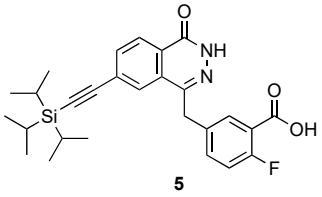


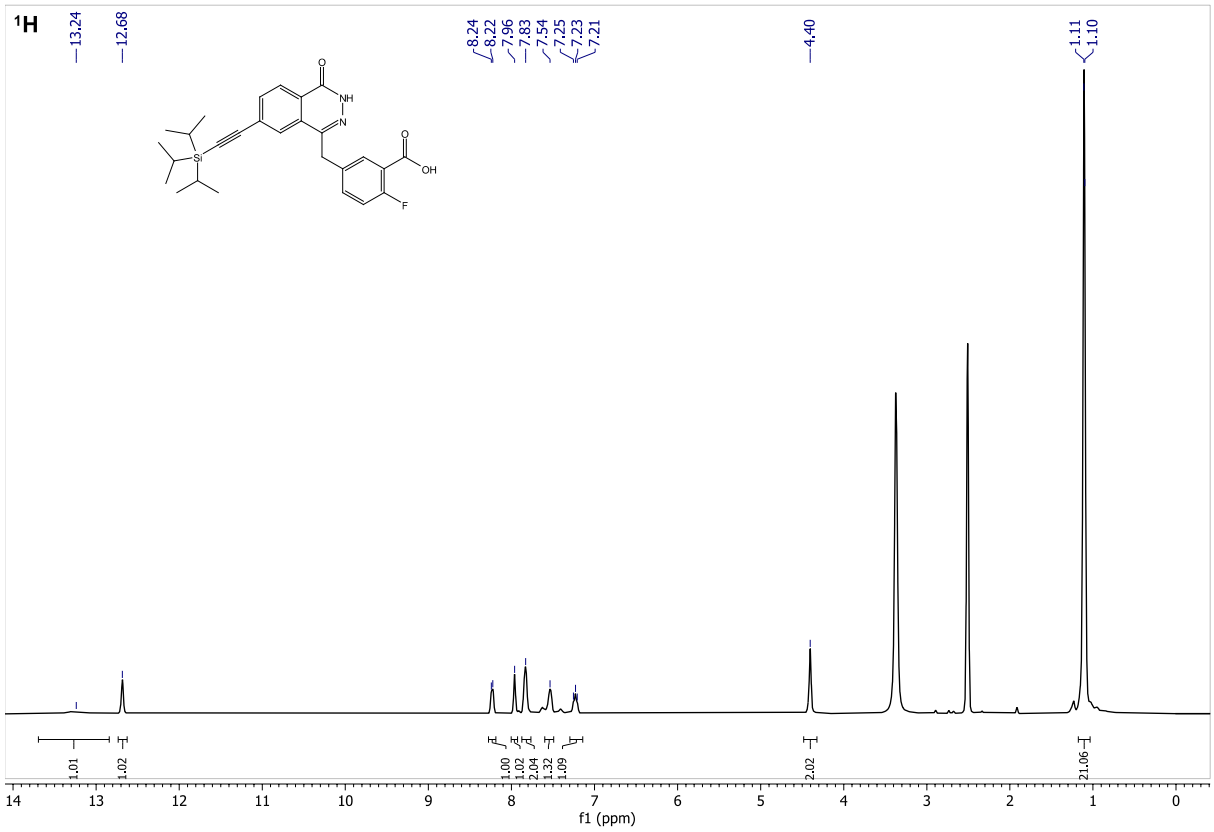
Figure S7. Scheme for DB008 synthesis

Synthesis of 2-fluoro-5-((4-oxo-7-((triisopropylsilyl)ethynyl)-3,4-dihydrophthalazin-1-yl)methyl)benzoic (**5**).

