Supplementary Information - Material & Methods

Library

The library screened contained 1664 compounds, arrayed in 96-well plates (Thermo Scientific™ Nunc™ 96-Well Polystyrene Conical Bottom) as single compounds at 10 mM in DMSO, sealed and stored at -20 °C (for more details, please refer to Hamon *et al.*, (1)). The quality of all compounds was assured by the vendors as greater than 90% pure with provided quality control data.

HTRF Setup Assays

Titration curves were optimized, keeping donor and donor-coupled proteins at constant concentration and increasing acceptor/acceptor-coupled proteins concentrations to avoid the 'hook effect' (2). Screening conditions were selected at 80% of the maximum observed signal when assessing the titration curve. This protocol ensures the optimization of a sufficient signal to noise ratio to perform HTS with adequate Z'. This optimization is crucial to achieve optimal sensitivity required for the detection of even weak small-molecule inhibitors of each protein-protein interaction. An estimated minimum of 2 to 3 value for signal to noise is necessary to ensure reliable HTS. Such signal to noise ratio was observed for each PPI complex at 80% of the maximum measured signal. The estimated time to reach equilibrium, the period of stability of the measured signal and the DMSO tolerance were evaluated to ensure stability of the signal during HTS (data not shown).

HTRF screen

HTRF assays were performed in white 384 Well Small Volume™ HiBase Polystyrene Microplates (Greiner) with a total working volume of 20 µL. Compounds were dispensed, with either 400 nL per well (2% final DMSO), from a concentration stock of 1mM in 100% DMSO for the primary screen, or with serial DMSO dilutions for secondary screening, counter screen and IC_{50} measurement assays, using a Mosquito Crystal pipetting robot platform (TTP labtech). For bromodomain selectivity assays, 200 nL of compounds were dispensed to obtain a 1% final concentration of DMSO. Primary screening assays have been performed in monoplicate while secondary screenings, counter screens and IC_{50} measurements were performed in triplicates.

All HTRF reagents were purchased from CisBio Bioassays and reconstituted according to the supplier protocols. For each assay 14.7 µL of mix1 (Table S2) is added in the assay wells, containing previously dispensed inhibitors, according to the final concentration and buffer described in Table S2, using a Biomek NX MC pipetting robot (Beckman). After 1 h incubation, 4.9 µL of protein B is added. HTRF signals were measured, after a final incubation, using a PHERAstar *FS* (BMG Labtech) with an excitation filter at 337 nm and

fluorescence wavelength measurement at 620 and 665 nm, an integration delay of 60 µs and an integration time of 400 µs. Results were analyzed with a two-wavelengths signal ratio: [intensity (665 nm)/intensity (620 nm)]*10^4. Percentage of inhibition was calculated using the following equation: % inhibition = [(compound signal) - (min signal)] / [(max signal) - (min signal)] * 100, where 'max signal' is the signal ratio with the compound vehicle alone (DMSO) and 'min signal' the signal ratio without protein B (Table S2). The Z' factor is calculated using the following equation: Z' = 1 – [3(SD of max) + 3(min SD)] /
[(mean max signal) - (mean min signal)], where SD is the standard deviation. For IC_{50} measurements, values were normalized and fitted with Prism (GraphPad software) using the following equation: Y = 100 / (1 + ((X / IC50)^Hill slope)).

Autofluorescence experiment

Autofluorescence experiments were performed at 20 µM final inhibitor concentration with 2% final concentration of DMSO. 400 nL of compounds were dispensed in 384 well plates has previously described, and 19.6 µL of buffer (Hepes 50 mM pH 7.5, 150 mM NaCl, 0.1% BSA (Sigma)) were then added. After overnight incubation at 4 °C HTRF signals were measured. Analyses were performed directly comparing the 620 and 665 nm fluorescence values with the fluorescence values at the same wavelength of the buffer + vehicle (DMSO). Thresholds have been set up at 10 times the control value measured at 665 nm and 50 times the control value at 620 nm.

SNAP-tag® labeling

The HTRF assay for the NS3/NS5 complex has been set-up using a SNAP-tag construct. This 20 KDa tag is based on a family of enzymes called « suicide enzymes » as they react covalently with their substrate which blocks the enzyme and prevents any subsequent enzymatic activity (3). SNAP-tag is a mutant version of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase that reacts specifically and rapidly with benzylguanine (BG) derivatives, leading to an irreversible covalent bond. A large variety of BG derivatives have been engineered to label proteins of interest with fluorophores compatible with *in vivo* imaging, microscopy or even immobilization on a specific support. We used one of these derivatives, 'BG Red', as acceptor for our HTRF assay. This type of labeling presents a main advantage compared to « classic » labeling since it does not require secondary labeled-antibodies to label the protein of interest with a HTRF compatible fluorophore.

SNAP-tag tagged protein were incubated at a final concentration of 20 µM in presence of 3 time excess of Tag-lite ® SNAP Red (CisBio Bioassays) in Hepes 50 mM pH 7.5, 150 mM NaCl overnight at 4 °C in the dark in low absorbent tubes. Labeled protein and unreacted dye were then separated using Ilustra NAP-5 Columns (GE healthcare). Protein concentration and labeling efficiency was evaluated by measuring protein and dye absorbance,

respectively at 280 and 650 nm using a NanoDrop 2000 (Thermo scientific). Proteins were stored at -80 °C after addition of BSA at a final concentration of 0.1%.

