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Design and chemoproteomic functional characterization of a chemical probe targeted to bromodomains of BET family proteins

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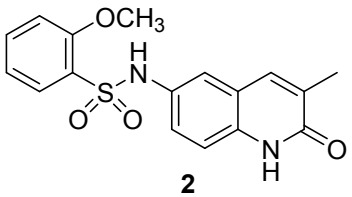
Supporting Information

Chemical Synthesis of Probes

Chemistry Methods and Compound Characterization

Starting materials and reagents were purchased from commercial sources and used as received. Tetrahydrofuran (THF) and acetonitrile were purchased from EMD anhydrous and were used without further drying. All air and moisture sensitive reactions were carried out under an atmosphere of dry nitrogen using heat-dried glassware and standard syringe techniques. All final compounds were isolated following flash chromatography which was performed using an Analogix Intelliflash 280 with Sepra Si 50 silica gel utilizing ethyl acetate/heptane mixtures as solvent unless otherwise indicated. All compounds reported in this manuscript were obtained in ≥95% purity unless otherwise noted. Proton (^{1}H NMR) and carbon (^{13}C NMR) magnetic resonance spectra where obtained in DMSO-d_6 at 400 MHz and 100 MHz, respectively unless otherwise noted. The following abbreviations were utilized to describe peak patterns when appropriate: br = broad, s = singlet, d = doublet, t = triplet and m = multiplet. High-resolution mass measurements were obtained on an Agilent ToF mass spectrometer. Accurate Mass Spectrometry analyses were conducted on an Agilent 6220 ToF mass spectrometer (Agilent Technologies, Wilmington, DE) in positive or negative electrospray mode. The system was calibrated to greater than 1ppm accuracy across the mass range prior to analyses according to manufacturer's specifications. The samples were separated using UHPLC on an Agilent 1200 (Agilent Technologies, Wilmington, DE) system prior to mass spectrometric analysis. The resulting spectra were automatically lockmass corrected and the target mass ions and any confirming adducts (Na^+, NH_4^+) were extracted and combined as a chromatogram. The mass accuracy was calculated for all observed isotopes against the theoretical mass ions derived from the chemical formula using MassHunter software (Agilent Technologies, Wilmington, DE). Certain compounds from the examples described were purified on an automated preparative Supercritical Fluid Chromatography (SFC) or reversed-phase High Performance Liquid Chromatography (RPHPLC). Purification by SFC was performed on a Waters SFC 80 and RPHPLC was performed on either a Gilson GX281, Shimadzu CL-2010C, or Agilent 1200 system. Specific columns, solvents and gradient conditions are described for each compound using either of these chromatographic techniques.

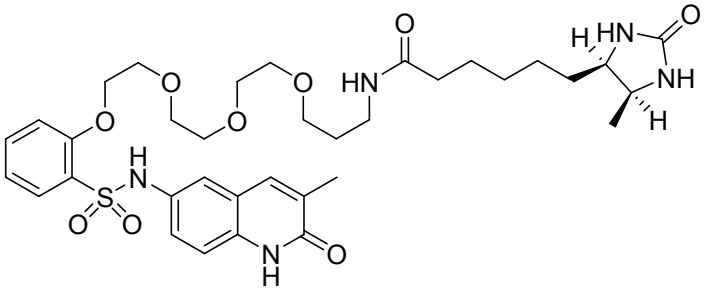


2

3-methyl-6-nitroquinolin-2(1H)-one (**4)**: 2-Chloro-5-nitrobenzaldehyde (7.50 g, 40 mmol), 2-ethyl-4,4-dimethyl-4,5-dihydroxazole (5.14 g, 40 mmol) and sodium bisulfate (0.4 g, 3.35 mmol) were dissolved in a mixture of NMP (21 mL) and xylenes (12 mL) in a flask equipped with Dean-Stark trap and condenser. The reaction mixture was slowly heated to 200 °C, while water and xylenes were collected in the Dean Stark trap. After 90 minutes at 200 °C, the temperature was further raised to 225 °C and maintained at that point for an additional 45 minutes. The mixture was then allowed to cool to ambient temperature and water was added. The precipitate was filtered and washed thoroughly with water, then dried in vacuo to afford 7.7 g (93%) of **4**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.31 (s, 1 H), 8.59 (d, 1 H), 8.27 (dd, 1 H), 7.99 (s, 1 H), 7.41 (d, 1 H), 2.12 (s, 3 H). LCMS: Rt = 2.61 min, m/z 203[M-H$^+$]