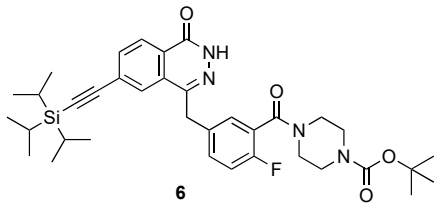


Compound **4** was ordered by custom synthesis from Atomwise. To a dry 10 mL microwave vial, compound **4** (0.5 g, 1.33 mmol) was added and dissolved in anhydrous DMF (1.6 mL), followed by addition of $PdCl_2(PPh_3)_2$ (51.2 mg, 0.073 mmol), CuI (13.9 mg, 0.073 mmol), triphenylphosphine (69.5 mg, 0.265 mmol), and triethylamine (2.8 mL, 20.0 mmol). After 10 min stirring at room temperature, (triisopropylsilyl)acetylene (0.44 mL, 1.99 mmol) was added dropwise and the microwave vial was sealed, purged with argon, and reacted in a microwave reactor for 25 min at 120°C. The reaction mixture was diluted with ethyl acetate (10 mL) and washed once with 1M HCl (10 mL). The aqueous layer was extracted with ethyl acetate (3 x 10 mL), and the combined layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The resulting crude residue was purified on a Teledyne ISCO CombiFlash (0-10% MeOH-containing 2% acetic acid, in CH_2Cl_2). The product was isolated as a white solid (0.482 g, 76% yield).

^{1}H NMR (400 MHz, DMSO-d6) δ 13.24 (brs, 1H), 12.68 (s, 1H), 8.23 (d, *J* = 7.0 Hz, 1H), 7.96 (s, 1H), 7.83 (s, 2H), 7.54 (s, 1H), 7.30 – 7.15 (m, 1H), 4.40 (s, 2H), 1.17 – 1.04 (m, 21H).



Synthesis of tert-butyl 4-(2-fluoro-5-((4-oxo-7-((triisopropylsilyl)ethynyl)-3,4-dihydrophthalazin-1-yl)methyl)benzoyl)piperazine-1-carboxylate (**6**).