Molecular biology

Dengue Virus Proteins

Dengue virus proteins were amplified from a synthetic gene (GeneArt, Life technologies) of dengue serotype 3 of Singapore. Non-structural protein 3 (NS3) fused, through G4-S-G4 linker, on the N-terminal part with hydrophilic core of 18 amino acid (43-60) of NS2B protein (NS3-NS2B) of dengue virus serotype 3 (DV3), amplified from the synthetic gene (forward primer : GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGTGCAGATC TGACCGTTGAAAAAGC, reverse primer: GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTACCAAATTGCGCCTTCGCTTTC), have been cloned in a pETG-30A *(*Arie Geerlof, EMBL, Hamburg, Germany) *by* Gateway® recombination (Life technologies) for GST fusion. For the cloning of methyltransferase domain of non-structural protein 5 (MTase NS5) we used a modified Gateway® pDEST™17 Vector (Life technologies) (insertion of an XhoI restriction site between His tag and attR1 site by site directed mutagenesis). SNAP-tag previously amplified from pSNAP-tag® (T7)-2 Vector (New England Biolabs) (forward primer: GGG GCT CGA GTT ATG GAC AAA GAT TGC GAA ATG AAA C, reverse primer: ATA TAC TCG AGT CCC AGA CCC GGT TTA CCC AG) was inserted by restriction ligation in XhoI site. The coding sequence of MTaseNS5 DV3 amplified (forward primer: GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGTGGTACAG GTAGCCAGGGTG, reverse primer: GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTAATGACGGGTGCCTGCACCCAG) from the synthetic gene was then sub cloned in this vector by Gateway® recombination (Life technologies) resulting in a MTase NS5 fused with His and SNAP-tag on the N-terminal part.

GRASP55 PDZ domains

To generate GRASP55 expression constructs, the full-length mouse GORASP2 cDNA encoding the mGRASP55 protein was amplified by polymerase chain reaction (PCR) using the oligonucleotides CTCGAGATGGGCTCCTCGCAGAGC and GGATCCCCAGAAGGCTCTGAAGCATCTGC.

The amplification product was cloned in the pGEM‐T Easy vector (Promega). Then, this mouse GORASP2 cDNA was used as template for PCR amplification of the open reading frame (ORF) for mGRASP55 PDZ12 (aa 2‐208) and mGRASP55 Full‐length (FL) (aa 2‐451) using forward and reverse oligonucleotides flanked by attb1 and attb2 recombination sites. The following primers pairs were used:

the forward primer was: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGCTCCTCGCAGAGCGTCGAGAT and the reverse primers were : GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTCAAAGGGGCGTGTAGGTATTCGGTGCA and GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAGAAGGCTCTGAAGCATCTGCATCAGAC for mGRASP55 PDZ12 and mGrasp55 FL respectively. The amplicons were cloned by the BP reaction into pDONRZeo to produce the corresponding entry vectors. The coding sequences were transferred by LR cloning in pDEST^{TM}15 prokaryotic expression vectors intended to produce the corresponding Nterminal GST-tagged fusion protein.

Syntenin PDZ domains

Human syntenin1 cDNA (4) was cloned in 3' into the pGEX-5X vector (Amersham Pharmacia Biotech AB) for the expression of recombinant GST-syntenin1 full length.

Protein expression and purification

Dengue Virus Proteins

The dengue virus proteins were expressed in T7 Express Competent *E. coli* (NEB). Cells were grown in LB medium (Euromedex) at 37 °C until OD_{600} = 0.6 was reached and then induced using 0.5 mM isopropylβD-thiogalactopyranoside (IPTG) and cultured overnight at 17 °C for NS2B-NS3-GST and 5 hrs at 25 °C for MTaseNS5 SNAP-tag fusion. Cells were pelleted by centrifugation (10 min at 7,000*g*), and pellets were re-suspended in 20 mM HEPES (pH 7,5), 500 mM NaCl, 10% glycerol, 5 mM Imidazole, 2 mM 2-Mercaptoethanol, 1 mg/mL lysozyme, 22 µg/mL DNase I and 0.2 mM of benzamidine. After sonication and centrifugation (30 min at 45,000*g*), the supernatant was purified on HisPur™ Cobalt or GST agarose resin (Thermo Scientific). Elution fractions containing protein were then loaded onto a Superdex 75 or 200 gel filtration column (GE Healthcare) and eluted with 50 mM Hepes (pH 7.5), 300 mM NaCl, 1 mM DTT. Purified proteins were further concentrated and stored at −80 °C.

GRASP55 PDZ domains

The production of N−terminal GST−tagged GRASP fusion proteins were accomplished by induction for 3 h at 37 °C or 18 h at 25 °C with 0.2 mM IPTG in E. coli BL21 (DE3) bacteria cells transformed with the purified pDEST^{TM}15 GRASP expression plasmids. Fusion proteins were recovered from the cell lysates by conventional affinity chromatography on Glutathione resin.

Syntenin PDZ domain

E.coli ER2566 cells were transformed with the human syntenin1 pGEX-5X expression vector.