6-amino-3-methylquinolin-2(1H)-one (5): To a solution of **4** (7.5 g, 36 mmol) in acetic acid (250 mL) was added 10% palladium on carbon (7.8 g) under a nitrogen atmosphere. The mixture was hydrogenated under 50 psig of hydrogen at 20 °C for 3 h, after which the mixture was filtered through a short pad of Celite®. The filtrate was evaporated under reduced pressure and the residue was triturated with ethyl acetate to afford 5.4 g (84%) of **5**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.36 (br s, 1 H), 7.53 (s, 1 H), 7.00 (d, 1 H), 6.76 (dd, 1 H), 6.65 (d, 1 H), 4.93 (br s, 2 H), 2.04 (s, 3 H). LCMS: Rt = 1.35 min , m/z 175[M+H]$^+$

2-methoxy-N-(3-methyl-2-oxo-1,2-dihydroquinolin-6-yl)benzenesulfonamide (2): A solution of **5** (0.2 g, 1.1 mmol) in 8 mL of pyridine was treated with 0.236 g (1.1 mmol) of 2-methoxybenzenesulfonyl chloride. The reaction mixture was stirred at 20 °C for 16 h. The mixture was then diluted with water and extracted with ethyl acetate. The ethyl acetate extract was washed with 1 M hydrochloric acid, brine, dried (Na_2SO_4) and concentrated. Column chromatography of the residue on silica gel eluting with 30% ethyl acetate in hexane afforded 100 mg (46%) of **2**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.02 (s, 3 H), 3.90 (s, 3 H), 6.97 (t, 1 H), 7.08 (d, 1 H), 7.13-7.16 (m, 2 H), 7.22 (s, 1 H), 7.53 (t, 1 H), 7.65-7.69 (m, 2 H), 9.86 (s, 1 H), 11.62 (s, 1 H). LCMS: Rt = 2.71 min; m/z 345 [M+H]$^+$



3

2-(benzylthio)phenol (6): 2-Mercaptophenol (2.50 g, 19.8 mmol) in 20 mL of dry DMF was treated with benzyl bromide (2.39 mL, 19.8 mmol) and potassium bicarbonate (2.18 g, 21.8 mmol) at 0 °C. The reaction mixture was allowed to warm to 20 °C and was stirred at that temperature for 16 h. The reaction mixture was diluted with water and extracted with diethyl ether. The ethereal extract was washed with water, brine, dried (Na_2SO_4) and concentrated. Column chromatography of the residue on silica gel eluting with 10% ethyl acetate in hexane afforded 2.51 g (60%) of **6.** ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.11 (s, 2 H), 6.72 (t, 1 H), 6.83 (d, 1 H), 7.02 (t, 1 H), 7.15 (d, 1 H), 7.21 (t, 1 H), 7.28 (t, 2 H), 7.32 (d, 2 H), 9.82 (s, 1 H). LCMS: Rt = 3.36 min; m/z 215 [M-H$^+$]

3-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)propanenitrile (7): Sodium hydroxide (13 mg, 0.32 mmol) was added to a mixture of triethylene glycol (37.9 mL, 283 mmol) and acrylonitrile (12.5 mL, 188 mmol). The mixture was stirred for 16 h at 20 °C, after which the mixture was acidified with dilute sulfuric acid and diluted with 200 mL of water. The mixture was extracted four times with 50 mL portions of 9/1 (v/v) dichloromethane – methanol. Evaporation of the solvent followed by column chromatography on silica gel eluting with 2% methanol in dichloromethane afforded 10.1 g (26%) of **7** as a colorless liquid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.74 (t, 2 H), 3.42 (t, 2 H), 3.47-3.62 (m, 12 H), 4.54 (t, 1 H). LCMS: Rt = 1.34 min; m/z 204 [M+H]$^+$

2-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)ethanol (8): To a solution of **7** (10.0 g, 49.3 mmol) in a mixture of ethanol (1000 mL) and chloroform (25 mL) was added Adam's catalyst (1.22 g) under a nitrogen atmosphere. The mixture was hydrogenated under 30 psig of hydrogen at 20 °C for 4 h, after which the mixture was filtered through a short pad of Celite®. The solvent was removed under reduced pressure to afford 10.0 g (99%) of **8**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.77-1.83 (m, 2 H), 2.78-2.86 (m, 2 H), 3.42 (t, 2 H), 3.46-3.50 (m, 12 H). LCMS: Rt = 0.59 min; m/z 208 [M+H]$^+$