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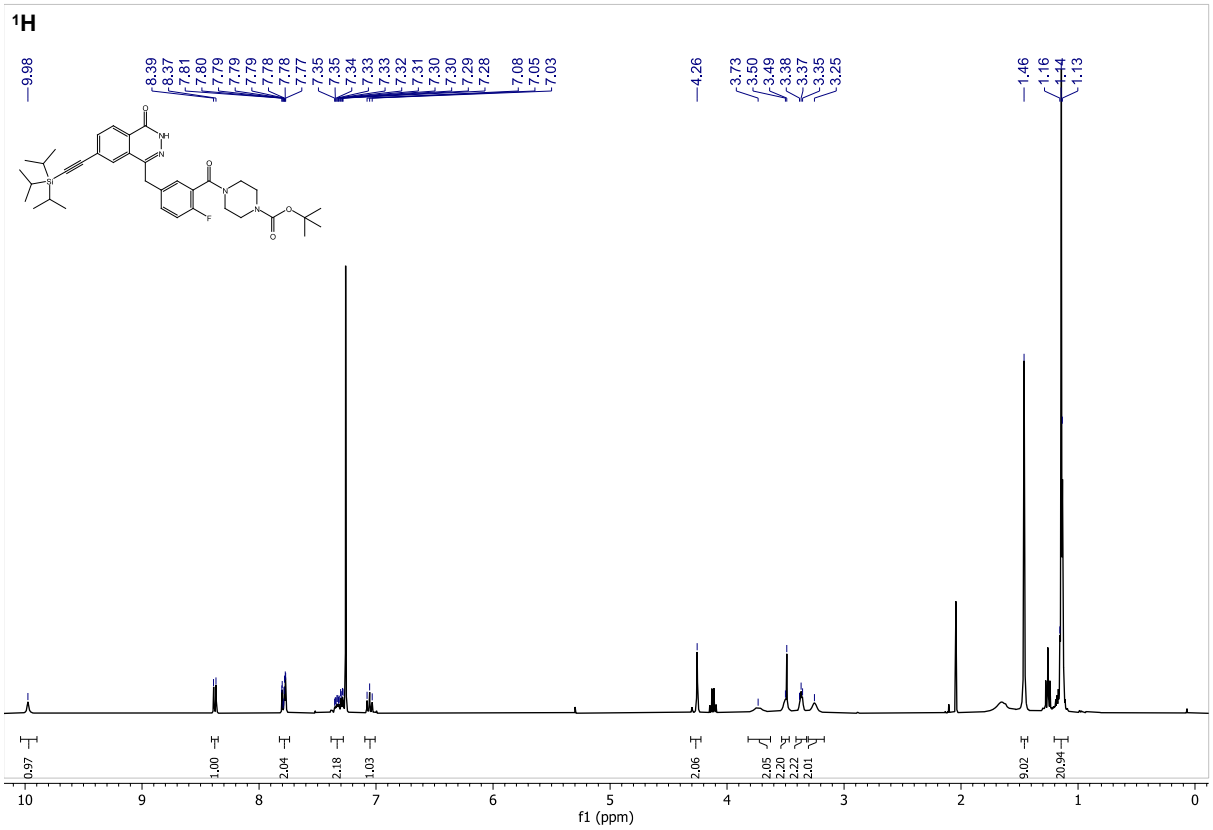
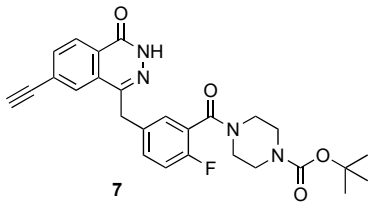
To a dry 4 mL dram vial, compound **5** (80 mg, 0.167 mmol) and tert-butyl piperazine-1-carboxylate (47 mg, 0.251 mmol) were combined, dissolved in dry DMF (0.33 mL), and cooled to 0°C. DIPEA (0.087 mL, 0.5 mmol) was added dropwise, followed by stirring for 5 min, then propylphosphonic anhydride solution (0.21 mL, 0.33 mmol) was added dropwise .The reaction mixture was stirred for 1.5 hr at room temperature, diluted with ethyl acetate (1 mL) and quenched with sat. sodium bicarbonate (1 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (3 x 1 mL). The combined organic layers were washed once with 1M HCl (3 mL), dried over sodium sulfate, filtered, co-evaporated with heptane (3 x 10 mL) and concentrated *in vacuo* to yield an off-white, semi-transparent solid (101.6 mg crude, 94% yield) that was used in the TIPS deprotection step without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.98 (s, 1H), 8.38 (d, *J* = 8.1 Hz, 1H), 7.85 – 7.74 (m, 2H), 7.40 – 7.27 (m, 2H), 7.06 (t, *J* = 8.8 Hz, 1H), 4.26 (s, 2H), 3.73 (br s, 2H), 3.50 (br s, 2H), 3.36 (t, *J* = 5.1 Hz, 2H), 3.25 (s, 2H), 1.46 (s, 9H), 1.21 – 1.09 (m, 21H).



Synthesis of tert-butyl 4-(5-((7-ethynyl-4-oxo-3,4-dihydrophthalazin-1-yl)methyl)-2-fluorobenzoyl)piperazine-1-carboxylate (**7**).



