Supporting Information:

Fluorinated Aromatic Amino Acids are Sensitive ^{19}F NMR Probes for Detecting Bromodomain-Ligand Interactions

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Protein Expression and Molecular Biology Materials:
For *E. coli* growth, LB agar, LB media, defined media components including unlabeled amino acids, uracil, thiamine-HCl, nicotinic acid, biotin and buffer components were purchased from RPI corp. 3-Fluorotyrosine, thymine, cytosine, guanosine were purchased from Alfa Aesar. Magnesium chloride, manganese sulfate, succinic acid, calcium chloride and 5-fluoroindole were purchased from Sigma-Aldrich. Miniprep plasmid purification kit was purchased from Clontech.

Methods:

Unlabeled Brd4(1), BrdT(1), and BPTF Protein Expression:

The pNIC28-BSA4 plasmid containing the Brd4(1) and BPTF genes were kind gifts from the laboratory of Stefan Knapp. For protein expression, either the *E. coli* Rosetta (DE3) strain (Novagen) was first transformed with the respective expression plasmid or the BL21(DE3) strain was cotransformed along with the pRARE (Novagen) plasmid and plated onto agar plates containing kanamycin (100 mg/L) and chloramphenicol (35 mg/L). Following overnight incubation at 37 ^{o}C, a single colony was selected from the agar plate and inoculated in 50 mL of LB media containing kanamycin (100 mg/L) and chloramphenicol (35 mg/L). The primary culture was grown overnight at 25 ^{o}C while shaking at 250 rpm. For secondary culture growth, 1 L of LB media containing kanamycin (100 mg/L) was inoculated with the primary culture and cultured at 37 ^{o}C while shaking at 250 rpm. When the O.D. of culture at 600 nm reached 0.6, the shaker temperature was reduced to 20 ^{o}C. After 30 minutes, the expression was induced with 1 mM IPTG overnight for 12-16 h. Cells were harvested by centrifugation and stored at -20 ^{o}C.

Bromodomain Purification:
To purify fluorinated and unlabeled Brd4(1), the cell pellet was thawed at room temperature followed by the addition of lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl and 10% v/v glycerol) containing protease inhibitor PMSF (5 mM). Cells were lysed by sonication and the cell lysate was centrifuged at 6500 g for 30 minutes followed by supernatant filtration over Whatman filter paper. Filtrate containing the histidine-tagged

Brd4(1) was loaded on to a nickel-NTA affinity column and eluted with an imidazole gradient on an AKTA FPLC system monitoring the O.D. at 280 nm. Imidazole was removed from the buffer using a HiPrep column (GE) for buffer exchange into either 50 mM potassium phosphate, pH 7.4, and 100 mM NaCl or 50 mM Tris pH 7.4, 100 mM NaCl. Purified and buffer exchanged protein was treated with TEV protease for either 2 hours at room temperature or alternatively at 4 ^{o}C overnight on a rotating carrousel. The cleaved His-tag, TEV protease and uncleaved Brd4 were removed using nickel-NTA affinity resin.

Site-directed mutagenesis:

To assign the fluorinated resonances in the ^{19}F NMR spectrum, we mutated single tyrosine and tryptophan residues to phenylalanine using a standard PCR amplification method. One common reverse primer and forward primers containing point mutation for (Y65F, Y98F, Y118F, Y137F, Y139F, W75F and W120F) and a primer pairs for (Y97F, Y119F and W81F) were used for PCR amplification. Primer sequences are shown in Table **S1**. Parent plasmid template was digested by Dpn1 nuclease followed by transformation into E. coli BL21(DE3). Three colonies for each mutant were picked and grown in LB media containing kanamycin followed by miniprep plasmid purification (kits from Clontech). Point mutations on plasmids were confirmed by Sanger sequencing. Auxotrophic E. coli DL39(DE3) + pRARE and E. coli BL21(DE3) + pRARE cells were transformed with tyrosine and tryptophan mutation containing plasmids respectively and subjected to protein expression and purification. After several attempts Y97, Y119 and W81 mutants did not express well, and were not purified. These assignments were either inferred (last resonance unassigned for W120) or validated by binding a known ligand (Y119 vs. Y97).

Table S1. Primer sequences for point mutation of tyrosine and tryptophan to phenylalanine:

S. No.	Residue Name	Primer	Sequence
1.	Y65F	Forward	CCAACTGCAATTCCTGCTCAGAG
2.	Y98F	Forward	CCTCCCTGATTACTTCAAGATCAT
3.	Y118F	Forward	GGAAAACAACTTCTACTGGAATGC
4.	Y137F	Forward	ACAAATTGTTTCATCTACAACAAGCC
5.	Y139F	Forward	TGTTACATCTTCAACAAGCCTGG
6.	W75F	Forward	CAAGACACTATTTAAACACCAGTT
7.	W120F	Forward	CAACTATTACTTTAATGCTCAGGAAT
8.	Common	Reverse	CAGCAGCCAACTCAGCTTCCT
9.	Y97F	Reverse	GATCTTATAGAAATCAGGGAGG
10.	Y119F	Reverse	CCTGAGCATTCCAGAAATAGTTG
11.	W81F	Reverse	CTGCTGGAAAGGAAATGCAAACT

Protein Mass Spectral Analysis

Product molecular weight was confirmed by electrospray ionization mass spectrometry (ESI-MS) using a Thermo Scientific Orbitrap Velos LC-MS, Table **S2**. To determine the percent incorporation for fluorinated proteins the integration values of the different deconvoluted

mass peaks are entered into the following equation to determine the relative incorporation e..g, FWBrd4:

$$\%_{incorporation} = \frac{(0FWBrd4)*0+(1FWBrd4)*1+(2FWBrd4)*2+(FWBrd4)*3}{(0FWBrd4)*3+(1FWBrd4)*3+(2FWBrd4)*3+(FWBrd4)*3} * 100$$

0FWBrd4 is 5FWBrd4 with no fluorine substitutions, 1FWBrd4 is 5FWBrd4 with one fluorine substitution, 2FWBrd4 has two fluorines substituted and FWBrd4 has 3 fluorines substituted.

Table S2. Deconvoluted mass spectral data of wild-type Brd4(1) as well as fluorinated variants and mutants. Only data for fully labeled or unlabeled proteins are shown.