***tert*-butyl (3-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)propyl)carbamate (9)**: A solution of **8** (10.0 g, 50.0 mmol) in THF (100 mL) was treated sequentially with di-t-butyl dicarbonate (13.1 mL, 60.0 mmol) and triethylamine (8.3 mL, 60.0 mmol). The reaction mixture was stirred for 4 h at 20 °C and the volatile components were removed in vacuo. The remaining residue was purified by column chromatography on silica gel eluting with 1% methanol in dichloromethane to afford 5.70 g (37%) of **9** as a colorless liquid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.37 (s, 9 H), 1.56-1.62 (m, 2 H), 2.95 (q, 2 H), 3.31-3.49 (m, 14 H), 4.55 (t, 1 H), 6.73 (br s, 1 H). LCMS: Rt = 0.72 min; m/z 308 $[M+H]^+$

***tert*-butyl (3-(2-(2-(2-(2-(benzylthio)phenoxy)ethoxy)ethoxy)ethoxy)propyl)carbamate (10)**: A solution of **6** (0.20 g, 0.925 mmol) and polymer bound triphenylphosphine (0.58 g, 0.925 mmol, 1.60 mmol/ g) in THF (5 mL) was treated sequentially with **9** (0.30 g, 0.97 mmol) and diisopropylazodicarboxylate (0.24 g, 1.11 mmol). The reaction mixture was stirred for 1 h at 20 °C. The reaction mixture was filtered through a short pad of Celite®, and the filter cake was rinsed thoroughly with ethyl acetate. The solvent was removed under reduced pressure and the remaining residue was purified by column chromatography on silica gel eluting with 5 - 50% ethyl acetate in heptane to afford 0.37 g (79%) of **10** as an oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.43 (s, 9 H), 1.69-1.75 (m, 2 H), 3.20 (t, 2 H), 3.49 (t, 2 H), 3.51-3.54 (m, 2 H), 3.60-3.62 (m, 2 H), 3.64-3.67 (m, 2 H), 3.76-3.78 (m, 2 H), 3.91 (t, 2 H), 4.11 (s, 2 H), 4.19 (t, 2 H), 6.83-6.87 (m, 2 H), 7.13-7.31 (m, 7 H). LCMS: Rt = 1.08 min; m/z 528 $[M+23]^+$

***tert*-butyl chloro(3-(2-(2-(2-(2-(chlorosulfonyl)phenoxy)ethoxy)ethoxy)ethoxy)propyl)carbamate (11)**: To a solution of benzyltrimethylammonium chloride (0.46 g, 2.49 mmol) in water (1 mL) was added trichloroisocyanuric acid (0.26 g, 1.10 mmol) in acetonitrile (2.5 mL). The clear solution was stirred for 0.5 h at 20 °C. The solution was added dropwise to an ice cooled solution of compound **10** (0.37 g, 0.73 mmol) in acetonitrile (5 mL) followed by the addition of 1 M sodium bicarbonate solution (0.73 mL, 0.73 mmol). The reaction mixture was stirred for 1 h at 5 °C. The reaction mixture was quenched with 1 M sodium bicarbonate solution (5 mL) and the resulting white precipitate was filtered and washed thoroughly with ethyl acetate. The layers were separated and the organic layer was washed with brine, dried over anhydrous magnesium sulfate and concentrated. Column chromatography of the residue on silica gel eluting with 50 - 100% ethyl acetate in heptane afforded 0.38 g (92%) of **11** as an oil that was used without further purification. LCMS: Rt = 2.07 min; m/z 538 $[M+23]^+$

***tert*-butyl(3-(2-(2-(2-(2-(N-(3-methyl-2-oxo-1,2-dihydroquinolin-6-yl)sulfamoyl)phenoxy)ethoxy)ethoxy)ethoxy)-propyl)carbamate (12)**: A solution of **5** (0.30 g, 1.7 mmol) in 8 mL of pyridine was treated with 0.67 g (1.3 mmol) of **11**. The reaction mixture was stirred for 18 h at 20 °C and the volatile components were removed in vacuo. The remaining residue was purified by column chromatography on silica gel eluting with 0 - 50% methanol in ethyl acetate to afford 0.81 g (68%) of **12** as a foam that was used without further purification. LCMS: Rt = 0.85 min; m/z 642 $[M+23]^+$.