Expression of N-terminally GST tagged syntenin 1 full length was induced at 30 °C and by the addition of IPTG (Fisher Scientific). Protein was purified using GSTrap4B columns 28-4017-45 (GE Healthcare) using an Akta Explorer system (GE Healthcare).

Nef protein & SH3 domain from Hck

For the Hck SH3 domain, the prokaryotic expression vector and the GST affinity chromatography purification protocol have been previously described in (5).

For the HIV-1 Nef WT protein, the prokaryotic expression vector and the His affinity chromatography purification protocol have been kindly provided by Dr. Sebastian Breuer as described in (6). For both Hck-SH3 and HIV-1 Nef WT, protein peak fractions were further purified using a Superdex 75 10/300 GL column (GE Healthcare) in 20 mM TRIS pH 8.0, 150 mM NaCl Buffer.

BRD4

For isothermal titration Calorimetry (ITC), thermal shift assay (TSA) and crystallogenesis, BRD4(1) was produced and purified using a histidine tag affinity chromatography as described by Filipakopoulos *et al.* (7). For these experiments, a pNIC28-BSA4 expression vector containing BRD4(1) and a Tobacco Etch Virus (TEV) protease cleavage site have kindly been provided by Stefan Knapp laboratory from the SGC at the University of Oxford. After size exclusion chromatography, the fractions presenting pure BRD4 after TEV cleavage of the histidine tag were pooled and concentrated to 25 mg/mL for crystallogenesis. For ITC assays, the protein was concentrated up to at 6 mg/mL and the DTT was removed using a buffer exchange column (PD10 from GE healthcare) equilibrated with 10 mM HEPES pH 7.5, 150 mM NaCl. For HTRF experiments, a BRD4(1) synthetic gene that includes a TEV cleavage site was purchased from LifeTechnology in a pDONR transport vector before cloning into a pDEST^{TM}15 expression vector for GST affinity purification. Protein production and purification was carried out using similar protocols and buffers as used for the His-BRD4(1) system. Purification was carried on GST affinity resin (Thermo Scientific) and reduced glutathione was used for protein release. GST-BRD4(1) was further purified by size exclusion chromatography on a Superdex 16/60 Hiload column (GE Healthcare) using 20 mM TRIS pH 8.0, 150 mM NaCl Buffer.

Bromodomain selectivity Profiles

Selectivity profiles of bromodomain inhibitors were performed as described in HTRF screen and HTRF Setup Assays sections. Concentration of histone peptide was optimized to ensure sufficient signal to noise ratio, sufficient sensitivity for detection of weak inhibitors and comparable data from one bromodomain to another. HTRF detection reagents

(EPIgeneous™ Binding Domain kits) were purchased from Cisbio Bioassays and used according to supplier's protocol. GST tagged bromodomain proteins were purchased from BPS Bioscience and histone peptide from Anaspec.

Fusion protein, counter screen assays

GST-biotinylation: Purified Glutathione S-transferase was biotinylated with Sulfo-NHS-LC-biotin (Thermofischer) at a 10 : 1 biotin : protein molar ratio in Phosphate Buffer Saline pH 8.3 for 40 min on ice followed by quenching with 100 mM glycine pH 8.3 and removal of free biotin by desalting on PD10 column.

GST-SNAP: GST-Snap fusion protein was provided as custom reagent from Cisbio Bioassays and labeled with BG-Red has previously described.

GST-His: GST-His fusion protein has been produced via Gateway plasmid pETG30A (Arie Geerlof, EMBL, Hamburg, Germany) in *E. coli* has previously described for dengue virus protein production.

Cells and Cell Culture

The human leukemia cell line Jurkat (*ATCC® TIB-152*) was maintained in RPMI-1640 medium supplemented with 10% FBS at 37 °C and 5% CO2. Human osteosarcoma cell line (U2OS, ATCC® HTB-96™) was maintained in DMEM supplemented with 10% FBS at 37 °C and 5% CO2.

Western blot

Non-treated Jurkat cells and Jurkat cells treated during 24 hours with compound #**1**, compound #**2** or DMSO were lysed in RIPA lysis buffer (Tris HCl pH 7.5 50 mM, NaCl 150 mM, Triton 1%, SDS 0.1%, sodium deoxycholate 1%) supplemented with protease inhibitor cocktail (P8340, Sigma-Aldrich) and 1 mM PhenylMethylSulfonyl Fluoride (Sigma-Aldrich). 50 µg of protein were loaded onto 10% acrylamide SDS/PAGE and then transferred to nitrocellulose Hybond C-extra membranes, 45 micron (GE Healthcare). The membranes were satured with 5% (wt/vol) skim milk in TBST [Tris-buffered saline/0.1% (vol/vol) Tween 20] 1 hour at room temperature and incubated with anti-myc antibody (clone 9E10, sc40 Santa Cruz) at a 1 : 500 dilution in 0.5% (wt/vol) skimmed milk in TBST overnight at 4 °C. Membranes were then washed with TBST, incubated with an HRP-conjugated anti-mouse secondary antibody (polyclonal goat anti mouse P0447, Pierce) at a 1 : 20,000 dilution in TBST 1 hour at room temperature. Immunoreactive bands were revealed using SuperSignal™ West Dura Extended Duration Substrate (Pierce) detection reagents. Quantification was performed using ImageJ software.