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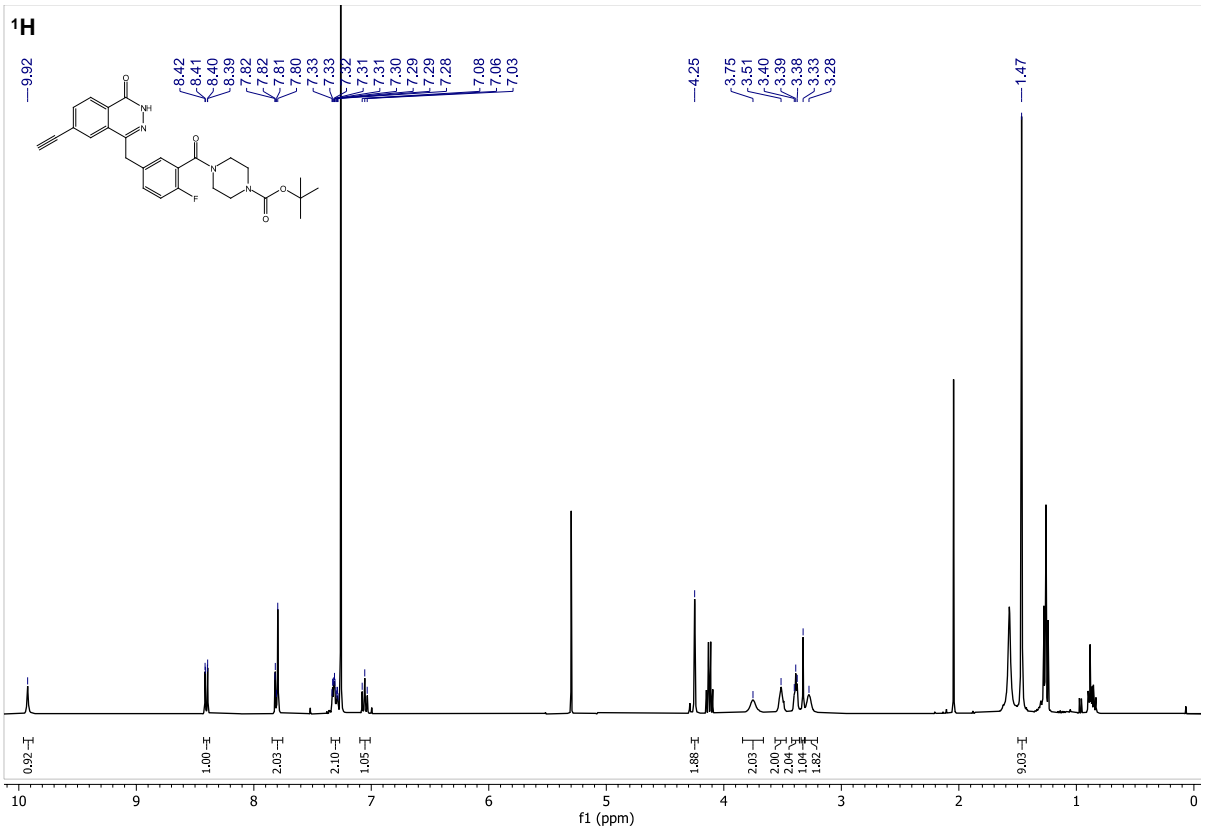
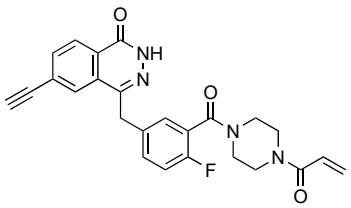
Compound **6** (67.8 mg, 0.10 mmol) was dissolved in anhydrous THF (1 mL) in a dry 4 mL dram vial and cooled to 0ºC. A 1M solution of TBAF in THF (0.12 mL, 0.12 mmol) was added dropwise and the solution was stirred for 15 min at 0ºC, then 45 min at room temperature. The reaction mixture was diluted with ethyl acetate (1 mL) and washed once with brine (1 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (2 x 1 mL). The combined organic layers were dried over sodium sulfate, filtered, concentrated *in vacuo*, and the resulting residue was purified on a Teledyne ISCO CombiFlash (0-75% ethyl acetate in hexanes). The produce was isolated as a white solid (36.0 mg, 70% yield).
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.92 (s, 1H), 8.40 (dd, *J* = 8.0, 0.9 Hz, 1H), 7.84 – 7.76 (m, 2H), 7.39 – 7.26 (m, 2H), 7.06 (t, *J* = 8.7 Hz, 1H), 4.25 (s, 2H), 3.75 (br s, 2H), 3.51 (s, 2H), 3.39 (t, *J* = 5.1 Hz, 2H), 3.33 (s, 1H), 3.28 (s, 2H), 1.47 (s, 9H).