2-(2-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)ethoxy)-N-(3-methyl-2-oxo-1,2-dihydroquinolin-6-yl)benzene-sulfonamide (13): To a solution of **12** (0.55 g, 0.89 mmol) in dichloromethane (10 mL) was added trifluoroacetic acid (0.5 mL) at 0 °C. The reaction mixture was allowed to warm to 20 °C and was stirred at that temperature for 16 h. Volatiles were removed under reduced pressure to afford 0.46 g (100%) of **13** as an oil that was used without further purification. LCMS: Rt = 0.56 min; m/z 520 $[M+H]^+$

N-(3-(2-(2-(2-(2-(N-(3-methyl-2-oxo-1,2-dihydroquinolin-6-yl)sulfamoyl)phenoxy)ethoxy)ethoxy)ethoxy)propyl)-6-((4*R*,5S)-5-methyl-2-oxoimidazolidin-4-yl)hexanamide (3): To a solution of **13** (0.25 g, 0.48 mmol) and 6-((*4R*,*5S*)-5-methyl-2-oxoimidazolidin-4-yl)hexanoic acid (desthiobiotin, 0.103 g, 0.48 mmol) in DMF (1 mL) was treated sequentially with diisopropylethylamine (0.25 mL, 1.44 mmol) and HATU (0.19 g, 0.48 mmol). The reaction mixture was stirred at 20 °C for 16 h and the volatile components were removed in vacuo. The remaining residue was purified by preparative SFC eluting with 5 – 100% methanol (0.1% formic acid) in water (0.1% formic acid) to afford 0.05 g (15%) of **3** as an off white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.94 (d, 3 H), 1.13 – 1.39 (m, 6H), 1.43 – 1.50 (m, 2 H), 1.52 – 1.59 (m, 2 H), 2.00 – 2.04 (m, 5 H), 2.99 – 3.07 (m, 2 H), 3.29 – 3.39 (m, 6 H), 3.44 – 3.51 (m, 3 H), 3.56 – 3.63 (m, 1 H), 3.64 – 3.66 (m, 2 H), 3.86 – 3.88 (m, 2 H), 4.32 – 4.34 (m, 2 H), 6.11 (brs, 1 H), 6.28 (brs, 1 H), 6.98(dt, 1 H), 7.09 (d, 1 H), 7.18 (dd, 1 H), 7.23 (dd, 1 H), 7.28 (d, 1 H), 7.50 – 7.55 (m, 1 H), 7.62 – 7.65 (m, 2 H), 7.69 (t, 1 H), 9.03 (brs, 1 H), 11.64 (brs, 1 H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 15.93, 16.90, 25.64, 26.01, 29.17, 29.83, 29.97, 35.78, 36.12, 50.85, 55.41, 68.50, 68.82, 68.98, 69.80, 70.08, 70.15, 70.38, 114.42, 115.70, 119.51, 119.89, 120.87, 124.07, 126.77, 130.48, 131.05, 131.64, 135.41, 135.54, 136.28, 155.89, 162.49, 163.23, 172.38. LCMS: Rt = 1.24 min; m/z 716 $[M+H]^+$. HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{35}H_{50}N_5O_9S$, 716.3324; found 716.3322.

Synthesis of **PF-411FP**: 2-((1E,3E,5E)-5-(3,3-dimethyl-1-(1-(2-(N-(3-methyl-2-oxo-1,2-dihydroquinolin-6-yl)sulfamoyl)phenoxy)-14-oxo-3,6,9-trioxa-13-azanonadecan-19-yl)-5-sulfoindolin-2-ylidene)penta-1,3-dienyl)-1-ethyl-3,3-dimethyl-3H-indolium-5-sulfonate

To a solution of 1.4 mg (2.2 µmol) of **13** in 100 µL DMSO was added 5.0 mg (6.3 µmol) Cy5 carboxylic acid succinimidyl ester (GE Healthcare, cat. PA15100) and 4 µL N,N-diisopropylethylamine. After 1 h, the reaction was purified directly by preparative high-performance liquid chromatography (HPLC). Preparative HPLC was performed using a XBridge BEH C18 Prep Column, 130Å, 10 µm, 10 mm X 150 mm (Waters # 186003890), eluting with a linear slope gradient at 10 mL/min flow rate. Solvent gradient: Acetonitrile/water/trifluoroacetic acid (TFA) (2:98:0.1) to (42:58:0.1) in 40 min. Collected fractions were analyzed by analytical LCMS, and the fractions at 28-29 minutes judged as having adequate purity were pooled. ESMS: calculated m/z for desired (MH+) = 1158.42, found = 1158.27. The solution was evaporated in a vacuum centrifuge, and the product was stored at 4 °C. The yield was determined by dry weight, recovery was calculated as 0.8 mg (35%)