Fluorescence Recovery After Photobleaching (FRAP)

FRAP studies were performed essentially as described (8). In brief, U2OS cells were transfected (Fugene HD; Roche) with mammalian over-expression constructs encoding GFP fused to the N-terminus of human full-length BRD4. The FRAP and imaging system consisted of a Zeiss LSM 710 laser-scanning and control system (Zeiss) coupled to an inverted Zeiss Axio Observer.Z1 microscope equipped with a high-numerical-aperture (N. A. 1.3) 40 x oil immersion objective (Zeiss). Samples were placed in an incubator chamber capable of maintaining temperature and humidity. FRAP and GFP fluorescence imaging were both carried out with an argon-ion laser (488 nm) and with a PMT detector set to detect fluorescence between 500-550 nm. Once an initial scan had been taken, a region of interest corresponding to approximately 50% of the entire GFP positive nucleus was empirically selected for bleaching. A time lapse series was then taken to record GFP recovery using 1% of the power used for bleaching. The image datasets and fluorescence recovery data were exported from ZEN 2009, the microscope control software, into Microsoft Excel to determine the average half-time for full recovery for 10-20 cells per treatment point. Where appropriate, inhibitor was added 1 hour before imaging, which was carried out 24 hours after transfection.

Isothermal titration calorimetry

ITC was used to evaluate the thermodynamics parameters of the binding between BRD4(1) and the selected compounds, using ITC conditions previously described by Filippakopoulos *et al.* (7). Purified BRD4(1) was extensively dialyzed in the ITC buffer containing 10 mM Hepes pH 7.5 and 150 mM NaCl. Compounds were diluted directly in the last protein dialysate prior to experiments. Titrations were carried out on a MicroCal ITC200 microcalorimeter (GE Healthcare, Piscataway, NJ). Each experiment was designed using a titrant concentration (protein in the syringe) set 10 to 15 times the analyte concentration (compound in the cell generally between 10 and 30 µM) and generally using 13 to 17 injections at 15 °C. A first small injection (generally 0.2 µL) was included in the titration protocol in order to remove air bubbles trapped in the syringe prior titration. Raw data were scaled to zero by subtracting a negative control experiment (buffer injected in the cell containing the same compound concentration) or after setting the zero to the titration saturation heat value when possible. Integrated raw ITC data were fitted to a one site non-linear least squares fit model using the MicroCal Origin plugin as implemented in Origin 9.1 (Origin Lab). Finally, ΔG and $T\Delta S$ values were calculated from the fitted ΔH and K_A values using the equations $\Delta G = -R.T.\ln K_A$ and $\Delta G = \Delta H - T\Delta S$.

Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) or Thermal Shift Assay (TSA) was performed as

described previously (9). A protein/Sypro® orange mix containing 2 µM BRD4(1) and a 1 : 1,000 dilution of dye in DMSO (as supplied by Life Technologies) was prepared just before plate setup in 50 mM HEPES pH 7.5, 200 mM NaCl. 19.5 µL of the protein/Sypro orange mix was aliquoted into a 96-well plate and 0.5 µL of compounds (2 mM 100% DMSO, final concentration of 50 µM 2.5% DMSO) or DMSO for control were dispensed. For experiment performed with DTT and tween, 5mM of DTT or 0.012% (0.3 CMC) were added to previously described buffer. After sealing with optical tape, the plates were centrifuged at 1,000 rpm for 1 min at room temperature. Each experiment was performed in triplicate. Thermal melting experiments were carried out using a CFX96 Real Time PCR machine (Biorad). The plates were first equilibrated at 25 °C for 2 min in the PCR machine before starting the thermal melting experiment. The plates were heated by 1 °C per 60 sec from 25 to 95 °C and the fluorescence intensity was recorded at each temperature step using the built-in FRET filter. Raw fluorescence data were treated using Excel files adapted from (9) and T_m measured using a Bolztmann fit equation from GraphPad Prism software.

Crystallography

BRD4(1)-Inhibitor co-crystallization was performed at 19 °C (292K) using the hanging drop vapor diffusion method. For the complex with 1 mM of compound #**2**, 12 mg/mL of BRD4(1) preparation was mixed at a 1 : 1 ratio with the precipitant solution (0.3 M Sodium Formate, 0.1 M NaCl, 22% (w/v) PEG 3350, 10% (w/v) ethylene glycol) and crystals grew to diffracting quality within 5-7 days. Co-crystallization experiments with 5 mM of compound #**1** were carried using the same protocol (Sodium Formate was replaced by 0.3 M $NaNO_3$) and crystals grew to diffracting quality within 3 weeks. Crystals were cryo-protected using the precipitant solution supplemented with 10% glycerol and were flash frozen in liquid nitrogen. Data for compound #**1** and compound #**2** were respectively collected at the ESRF beamlines ID23-2 and ID30A-3. Indexing, integration and scaling were performed using XDS (10). Initial phases were calculated by molecular replacement with Phaser MR (CCP4 suite) (11) using a model of the first domain of BRD4 (extracted from the Protein Data Bank accession code: 2OSS). Initial models for the protein and the ligands were built in COOT (12). The cycles of refinement were carried out with Refmac5 (CCP4 suite (11)). Data collection and refinement statistics can be found in Table S1. The models and structure factors have been deposited with Protein Data Bank accession code for compound #**1**: 5DLZ and compound #**2**: 5DLX.