Protein	Calculated	Observed
wtBrd4	15083.33	15083.2
5FW-Brd4	15137.3	15136.8
3FY-Brd4	15209.26	15206.6
Y98F Brd4	15175.27	15173
Y118F Brd4	15175.27	15174.2
Y137F Brd4	15175.27	15172.8
Y139F Brd4	15175.27	15174.3
W75F Brd4	15080.27	15079.4
W120F Brd4	15080.27	15080.1
wtBPTF	14437.40	14434.4
5FW-BPTF	14455.40	14454.3
wtBrdT	14148.44	14153.3
5FW-BrdT	14184.42	14188.5

Circular Dichroism:
To check the secondary structural content, far-UV CD spectra (200-260 nm) of unlabeled and labeled proteins were collected using a peltier equipped temperature controlled Jasco J-815 spectropolarimeter at 25 ^{o}C. For all measurements, 20 μM (50 mM Tris buffer pH 7.4 containing 100 mM NaCl) of protein and a 1 mm cuvette path-length were used. Spectral data were collected at a scan rate of 50 nm/min with averaging of 5 spectra. Processed data were baseline corrected against spectra taken with buffer alone.

Thermal Melting: Thermal stabilities of labeled and unlabeled proteins were measured by the change in ellipticity at 222 nm with the increase in temperature from 20 ^{o}C to 80 ^{o}C at the scan rate of 60 degrees/h. The Mid-point of transition was calculated by a sigmoidal fit to determine the T_m.

Isothermal titration calorimetry of unlabeled, 3FY and 5FW labeled Brd4(1):
Auto ITC200 (GE) and Nano-ITC were used to perform isothermal titration calorimetry experiments. A 3FY-Brd4(1) solution was loaded in the Auto ITC200 calorimeter and titrated with (+)-JQ1 diluted in the same buffer as protein (50 mM potassium phosphate pH

7.4, 100 mM NaCl). Note: to facilite solubility, JQ1 solutions were sonicated immediately prior to use. We used 47 μM of 3FY-Brd4(1). A 50 mM (+)-JQ1 stock solution was prepared in DMSO and diluted in the same buffer as protein at concentration of 500 μM and used for the titration. Titrations were carried out by using 2 μL per injection volume of 500 μM (+)-JQ1. Heat liberated by each injection was integrated (ΔH) and plotted against molar ratio of ligand and protein. Obtained data was fitted by using predefined one and two binding site modes. The fitting of data was not sufficient with one binding site whereas a two binding site model produced an appropriate fit, indicating a potential low affinity binding (K_d = 1.6 mM) site which could be from DMSO. The reported K_d is an average value from of two separate titrations.

For binding of (+)-JQ1 (50 μM) to unlabeled Brd4(1) at 25 °C, the experiments were performed using Nano-ITC (TA Instruments). In this experiment, 550 μM unlabeled in 50 mM Tris pH 7.4, 100 mM NaCl and 4 mM DTT and 500 μM 5FW labeled proteins in 50 mM HEPES pH 7.5, 150 mM NaCl and 4 mM DTT were used as a ligand and loaded to the microsyringe (50 μL). The first injection of 0.5 μL was followed by 20 and 24 identical injections of 2 and 2.5 μL respectively with a releasing period of 8 seconds per injection with 200-300 second spacing time between injections. Control heat of dilution was measured by independent protein titration into buffer and was subtracted from the protein-ligand binding experimental data. Data was analyzed by NanoAnalyze software to calculate enthalpy of binding (ΔH) and dissociation constants (K_d). In all cases an independent binding model was used for data fitting. See **Fig. S29** and **Table S22**

Secondary structure measurement by circular dichroism:

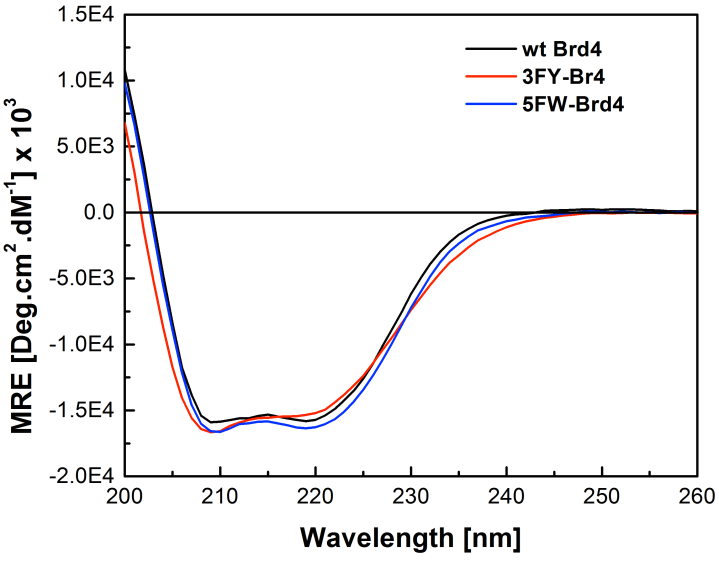

Fig. S1: Far-UV spectra of unlabelled (black), 3FY (red) and 5FW (blue) labelled Brd4(1). Each spectrum was measured at 25 ^{o}C and protein concentration for each spectrum was 20 μM in 50 mM Tris buffer pH 7.4 containing 100 mM NaCl.

Table S3: CD data of unlabeled and fluorine-labeled Brd4(1)

Protein	T_m (^{o}C)	MRE at 222 nm
Unlabeled Brd4 (1)	52.40 ± 0.1	-14893
3FY labeled Brd4 (1)	49.57 ± 0.3	-14413
5FW labeled Brd4 (1)	49.90 ± 0.2	-15677

Effect of 3FY and 5FW labelling on thermal stability of Brd4(1):

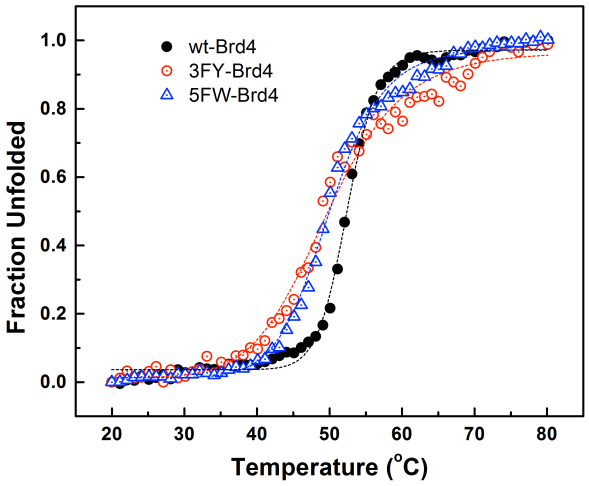

Fig. S2: Thermal denaturation curve of unlabeled (black filled circle), 3FY labeled (red open circle) and 5FW labeled (open blue triangle). The change in ellipticity at 222 nm was measured with increasing temperature from 20 ^{o}C to 80 ^{o}C. 20 μM protein was used in 50 mM Tris buffer pH 7.4 and 100 mM NaCl.