Culture of THP-1 cells and SILAC labeling

THP-1 cells were cultured in suspension in RPMI 1640 media containing GlutaMAX and supplemented with 10% fetal bovine serum (FBS), 10 mM Hepes, 1 mM sodium pyruvate, 100U/mL penicillin with 100 µg/mL streptomycin, and 55 µM 2-mercaptoethanol. Cells were passaged after 3 days. After two passages, cells were transferred to either Light or Heavy SILAC medium (RPMI 1640 initially lacking L-arginine and L-lysine, but supplemented with the components listed above (except that dialyzed FBS was now used) and either isotopically standard L-arginine HCl and L-lysine 2HCl or $^{13}C_{6,}{}^{15}N_4$-L-arginine HCl and $^{13}C_{6,}{}^{15}N_2$-L-lysine 2HCl) (Cambridge Isotope Laboratories).

Cells were seeded at 2 x 10^5 cells/ml in their respective media and were passaged every 3 days for 7 passages to ensure almost complete L-arginine and L-lysine incorporation (checked as described below). Cells were harvested from the media by centrifugation at 450 x *g* for 5 min, washed in phosphate buffered saline (PBS), separated by centrifugation, and used fresh for the preparation of nuclear extracts.

Nuclear fractionation

SILAC-labeled THP-1 cells were fractionated using the Qiagen Qproteome Nuclear Protein Kit. In brief, THP-1 cells were collected and washed twice with ice-cold PBS, then separated by centrifugation for 5 min at 450 x *g*. All centrifugations were performed in a microcentrifuge pre-cooled to 4 °C. The cell pellet was kept on ice and then gently resuspended in the hypotonic buffer provided in the kit in order to lyse the cells. Cells lysate was separated by centrifugation at 10,000 x *g* for 5 min to separate the nuclear pellet from the supernatant containing membrane and cytosolic proteins. Membrane and cytosolic fractions were transferred to a new microcentrifuge tube and stored at -80 °C. The pellet containing the isolated cell nuclei was washed with hypotonic buffer to remove cytosolic contaminants and separated by centrifugation at 10,000 x *g* for 5 min. The nuclear pellet was resuspended in the high salt extraction buffer and gently agitated at 4 °C for 30 min on an Eppendorf Thermomixer set to 750 rpm. Nuclear fractions were then separated by centrifugation at 12,000 x *g* for 10 min to separate soluble nucleic-acid binding proteins from insoluble nuclear debris. Products of all fractionations were stored at -80 °C until further experimentation. A sample of each labeled soluble nuclear fraction was sent for LC-MS analysis to confirm the degree amino acid incorporation.

Fluorescence polarization binding assay

His-tagged versions of BRD2 BD1 (65-187), BRD2 BD2 (65-187), BRD4 BD2 (342-460) and BRDT BD1 (22-138) were purchased from BPS Bioscience. His-tagged versions of BRD3 BD1 (24-144) and BRD3 BD2 (306-416) were purchased from Active Motif, Carlsbad, CA. A His-tagged version of BRD4 BD1 (44-160) was prepared as previously described.[1] FP assays were performed by adding various concentrations of test compound (diluted in 100% DMSO) to a low volume 384-well black flat bottom microtiter plate (Corning 3820). Each plate also contained both positive (10 µM compound **2** giving100% inhibition of FP probe binding (PF-411FP) and negative (DMSO, 0% effect on FP probe binding) control wells. The indicated BET protein (diluted in 50 mM HEPES pH 7.4 and 0.08% bovine serum albumin (assay buffer) was added to the assay plate containing test compound and incubated at room temperature (RT, 20^{o}C). After 15 minutes, FP probe diluted in assay buffer was added and the plate was incubated in the dark at RT. Final assay concentration of FP probe was 2 nM, final DMSO concentration was 0.4% and final concentration of BRD2 BD1, BRD2 BD2, BRD3 BD1, BRD3 BD2, BRD4 BD1 and BRD4 BD was 50 nM. Final concentration of BRDT BD1 was 200 nM. After 60 min, polarization values were measured with an Envision 2103 multilabel reader (Perkin Elmer) at an excitation wavelength at 620 nm and an emission wavelength at 688. The K_i values of competitive inhibitors were calculated using the equation described by Nikolovska-Coleska et al.[2]