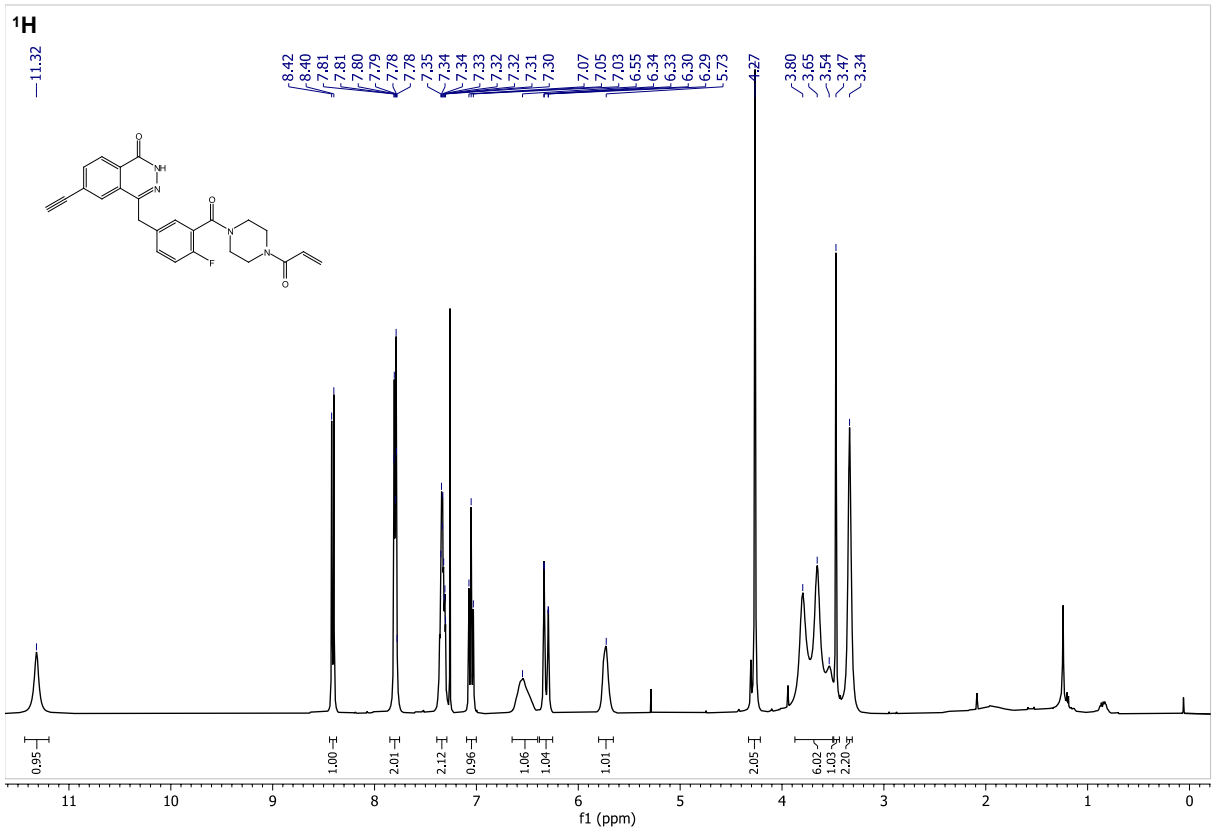
Synthesis of 4-(3-(4-acryloylpiperazine-1-carbonyl)-4-fluorobenzyl)-6-ethynylphthalazin-1(2H)-one (**8, DB008**).