PAINS" and Aggregators predictions

Pan-assay interference compounds ("PAINS") predictions are indicated based on FAF*Drugs*[3] (http://fafdrugs3.mti.univ-paris-diderot.fr/index.html) (13–15). Default parameters and "Filter Pan Assay Interference Compounds (PAINS) Filter A" have been chosen for analysis.

Aggregator predictions are indicated based on Aggregator advisor website (http://advisor.bkslab.org/) (16).

SI References

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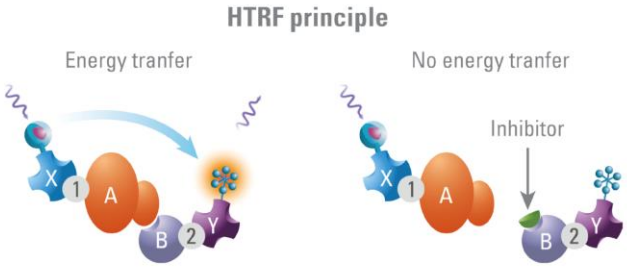
Supplementary Information - Figures

a



b

Protein/Protein Complex	Interface type	Kd (µM)	Protein 1 MW (Including tag, kDa)	Proteins 2 MW (Including tag, kDa)	Signal to noise	Tag 1	Tags 2	HTRF combinaison donnor/ acceptor	Length and temperature of incubation	Z'
NS3/NS5 dengue virus	Protein/protein	1.0	100	50	6	GST	SNAP Tag	Tb-Bg Red	4h RT	0.70
Nef /SH3-Hck (HIV virus)	Protein/domain	1.5	35	33	44	GST	His	KEu-d2	ON 4°C	0.88
Bromodomain/H4 ($K_{ac\ 5/8/12/16}$)	Protein/peptide	2.8	42	3	18	GST	Biotin	KEu-d2	4h RT	0.78
Grasp55/ JamB (PDZ domain)	Protein/peptide	1.0	48	3	26	GST	Biotin	Tb- d2	ON 4°C	0.81
Syntenin/ Syndecan (PDZ domain)	Protein peptide	1.8	60	2	22	GST	Biotin	Tb- d2	ON 4°C	0.92

c

HTRF Autofluorescence (%)	
665nm (10x blank buffer value)	620nm (50x blank buffer value)
3.18%	2.34%

Figure S1*: Principle of the HTRF assays and characterization of the 2P2I$_{3D}$* chemical database.

(a) HTRF assay principle (b) HTRF assays parameters (c) Summary of the autofluorescence experiments. Compounds have been considered as autofluorescent, when fluorescence values were respectively higher than 10 and 50 times the values of the buffer alone at 665 and 620 nm.

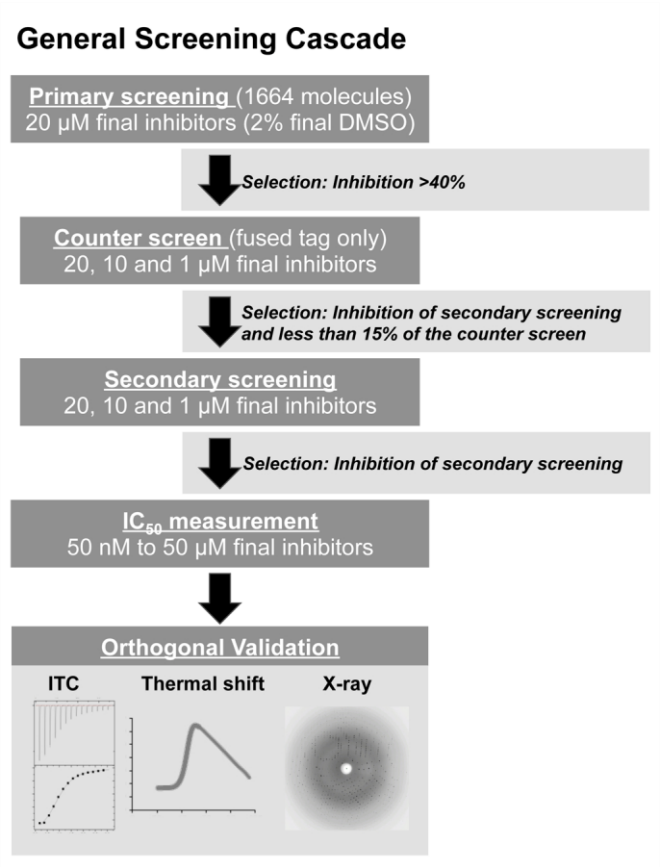


Figure S2: General screening cascade. Primary screenings were performed at 20 µM final concentration of each compounds. We selected compounds with a percentage of inhibition higher than 40% for the second step (secondary screenings and counter screens). We selected for the subsequent IC_{50} measurements, only compounds inhibiting the secondary screening and less than 15% of the counter screen signal. Compounds with suitable dose response curves were validated by orthogonal experiments (isothermal titration calorimetry, thermal shift assays and X-ray crystallography).