Isothermal Titration Calorimetry (ITC) binding studies

ITC experiments were performed on a Microcal VP-ITC instrument (GE Healthcare), in a buffer containing 50mM TRIS-HCl, pH 7.5, 150mM NaCl, 0.5mM TCEP and 1% DMSO. The concentration of BRD4-BD1 protein in the cell was 5µM. The compounds were dissolved in the same buffer to a concentration of 75µM. Each titration experiment consisted of 28 injections of compound solution that were spaced at 3 minute intervals. During the course of the titrations, the temperature was maintained at 25 ^{o}C and the solution in cell was stirred at 300RPM. The binding affinities and thermodynamic parameters were calculated by fitting the ITC data to a simple 1:1 interaction model in Microcal Origin 6.0 software.

Affinity capture and selectivity assay design (Affinity capture methodology)

PFI-1 in solution binds with sub-micromolar affinities to the two bromodomains of BRD4. As measured by isothermal titration calorimetry, its dissociation constants (K_d) from BD1 and BD2 of BRD4 were 47

nM and 195 nM, respectively.[3] Estimates by SPR for binding of PFI-1 to BD1 (present work) indicated comparable, although somewhat weaker, affinity (see below). Some discrepancy between values obtained in these two methods is not unusual.[4,5]

The ability of its linker-coupled derivative, **3,** to capture BRD4 or a recombinant His-tagged form of its BD1 from solution was first assessed using targeted immunodetection on western blots. Protein capture was attempted using the SILAC-labeled nuclear extracts (light and heavy) from THP-1 cells, a HeLa whole cell lysate (Santa Cruz Biotechnology), and the purified recombinant protein diluted into incubation buffer containing 1% NP-40 in PBS with 1x protease inhibitor cocktail (Roche). The THP-1 nuclear extracts and HeLa whole cell lysates were also diluted in incubation buffer to a protein concentration of 1 mg/ml.

For the affinity capture pull down experiments destined to be analyzed by LC-MS, a protein-only sample was included as a positive control along with samples in the presence and absence of the soluble competitor **2**. Soluble nuclear fractions grown in the light media were incubated with 1% dimethyl sulfoxide (DMSO) while the soluble heavy amino acid nuclear fractions were incubated with compound **2** at 10µM, 1% DMSO. Biotinylated probe **3** at a concentration of 3 µM was added to both samples and followed by a 3 h incubation at room temperature in order to establish equilibrium. Following this, 25 µL of high capacity MagnaLink streptavidin magnetic bead (Solulink, San Diego, CA, USA) slurry was added to each sample. Samples were incubated for 1 h with rotation at room temperature. A DynaMag-2 magnet was then used to separate the magnetic beads from the supernatant. Beads were resuspended in 100 µL of incubation buffer, mixed and then separated using the magnet. Beads were next resuspended in 25 µL of 1x elution buffer containing NuPAGE LDS Sample Buffer and Sample Reducing Agent and boiled at 60 ^{o}C for 10 min. The boiling step was repeated to ensure complete elution of proteins. After magnetic removal of the beads, the remaining sample was transferred to a clean microfuge tube and stored at -80 ^{o}C until protein detection by immunoblotting. Aliquots of the soluble light and heavy THP-1 samples were combined in a 1:1 ratio and processed for mass spectrometry analysis.

SDS-PAGE and Western Blot

Samples were re-heated at 60^{o}C before proceeding with the western blot. 20x NuPAGE MES-SDS running buffer was diluted in sterile water to obtain a 1x running buffer solution. 12 µL of each sample

was loaded onto a NuPAGE 4-12% BisTris 15 well gel. MagicMark XP Western Protein Standards and Benchmark PreStained Protein Ladder were used as reference markers. Electrophoretic separated proteins were transferred to a PVDF membrane using the iBlot dry transfer method. Transfer membrane was subsequently blocked for 1 h with 10 mL of 100% Superior Blocking Buffer. Detection antibodies were diluted in a 10% Superior Blocking Buffer that was diluted in Tris-Buffered Saline and 0.1% Tween20 (TBST). Horseradish peroxidase (HRP)-labeled monoclonal anti-6X polyHistidine antibody was used to detect recombinant his-tagged BD1. Membrane transferred THP-1 and HeLa protein samples were probed with the primary BRD4 (H-250) rabbit polyclonal antibody overnight at 4°C. Membranes were washed five times in TBST and then incubated for one hour with secondary antibody, goat anti-rabbit IgG-HRP. After further washing in TBST membranes were incubated in a 1:1 mixture of Pierce ECL western blotting substrate for 5 min before using enhanced chemiluminescence (ECL) imaging to detect proteins on the Kodak imaging system.