Assignment of 3FY tyrosine chemical shifts on 1D ^{19}F NMR spectra:

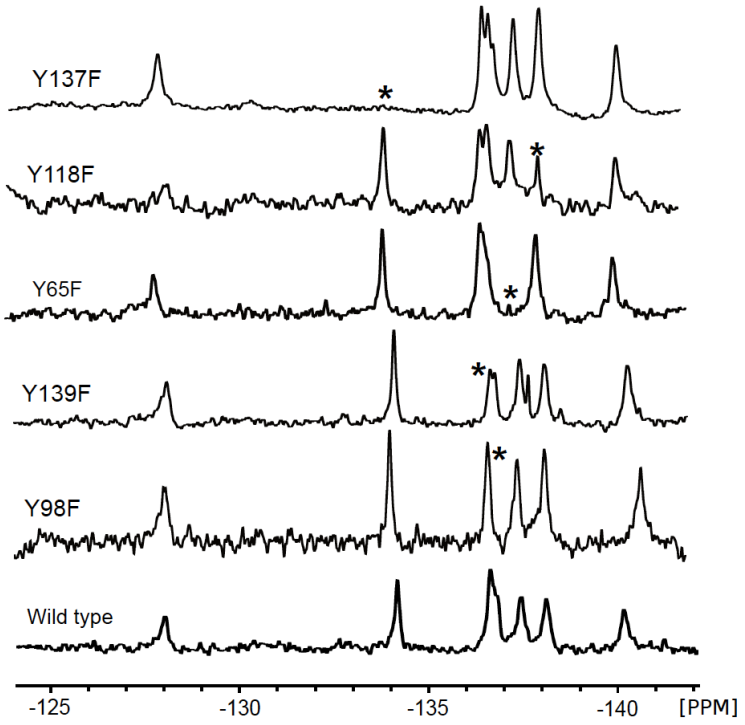

Fig. S3: 1D ^{19}F NMR spectra of 3FY labeled wild-type and tyrosine to phenylalanine point mutated Brd4(1). Asterisks represent the position of missing peaks of corresponding mutated tyrosine. The Y118F expressed poorly, and had low stability. In the Y118F spectrum a partial resonance (which integrates to 0.49) grows in obscuring the absence of the resonance at this position. We have identified this resonance as either a degradation product or resonance from aggregation of the protein see Fig. S5. The two rotamers for Y98, as seen in the Y139F spectrum, aided in the assignment of the shifted Y139 resonance in the acetaminophen binding experiment. (see Fig. **S8**)

Assignment of tryptophan chemical shifts on 1D ^{19}F NMR spectra:

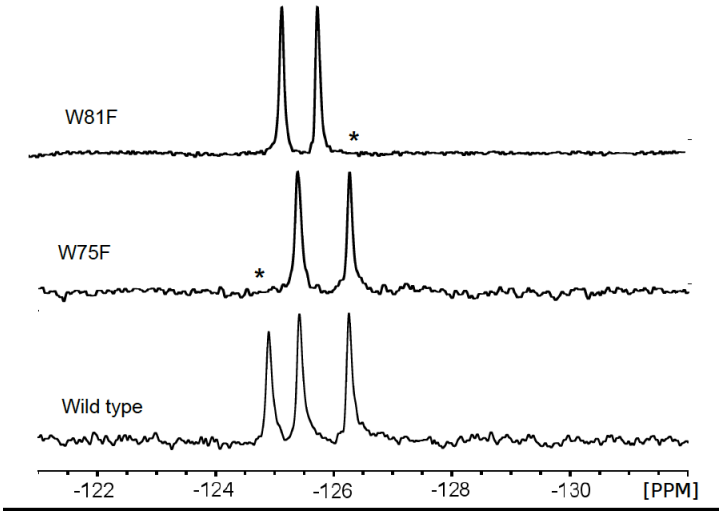

Fig. S4: 1D ^{19}F NMR spectra of 5FW-labeled wild type and tryptophan to phenylalanine point mutated Brd4(1). Asterisks represent the position of missing peaks corresponding of the mutated tryptophan.

Time dependent degradation of a phenylalanine 3FY-Brd4 mutant:

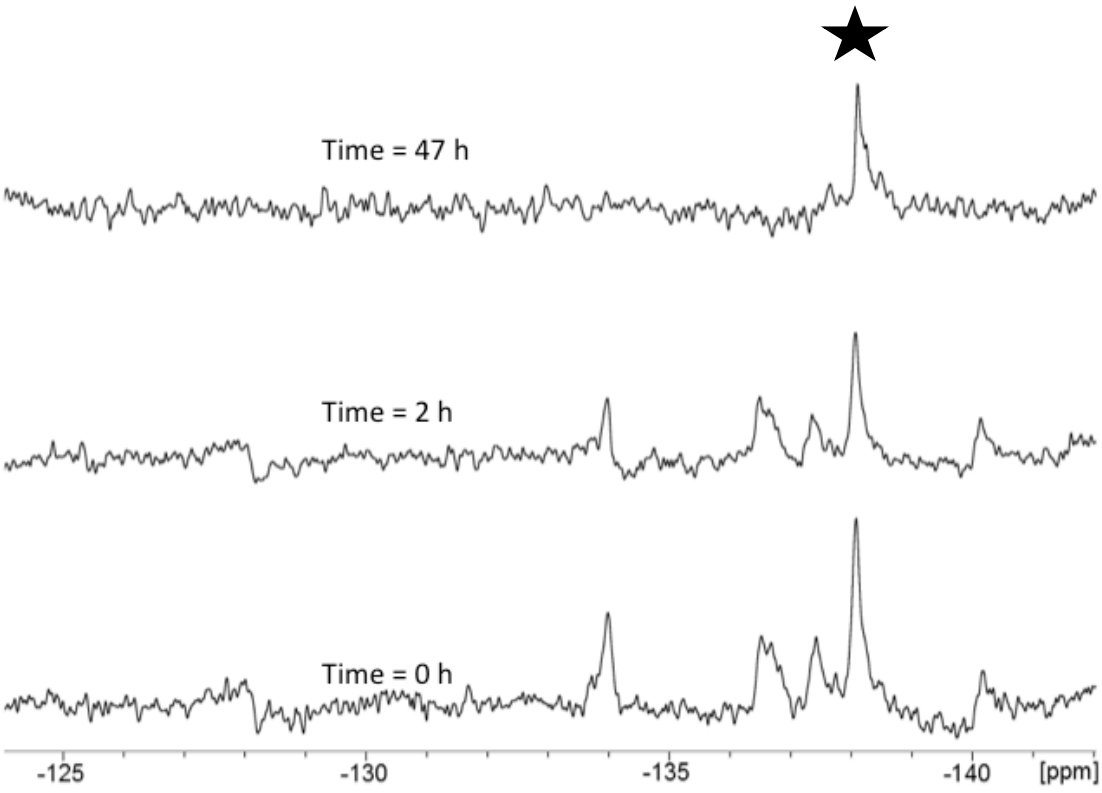

Fig. S5: ^{19}F NMR spectra collected over time of a 3FY-Brd4 mutant. A new resonance gradually increases in intensity at -138 ppm indicated by a black star. The narrowness of the resonance is consistent with degradation of the protein, but may also result from aggregation.

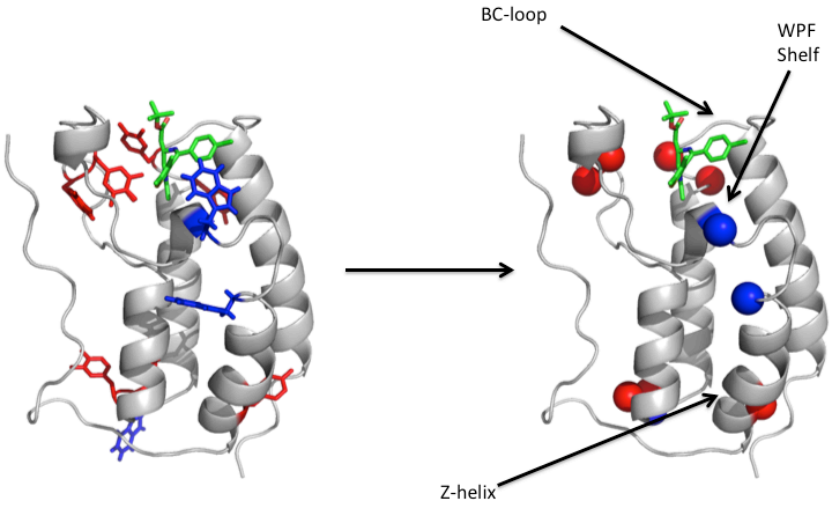

Fig. S6: Left) The crystal structure of (+)-JQ1 bound 3FY-Brd4 is shown on the left with aromatic side chains displayed as colored sticks. To simplify figures and make them more accessible, amino acids are represented as spheres at their respective α-carbons. Right) 3-Fluorotyrosine residues are shown in red, while tryptophan residues are shown in blue.

Aromatic amino acid analysis across all 61 Bromodomains Relative to Brd4(1)

Table S4 Top) Percent conservation of aromatic amino acids within bromodomains at the same site as Brd4(1). Additional aromatic amino acids are all present in bromodomains but found at sites distinct from Brd4(1). (e.g., see the BPTF sequence below). Data was compiled from the bromodomain sequence alignments from Filippakopoulos, P et al. *Cell* **2012**, *149,* 214-231. Residues near the histone binding site are highlighted in red Bottom) Alignment of aromatic amino acids in BET bromodomains and BPTF.

Brd4(1) Sequence	# Tyr	# Trp	# Phe	Sum	Percent
Y65	6		2	8	13
Y97	59			59	97
Y98	29		2	31	51
Y118	9		3	12	20
Y119	59			59	97
Y137	8	2	1	11	18
Y139	40		16	56	92
W75	10	6	4	20	33
W81		18	1	19	31
W120		3		3	5
F79	6		7	13	21
F83			57	57	93
F129			13	13	21
F133			37	37	61
F157	2		36	38	62

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BRD2(1)  97 WPFRQPV...LPDYHKIIKQPMDMGT..ENNYYWAASECMQDFNTMFTNCYIYNK
BRD2(2) 370WPFYKPV...LHDYHDIIKHPMDLST..ENRDYRDAQEFAADVRLMFSNCYKYNP
BRD3(1)  57 WPFYQPV...LPDYHKIIKNPMDMCT..ENNYYWSASECMQDFNTMFTNCYIYNK
BRD3(2) 332WPFYKPV...LHDYHDIIKHPMDLST..DGREYPDAQGFAADVRLMFSNCYKYNP
BRD4(1)  81 WPFQQPV...LPDYYKIIKTPMDMGT..ENNYYWNAQECIQDFNTMFTNCYIYNK
BRD4(2) 374WPFYKPV...LHDYCDIIKHPMDMST..EAREYRDAQEFGADVRLMFSNCYKYNP
BRDT(1)  50 WPFQRPV...LPDYYTIIKNPMDLNT..ENKYYAKASECIEDFNTMFSNCYLYNK
BRDT(2) 293WPFYNPV...LHNYYDVVKNPMDLGT..DNQEYKDAYKFAADVRLMFMNCYKYNP
BPTF       WPFLEPV...APDYYGVIKEPMDLAT..QRRYYEKLTEFVADMTKIFDNCRYYNP
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5FW-Brd4 acetaminophen titration.