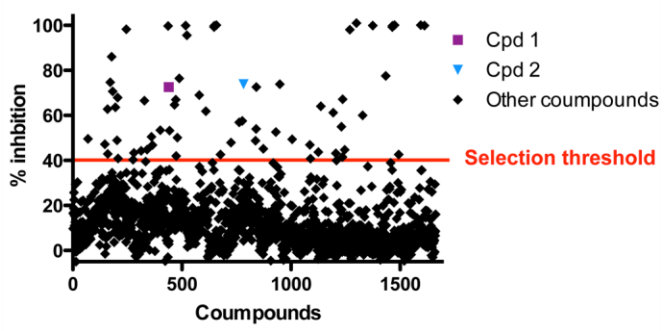


Figure S3: Overall quality-control (distribution) of the HTRF screening. Distribution of the percentage of inhibition values obtained during the bromodomain primary screening. Compound #**1** is highlighted in purple and compound #**2** in blue.

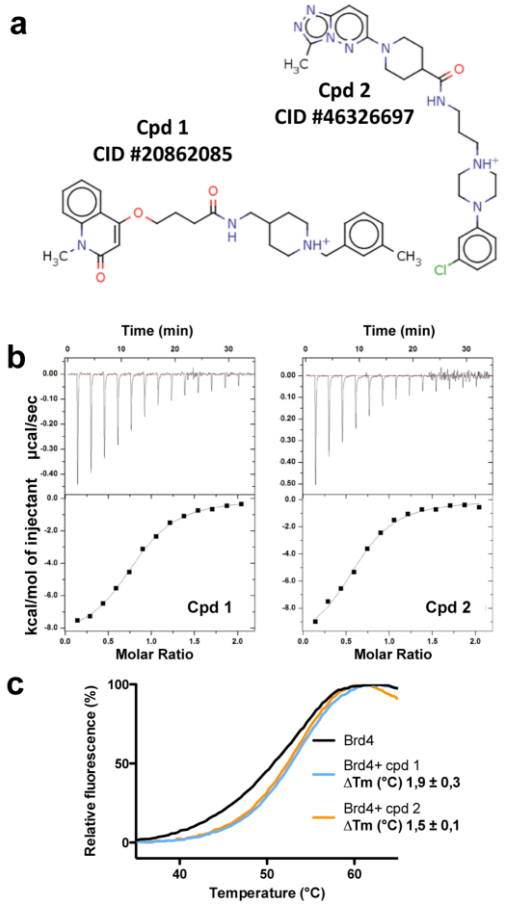
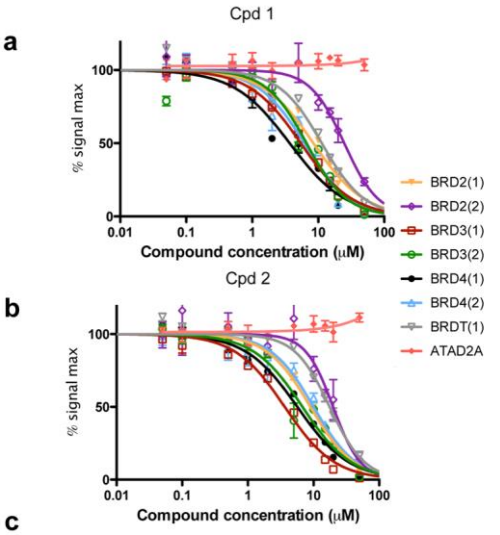


Figure S4: Chemical structures and biophysical characterization of compounds #1 and #2. (a) Chemical structures of compounds #1 and #2. (b) ITC thermograms for the binding of compounds #1 (Kd = 1,8 µM) and #2 (Kd = 2.0 µM) to BRD4(1). (c) Thermal shift experiments performed as 3 independent experiments with compounds #1 and #2 on bromodomain BRD4(1). The black curve represents BRD4(1) alone in presence of 2.5% DMSO, the blue curve BRD4(1) with a 25 fold excess of compound 1 (50 µM 2.5% DMSO, ΔTm = 1.9 °C) and orange curve with compound 2 (ΔTm = 1.5 °C).



Bromodomain	Cpd 1 IC_{50} (μM)	Cpd 2 IC_{50} (μM)
BRD2(1)	8.4 ± 1.1	8.9 ± 0.6
BRD2(2)	24.8 ± 3.1	19.9 ± 2.4
BRD3(1)	5.4 ± 0.4	3.6 ± 0.3
BRD3(2)	6.6 ± 1.1	6.8 ± 1.1
BRD4(1)	3.7 ± 0.6	5.7 ± 0.5
BRD4(2)	6.2 ± 0.8	10.0 ± 0.9
BRDT(1)	11.3 ± 1.4	17.6 ± 1.3
ATAD2A	Not converged	Not converged

Figure S5: *S*electivity of compounds #1 and #2. (a) Selectivity profiles of compounds #1 and (b) #2 on BET bromodomains, BRD2(1) (yellow), BRD2(2) (purple), BRD3(1) (red), BRD3(2) (green), BRD4(1) (black), BRD4(2) (blue), BRDT(1) (grey), ATAD2A (orange), using HTRF assays (c) IC_{50} values (± SEM, N=3) of bromodomain selectivity experiments with compound #1 and compound #2.