Bioactivity Test (Inhibition of Interleukin-6 Release from PBMC's)

PFI-1 and compound **2** were tested in a human PBMC functional assay to determine the potency at which they were able to inhibit toll-like receptor activated cytokine production. Blood was collected from healthy volunteers (all permissions secured) in 10 mL spray-coated sodium heparin tubes. PBMCs were isolated by Ficoll separation and challenged with an EC_{80} concentration of LPS (approximately 3 ng/mL) in the absence and absence of increasing concentrations of compound. Assay was incubated at 37°C, 5% CO_2 for 18 hrs. LPS induced IL-6 production at each compound concentration was measured using the Cisbio Homogenous Time Resolved Fluorescence detection system. Compound concentration response were analyzed GraphPad Prism using non-linear regression to calculate IC_{50} values.

Assessment of SILAC labeling in nuclear extracts.

After thawing, 12.5 μL of each nuclear sample was mixed with 37.5 μL of 8 M urea, 0.4 M NH_4HCO_3, 4.5 mM DTT, and the mixture was incubated for 20 min at 50 °C. Samples were then allowed to cool, treated with 5.8 μL each of 0.1 M iodoacetamide, and incubated for 30 min at room temperature. Each sample was next treated with 110 μL of water and 1.5 μg trypsin (Promega Sequencing Grade), then incubated overnight at 37 °C. Next, 4 μL of each sample was analyzed by capillary reversed-phase LC-MS using an LTQ Orbitrap XL mass spectrometer. Database searches to identify peptides were performed using the Mascot[6] search engine (licensed from Matrix Science, Boston, MA, USA) against the UniProtHuman database and an additional database of common contaminant proteins with

carbamidomethyl (C) as a fixed modification and three variable modifications as follows: Label:13C(6)15N(2) (K), Label:13C(6)15N(4) (R), Oxidation (M). This protocol allowed unlabeled or "light" peptides to be detected in the search of the "heavy" digest as an initial gauge of label incorporation. Further evaluation was performed by directly inspecting mass spectra.

SDS-PAGE separation and in-gel digestion

Proteins pulled down from +/- compound treated samples were mixed, and samples were reduced by incubation with 5 mM of dithiothreitol (DTT) for 1 h at 60 °C and subsequently alkylated with 15 mM iodoacetamide for 30 min at room temperature in the dark. The proteins were separated by a 4–12% NuPAGE Novex Bis-Tris precast mini gel (Invitrogen) and visualized by Coomassie Blue staining. The gel lane was cut into 12 slices; each was subjected to in-gel digestion by incubation with 40 μL of the sequence-grade modified trypsin (0.25 μg/slice, Promega) in 25 mM ammonium bicarbonate at 37 °C overnight. Resulting peptides were extracted with 40 μL of 1% formic acid in acetonitrile, concentrated in a SpeedVac until full evaporation of organic solvent.

LC-MS/MS analysis and data analysis

An aliquot (7 μL) of each sample was loaded onto a Pico-frit column (New Objective, Woburn, MA) packed with reversed-phase Reprosil-Pur Basic C_{18} particles (75 μm × 15 cm) and coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher, Waltham, MA). Peptides were separated at a flow rate of 0.25 μL/min using a 120-min linear gradient ranging from 8% to 35% B (mobile phase A: 0.1% formic acid/2% ACN; mobile phase B: 98% ACN/0.1% formic acid). Electrospray voltage was 2.3 kV. The instrumental method consisted of a full MS scan (scan range 300-1650 *m*/*z*, with 60 K fwhm resolution @ *m*/*z* 400, target value 1×10^6) followed by data-dependent CID scans of the 20 most intense precursor ions. Peptide precursor ions were selected with an isolation window of 2.0 Da. Target ion quantities for MS and MS^2 were 1×10^6 and 5×10^4 respectively. Dynamic exclusion was implemented with a repeat count of 1 and exclusion duration of 60 s. The mass spectra were searched against the UniProtHuman database using Proteome Discoverer 1.4 (Thermo Fisher, Waltham, MA, USA) with the Mascot[6] search engine at a false discovery rate (FDR) of 0.1%. The mass accuracy was set to 10 ppm for precursor and 0.6 Da for fragment ions. The search parameters took into account static modification of carbamidomethylation at Cys (+57.0215 Da), and differential modifications of oxidation on Met (+15.9949 Da), Heavy Lys (+8.01414 Da), and heavy Arg (+10.0083 Da). Maximally two missed cleavages and three labeled amino acids were allowed. Quantification of SILAC pairs was carried out by the search

algorithm using only peptides uniquely mapped to a given protein. The SILAC ratios were normalized using ratio of proteins identified with two or more peptides.