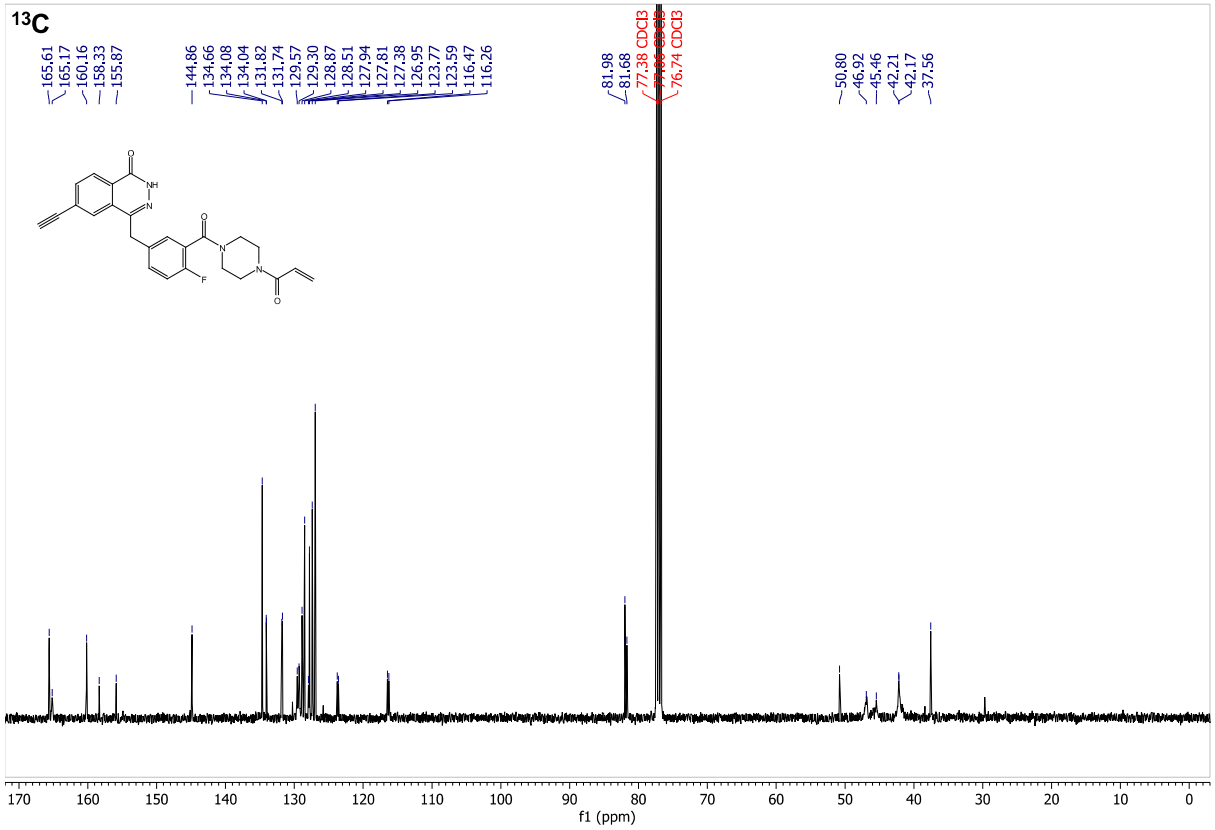


8 (DB008)

Compound **7** (34.5 mg, 0.07 mmol) was dissolved in anhydrous CH_2Cl_2 (1 mL) and cooled to 0ºC. TFA (0.21 mL, 2.8 mmol) was added dropwise, and the reaction was stirred for 1 hr at room temperature. Next, CH_2Cl_2 (0.5 mL) was added and the reaction mixture was cooled to -10ºC, followed by the dropwise addition of DIPEA (0.61 mL, 3.5 mmol). The reaction mixture was stirred for 10 minutes, then acryloyl chloride (6.8 µL, 0.084 mmol) was added dropwise and the solution was stirred for 15 min at -10ºC. The reaction mixture was quenched with water (2 mL) and the organic layer was separated, washed with brine (1 x 2 mL), dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was co-evaporated with toluene (4 x 5 mL), then purified on a Teledyne ISCO CombiFlash (0-5% MeOH in CH_2Cl_2). The product was isolated as a white solid (19.5 mg, 62.5% over two steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.32 (s, 1H), 8.41 (d, *J* = 8.5 Hz, 1H), 7.83 – 7.74 (m, 2H), 7.40 – 7.26 (m, 2H), 7.05 (t, *J* = 8.7 Hz, 1H), 6.55 (br s, 1H), 6.36 – 6.27 (m, 1H), 5.73 (s, 1H), 4.27 (s, 2H), 3.87 – 3.51 (m, 6H), 3.47 (s, 1H), 3.34 (s, 2H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 165.61-165.17 (rotamers), 160.16, 158.33-155.87 (d, *J* = 247.7 Hz), 144.86, 134.66, 134.08-134.04 (d, *J* = 3.4 Hz), 131.82-131.74 (d, *J* = 8.1 Hz), 129.57, 129.30, 128.87, 128.51, 127.94, 127.81, 127.38, 126.95, 123.77-123.59 (d, *J* = 17.9 Hz), 116.47-116.26 (d, *J* = 21.8 Hz), 81.98, 81.68, 50.80, 46.92, 45.46, 42.21, 42.17, 37.56.
HRMS (ESI) m/z $[M+H]^+$ calculated for $C_{25}H_{21}FN_4O_3$: 444.1592, observed: 444.1658