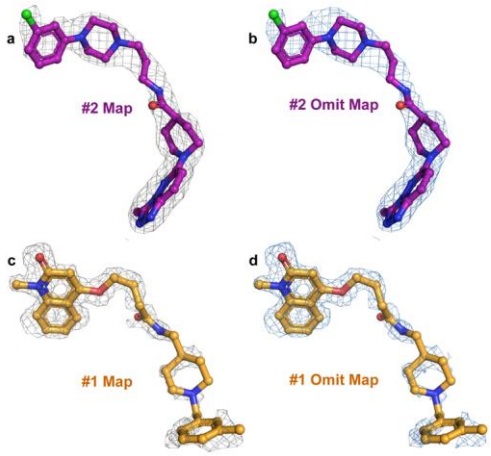


Figure S5: 2Fo-Fc map and omit map around compounds #1 and #2. (a) 2Fo-Fc map contoured at 1σ around compound #2. This map is very well defined for the entire molecule.

(b) 2Fo-Fc omit map contoured at 1σ around the compound #2. (c) 2Fo-Fc map around compound #1. This map indicated a lack of electron density for a part of the compound located outside of the N-acetylated binding pocket. (d) 2Fo-Fc omit map contoured at 1σ sigma around compound #1.

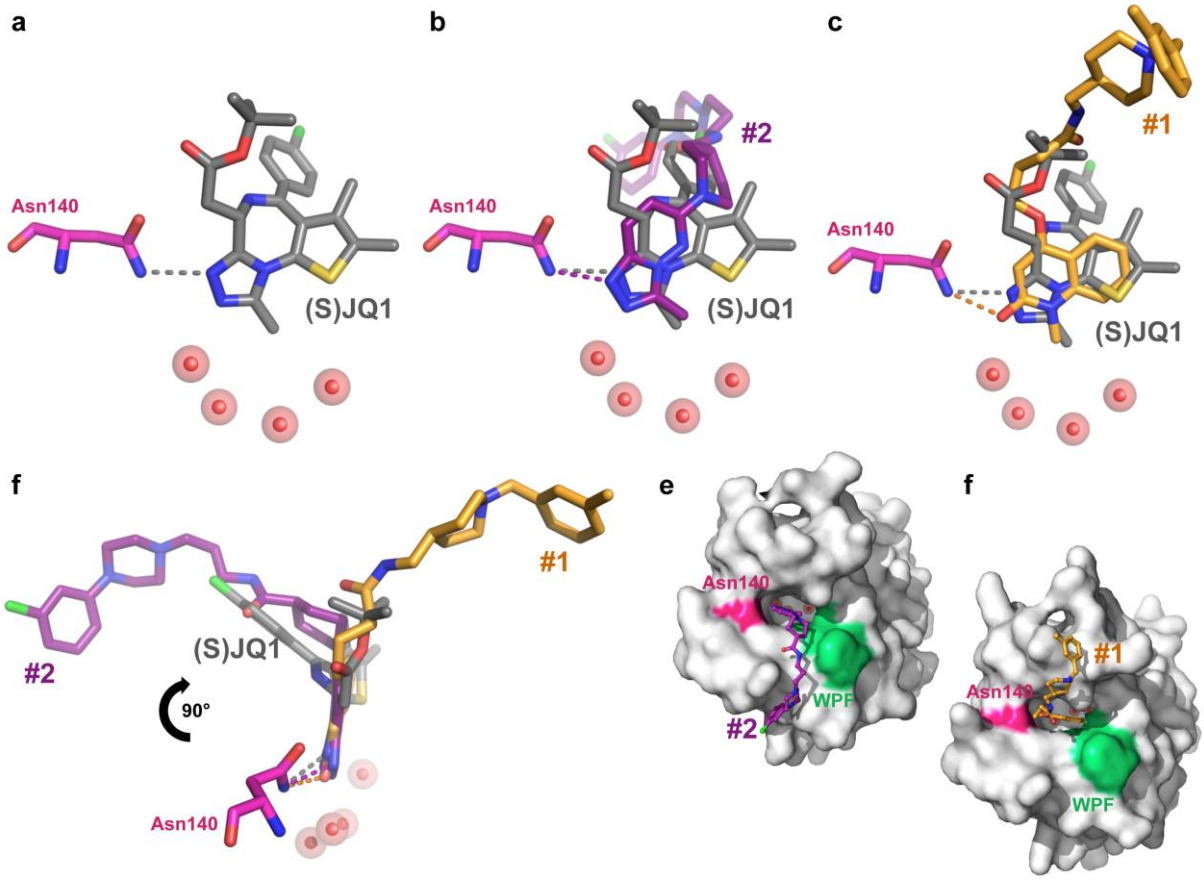


Figure S6: Binding mode of (S)JQ1 compared to compounds #1 and #2. (a) (S)JQ1, a pan-BET inhibitor (grey) in complex with BRD4(1) and detailed view of the the N140 residue (pink) hydrogen bond interaction (dash line). The four highly conserved water molecules (red spheres) are located at the bottom of the cavity. (b) Superimposition of (S)JQ1 with compound #2 (purple). The triazolo moieties of these two compounds are superimposed, with a slight shift. (c) Superimposition of (S)JQ1 (grey) with compound #1 (orange). The oxoquinoline moiety of compound #1 is in the same plane as the thieno-triazolo-diazepine moiety of (S)JQ1. (d) Superimposition of (S)JQ1, compound #1 and compound #2 with a 90° rotation. The chlorophenyl-piperazinyl-piperidinecarboxamide substituents of compound #1 are oriented in the same direction as the (S)JQ1 chlorophenyl moiety, engaging the shelf between the BC loop and the WPF motif. The methylphenyl-piperidinyl-butanamide substituents of compound #2 are oriented in the opposite direction. (e) Surface representations of BRD4(1) in complex with compound #2 and (f) with compound #1. The surface of N140 is colored in pink while the surface of the WPF shelf is highlighted in green.