Table S1. Bromodomain-containing proteins detected in whole-fraction analysis of THP-1 nuclear extract

Protein	Accession	Mascot Score	Matches	Sequences
Transcription intermediary factor 1-□	Q13263	786	46	19
BRD adjacent to zinc finger domain protein 1A	Q9NRL2	350	19	13
BRD-containing protein 3	Q15059	243	10	9
Transcription activator BRG1	F5H0X5	173	12	8
Protein polybromo-1	Q86U86	155	10	8
BRD-containing protein 4	Q4G0X8	108	10	7
Probable global transcription activator SNF2L2	P51531	106	10	9
PH-interacting protein	Q8WWQ0	98	7	5
ATPase family AAA domain-containing protein 2	Q6PL18	64	7	6
Tyrosine-protein kinase BAZ1B	Q9UIG0	42	2	2
Protein kinase C-binding protein 1	Q2HXV1	44	3	3
CREB-binding protein	Q4LE28	33	1	1
Nuclear body protein SP140-like protein	H7BYP4	29	1	1
Histone-lysine N-methyltransferase	Q9NR48	23	1	1
Sp110 nuclear body protein	F5H1M1	22	2	1

Table S2 DiscoveRx BROMOscan™ data for **1** and compound **3**

Bromodomain	PFI-1 K_d (nM)	Compound 3 K_d (nM)
BRD2(1)	62	9
BRD4(1)	63	7
BRD3(2)	72	8
BRD3(1)	87	9
BRDT(1)	94	14
BRD2(2)	99	12
BRD4(2)	180	55
BRDT(2)	220	57
BRPF1	1800	450
EP300	2100	4400
CREBBP	2500	4900
BRD1	4900	8200
TRIM24(PHD,Bromo.)	31000	5500
TAF1(2)	32000	8700
BAZ2A	68000	>30000
BRPF3	~50000	26000
ATAD2A	>150000	>30000
ATAD2B	>50000	>30000
BAZ2B	>50000	>30000
BRD9	>50000	>30000
FALZ	>50000	>30000
PBRM1(2)	>50000	>30000
TAF1L(2)	>50000	17000
TRIM33(PHD,Bromo.)	>50000	>30000
WDR9(2)	>50000	>30000

References

1. **P. Filippakopoulos, S. Picaud, M. Mangos, T. Keates, J.-P. Lambert, D. Barsyte-Lovejoy, I. Felletar, R. Volkmer, S. Müller, T. Pawson, A.-C. Gingras, C. H. Arrowsmith and S. Knapp, *Cell*, 2012, 149, 214-231.**
2. **Z. Nikolovska-Coleska, R. Wang, X. Fang, H. Pan, Y. Tomita, P. Li, P. P. Roller, K. Krajewski, N. G. Saito, J. A. Stuckey and S. Wang, *Anal. Biochem.*, 2004, 332, 261-273.**
3. **S. Picaud, C. D. Da, A. Thanasopoulou, P. Filippakopoulos, P. V. Fish, M. Philpott, O. Fedorov, P. Brennan, M. E. Bunnage, D. R. Owen, J. E. Bradner, P. Taniere, B. O'Sullivan, S. Muller, J. Schwaller, T. Stankovic and S. Knapp, *Cancer Res.*, 2013, 73, 3336-3346.**
4. **Y. S. N. Day, C. L. Baird, R. L. Rich and D. G. Myszka, *Protein Sci.*, 2002, 11, 1017-1025.**
5. **M. C. Jecklin, S. Schauer, C. E. Dumelin and R. Zenobi, *J. Mol. Recognit.*, 2009, 22, 319-329.**
6. **D. N. Perkins, D. J. C. Pappin, D. M. Creasy and J. S. Cottrell, *Electrophoresis*, 1999, 20, 3551-3567.**