13C
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134.04
131.82
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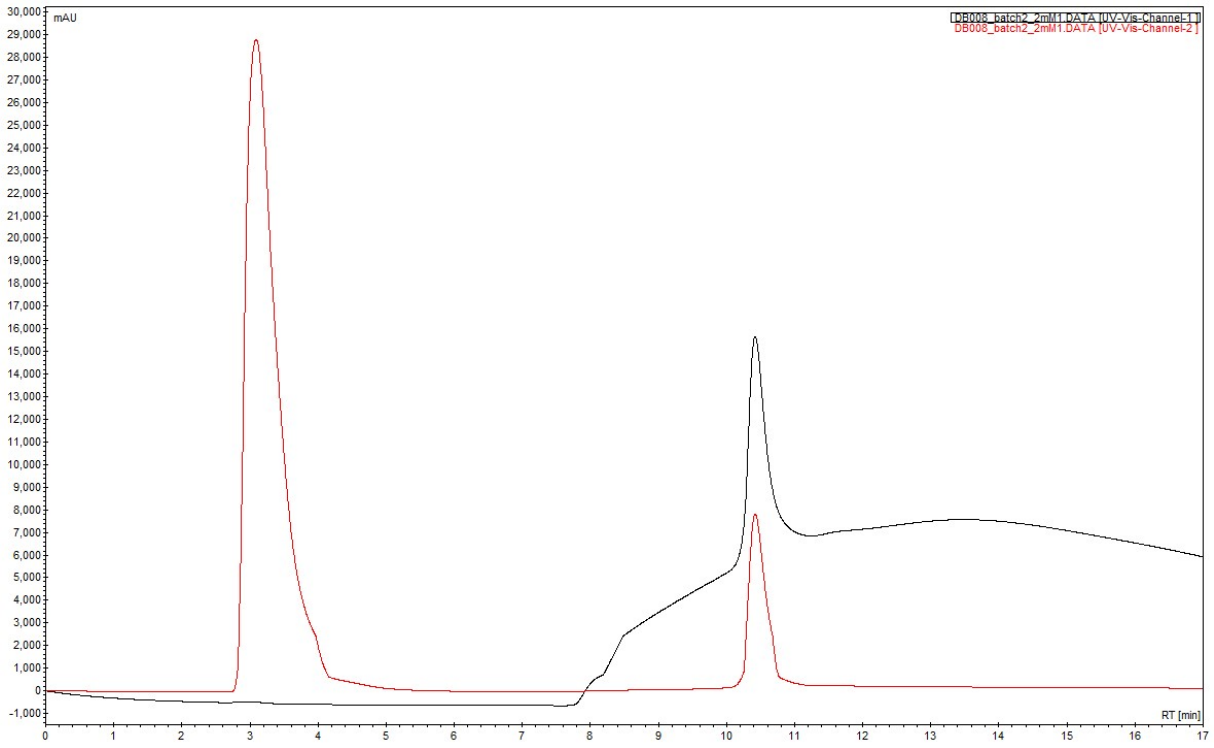
129.30
128.87
128.51
127.94
127.81
127.38
126.95
123.77
123.59
116.47
116.26
81.98
81.68
77.38 CDCl3
76.74 CDCl3
50.80
46.92
45.46
42.21
42.17
37.56
f1 (ppm)



HPLC chromatogram of 2 mM **DB008** dissolved in methanol (4% DMSO final) run on a Varian ProStar 210 (Agilent) using a Polaris 5 C18-A 150 x 4.6 mm column. The mobile phase was composed of solvent A (water) and solvent B (acetonitrile), run on a gradient with a flow rate 1.0 ml/min as follows: 1 min pre-run A =100% B = 0%, 0.1 min A = 95% B = 5%, 2.0 min A = 90% B = 10%, 5.0 min A = 70%, B = 30%, 7.1 min A = 50%, B = 50%,10.0 min A = 30% B = 70%. UV-Vis detection: channel 1 = 254 nm, channel 2 = 214 nm. The retention time (RT) for DB008 is 10.4 min. For UV-vis channel 2, DMSO RT is at 3.3 min.

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