Supplementary Information – Tables

Table S1: X-ray data collection and refinement statistics

	Cpd 1 (pdb: 5DLZ)	Cpd 2 (pdb: 5DLX)
Crystal parameters		
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell dimensions		
a, b, c (Å)	37.26, 44.24, 78.44	41.72, 48.51, 58.09
α, β, Υ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Data collection		
Beamline	ESRF – ID23-2	ESRF – ID30A-3
Wavelength (Å)	0.8726	0.9677
Resolution range (Å)	50 - 1.70	50 - 1.90
Total reflections	64144 (14342)	46717 (6904)
Unique reflections	9741 (2221)	9664 (1505)
Completeness (%)	96.3 (95.2)	99.3 (97.8)
Average I/σ	7.54 (4.15)	20.09 (3.73)
Rfactor obs (%)	14.9 (42.1)	4.2 (37.9)
Redundancy	4.47 (4.38)	4.83 (4.59)
Structure Refinement		
Starting model for Molecular Replacement	2OSS	2OSS
Resolution range (Å)	39.22 - 1.70	37.25 - 1.80
Rfree	0.3339	0.20707
R value		
Working + test set	0.25529	0.12959
Working set	0.25122	0.12547
Solvent (%)	42.92	37.31
R.m.s.d.		
Bond lengths (Å)	0.028	0.028
Angles (°)	2.329	2.316

Table S2: HTRF assay pipetting procedures

Protein A		Protein B		HTRF reagent A (CisBio)		HTRF reagent B (CisBio)		Buffer assay	Final incubation	Final DMSO concentration	N between partners
Name	**Final Concentration (nM)**	**Name**	**Final Concentration (nM)**	**Name**	**Final Concentration (nM)**	**Name**	**Final Concentration (nM)**				
Mix1		Mix 2		Mix1							
Syntenin1FL-GST	2.5	Syndecan 2 peptide-Biot	47.5	MAb Anti GST-Tb	0.8	Streptavidin d2	10	Buffer A	ON 4°C	2%	19
GRASP55-GST	1.6	JamB peptide-Biot	5.9	MAb Anti GST-Tb	0.8	Streptavidin d2	1.25	Buffer A	ON 4°C	2%	3.7
NS3-GST	5	NS5Mtase-SNAP	15	MAb Anti GST-Tb	0.8	SNAP Red	-	Buffer A	4h RT	2%	3
NEF FL-His	2.5	GST-Hck-SH3	2.5	MAb Anti His-K	1	GST-d2	1.25	Buffer B	ON 4°C	2%	1
Mix 1				Mix 2							
Bromodomaln BRD4(1)-GST	5	H4 Kac 5/8/12/16 peptide	100	MAb Anti GST-K	0.5	Streptavidin d2	12.5	Buffer B	3h RT	2%	20
BRD4(1)-GST	5	H4 KAc 5/8/12/16 peptide	25	MAb Anti GST-K	0.5	Streptavidin d2	3.1	Buffer B	3h RT	1%	5
BRD4(2)-GST	5	H4 KAc 5/8/12/16 peptide	40	MAb Anti GST-K	0.5	Streptavidin XL665	5	Buffer B	3h RT	1%	8
BRD2(1)-GST	5	H4 KAc 5/8/12/16 peptide	15	MAb Anti GST-K	0.5	Streptavidin d2	1.9	Buffer B	3h RT	1%	3
BRD2(2)-GST	5	H4 KAc 5/8/12/16 peptide	15	MAb Anti GST-K	0.5	Streptavidin XL665	1.9	Buffer B	3h RT	1%	3
BRD3(1)-GST	5	H4 KAc 5/8/12/16 peptide	10	MAb Anti GST-K	0.5	Streptavidin d2	1.25	Buffer B	3h RT	1%	2
BRD3(2)-GST	5	H4 KAc 5/8/12/16 peptide	200	MAb Anti GST-K	0.5	Streptavidin XL665	25	Buffer B	3h RT	1%	40
BRDT(1)-GST	5	H4 KAc 5/8/12/16 peptide	20	MAb Anti GST-K	0.5	Streptavidin d2	2.5	Buffer B	3h RT	1%	4
ATAD2A-GST	5	H4 KAc 5/8/12/16 peptide	80	MAb Anti GST-Tb	0.5	Streptavidin XL665	10	Buffer B	3h RT	1%	16
GST-Biotin	6.25	-		MAb Anti GST-Tb	0.8	Streptavidin d2	5	Buffer A	ON 4°C	2%	-
GST-SNAP	10	-		MAb Anti GST-Tb	0.8	SNAP Red	-	Buffer A	4h RT	2%	-
GST-His	2.5	-		MAb Anti His-K	1	GST-d2	1.25	Buffer B	ON 4°C	2%	-

Buffer A 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% BSA

Buffer B 50mM KPO4, pH7, BSA 0,1%, 0.1 M KF