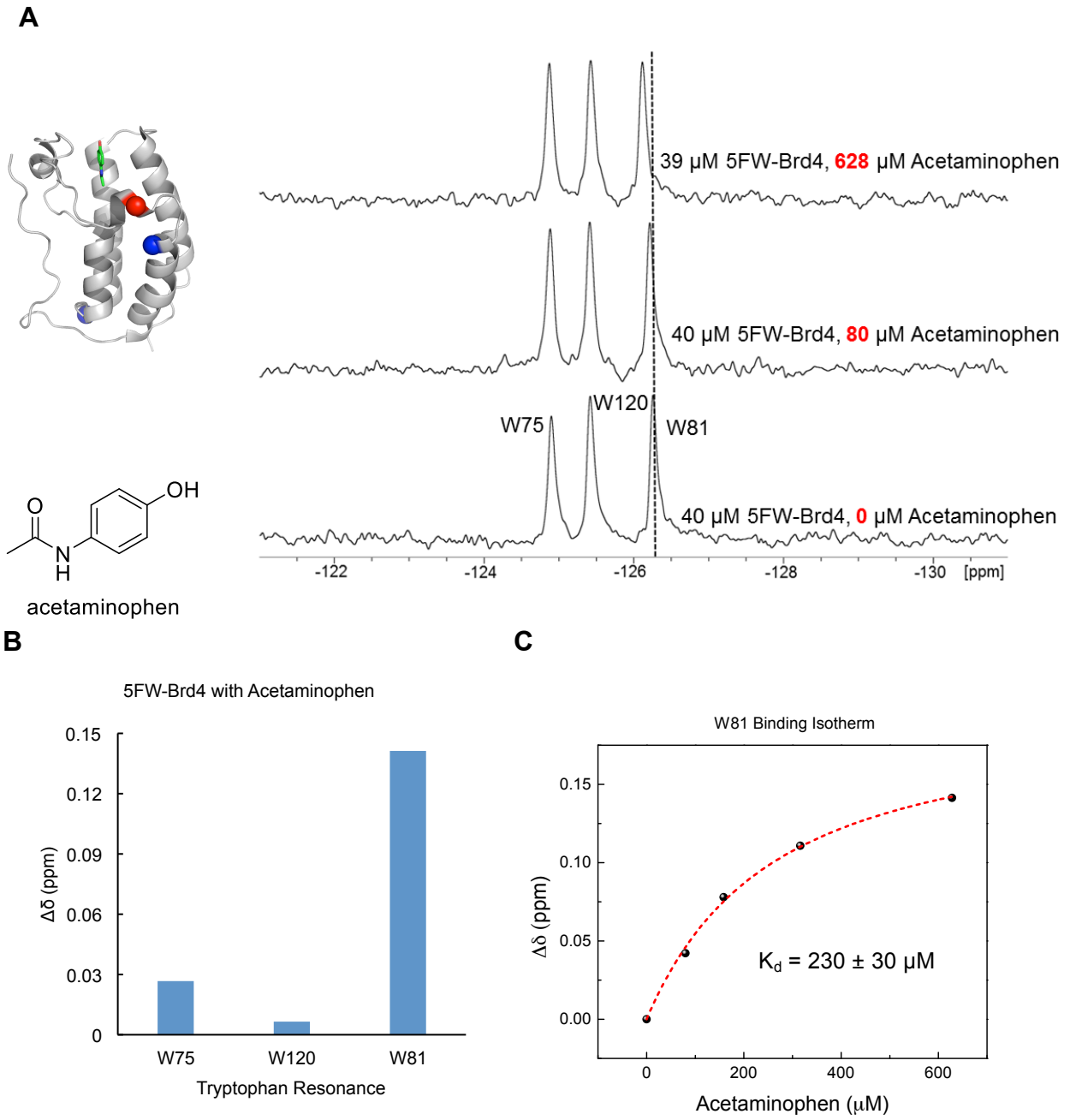


Fig. S7: ^{19}F NMR spectral analysis of 5FW-Brd4 demonstrating chemical shift perturbation of the W81 resonance in response to increasing concentration of acetaminophen. **A)** Representative ^{19}F NMR spectra from titration with acetaminophen. Ribbon diagram of acetaminophen bound to Brd2(1) (PDB Code: 4A9J) was aligned to Brd4(1) (PDB Code: 3MXF), Brd2(1) was removed to indicate probable binding mode of acetaminophen to Brd4(1). The α-carbon of tryptophan are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 5FW-Brd4 tryptophan resonances at 628 μM acetaminophen. **C)** A binding isotherm of W81 perturbation for the acetaminophen titration using equation:

$$\Delta_{obs} = \Delta_{max}\frac{(K_D + [L]_0 + [P]_0) - \sqrt{(K_D + [L]_0 + [P]_0)^2 - (4[P]_0[L]_0)}}{2[P]_0}$$

3FY-Brd4 acetaminophen titration:

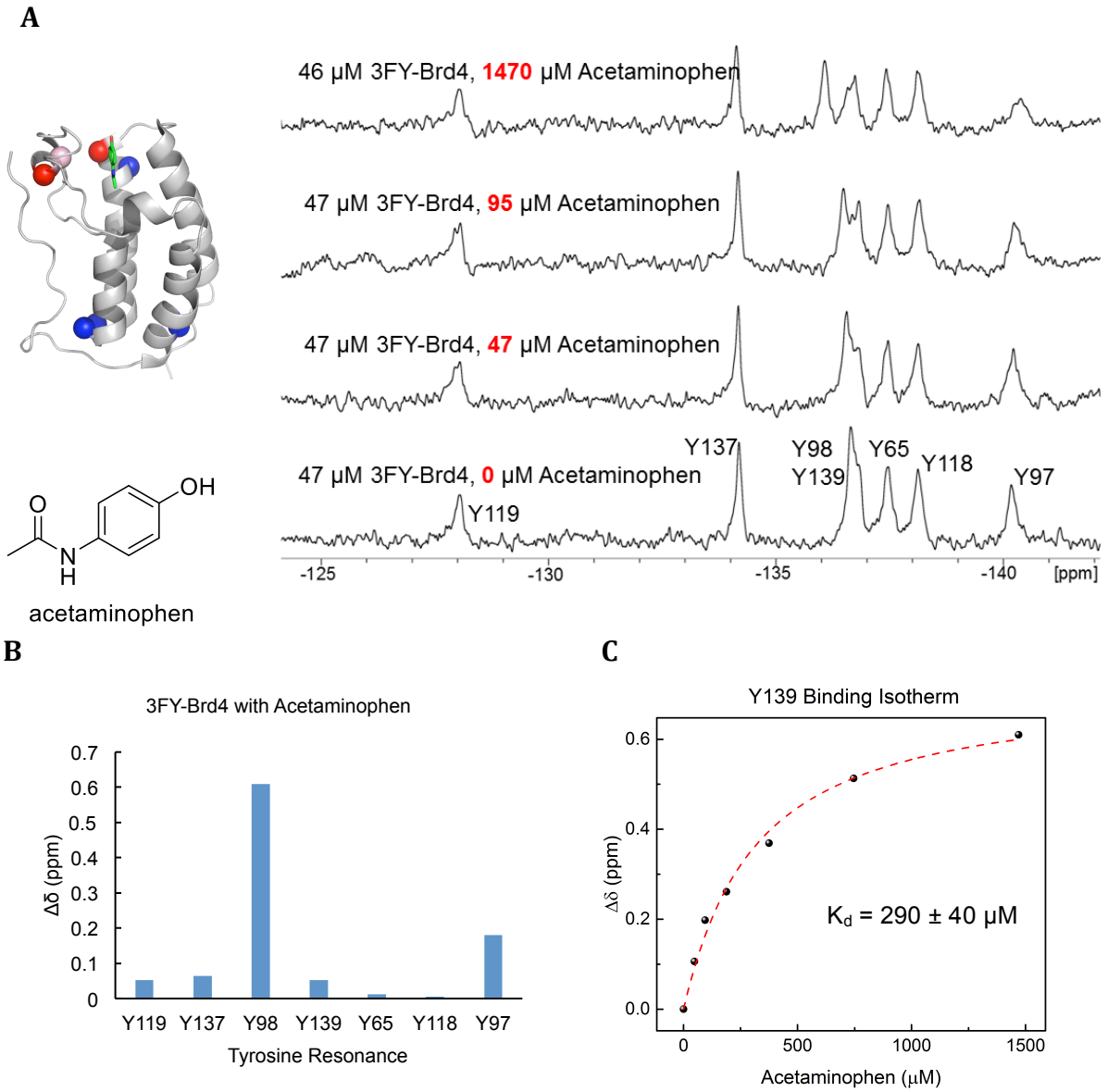


Fig. S8: ^{19}F NMR spectral analysis of 3FY-Brd4 demonstrating chemical shift perturbation of the Y139 resonance in response to increasing concentration of acetaminophen. **A)** Representative ^{19}F NMR spectra from titration with acetaminophen. Ribbon diagram of acetaminophen bound to Brd2(1) (PDB Code: 4A9J) was aligned to Brd4(1) (PDB Code: 3MXF), Brd2(1) was removed to indicate probable binding mode of acetaminophen to Brd4(1). The α-carbon of tyrosine are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 3FY-Brd4 tyrosine resonances at 1470 μM acetaminophen. **C)** A binding isotherm of Y139 perturbation for the acetaminophen titration using equation:

$$\Delta_{obs} = \Delta_{max}\frac{(K_D + [L]_0 + [P]_0) - \sqrt{(K_D + [L]_0 + [P]_0)^2 - (4[P]_0[L]_0)}}{2[P]_0}$$

Table S5. 5FW-Brd4 ^{19}F NMR chemical shift perturbations at different acetaminophen concentrations:

Acetaminophen Concentration	Chemical Shift	W75	W120	W81
0 μM	Chemical shift (ppm)	-124.901	-125.420	-126.259
80 μM	Δδ (ppm)	0.015	0.005	0.042
158 μM	Δδ (ppm)	0.020	0.005	0.07
316 μM	Δδ (ppm)	0.022	0.001	0.110
628 μM	Δδ (ppm)	0.026	-0.006	0.141

Table S6. 3FY-Brd4 ^{19}F NMR chemical shift perturbations at different acetaminophen concentrations:

Acetaminophen Concentration	Chemical Shift	Y119	Y137	Y98/139	Y98/139	Y65	Y118	Y97
0 μM	Chemical shift (ppm)	-128.023	-134.007	-136.603	-136.603	-137.428	-138.049	-140.092
48 μM	Δδ (ppm)	0.035	0.017	0.106	-0.076	-0.010	-0.015	-0.064
95 μM	Δδ (ppm)	0.043	0.041	0.198	-0.045	0.009	-0.015	-0.275
188 μM	Δδ (ppm)	0.094	0.033	0.261	-0.056	-0.009	-0.086	-0.127
375 μM	Δδ (ppm)	0.022	0.043	0.369	-0.021	-0.018	-0.050	-0.148
746 μM	Δδ (ppm)	0.121	0.068	0.513	0.039	0.030	-0.008	-0.220
1470 μM	Δδ (ppm)	0.053	0.064	0.610	0.052	0.013	-0.005	-0.181

5FW-Brd4 (+)-JQ1 titration:

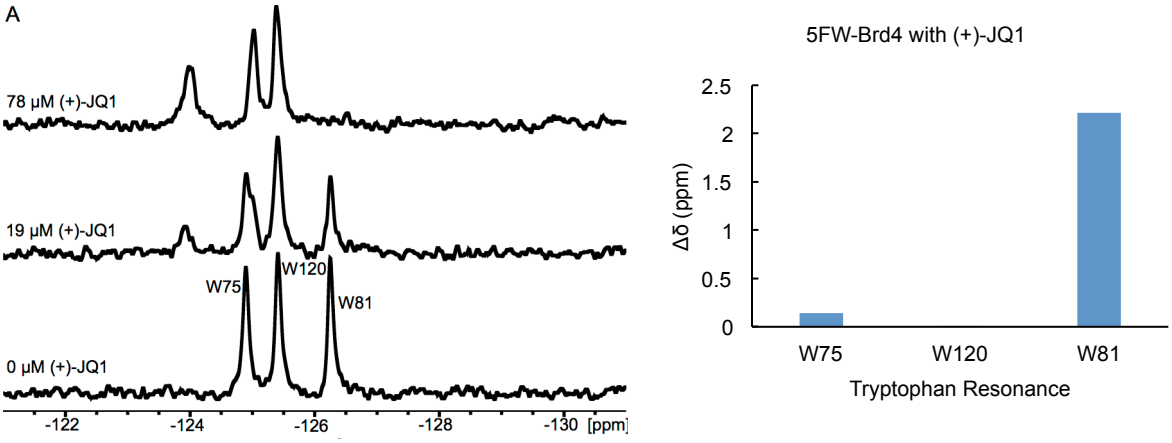


Fig. S9: Absolute value of chemical shift perturbation for the 5FW-Brd4 tryptophan resonances at 78 μM (+)-JQ1. Concentration of 5FW-Brd4 is 39 μM.

Table S7. 5FW-Brd4 ^{19}F NMR chemical shift perturbations at different (+)-JQ1 concentrations:

(+)-JQ1 Concentration	Chemical Shift	W75	W120	W81
0 μM	Chemical shift (ppm)	-124.897	-125.412	-126.252
78 μM	Δδ (ppm)	-0.142	0.000	2.214

3FY-Brd4 (+)-JQ1 titration:

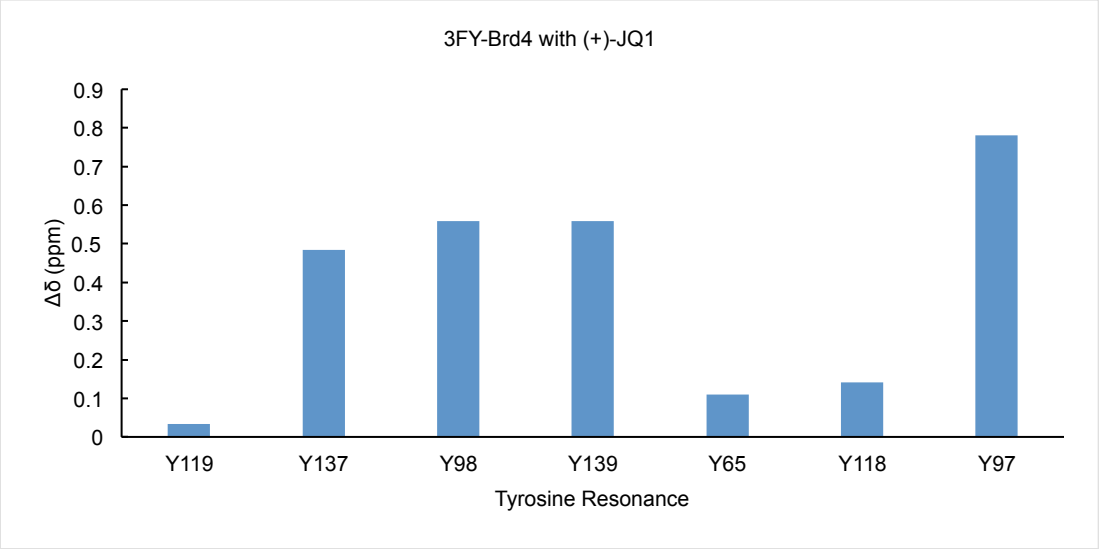


Fig. S10: Absolute value of chemical shift perturbation for the 3FY-Brd4 tyrosine resonances at 94 μM (+)-JQ1. The chemical shifts of the resonances corresponding to Y98 and Y139 were estimated as the midpoint between the peak cluster ranging from -135.804 to -136.363 ppm. Concentration of 3FY-Brd4 is 47 μM.

Table S8. 3FY-Brd4 ^{19}F NMR chemical shift perturbations at different (+)-JQ1 concentrations:

(+)-JQ1 Concentration	Chemical Shift	Y119	Y137	Y98/139	Y98/139	Y65	Y118	Y97
0 μM	Chemical shift (ppm)	-128.044	-134.186	-136.643	-136.643	-137.469	-138.120	-140.172
94 μM	Δδ (ppm)	0.034	0.484	0.559	0.559	0.109	0.141	-0.781

5FW-Brd4 dinaciclib titration:

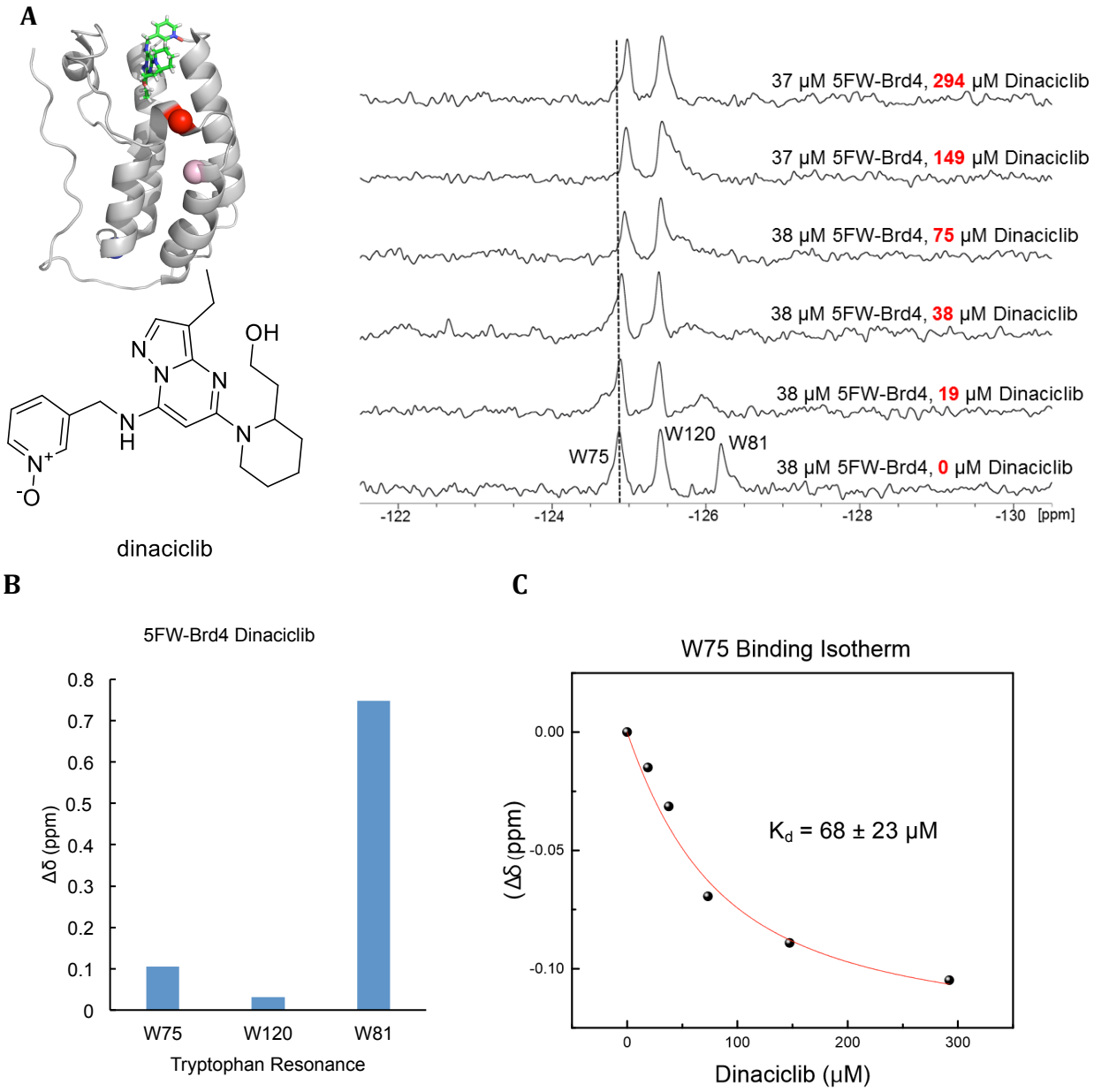


Fig. S11: ^{19}F NMR spectral analysis of 5FW-Brd4 demonstrating chemical shift perturbation of the W75 resonance in response to increasing concentration of dinaciclib. **A)** Representative ^{19}F NMR spectra from titration with dinaciclib. Ribbon diagram of dinaciclib bound to Brd4(1) (PDB Code: 4O70). The α-carbon of tryptophan are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 5FW-Brd4(1) tryptophan resonances at 294 μM dinaciclib. **C)** A binding isotherm of W75 perturbation for the dinaciclib titration.

3FY-Brd4 dinaciclib titration:

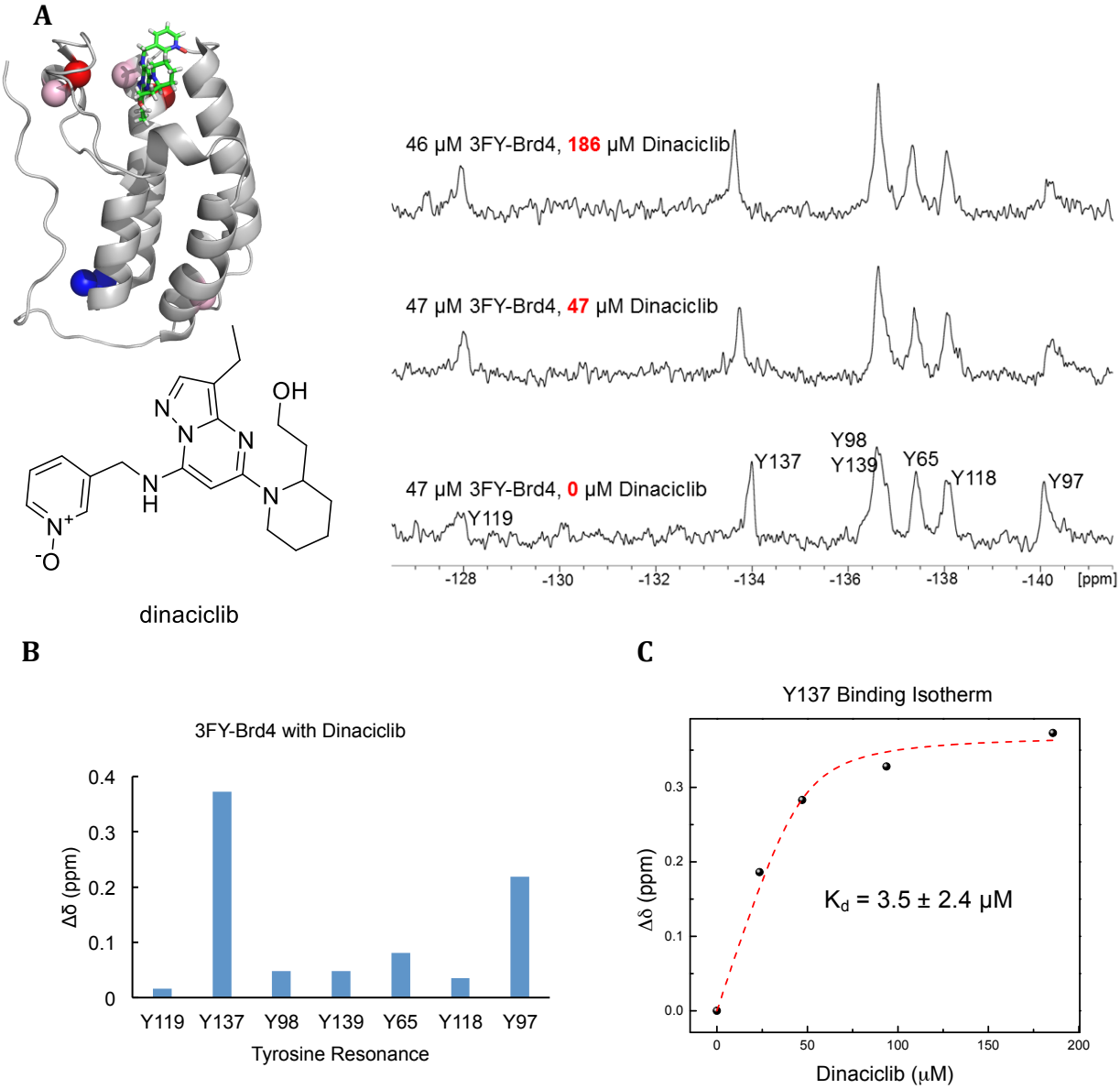


Fig. S12: ^{19}F NMR spectral analysis of 3FY-Brd4 demonstrating chemical shift perturbation of the Y137 resonance in response to increasing concentration of dinaciclib. **A)** Representative ^{19}F NMR spectra from titration with dinaciclib. Ribbon diagram of dinaciclib bound to Brd4(1) (PDB Code: 4O70). The α-carbon of tyrosine are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 3FY-Brd4(1) tyrosine resonances at 186 μM dinaciclib. **C)** A binding isotherm of Y137 perturbation for the dinaciclib titration. These values are slightly different than obtained from the 5FW-Brd4(1) titration, suggesting potential effects from fluorine incorporation and/or the error in the affinity estimate.

Table S9. 5FW-Brd4 ^{19}F NMR chemical shift perturbations at different dinaciclib concentrations:

Dinaciclib Concentration	Chemical Shift	W75	W120	W81
0 μM	Chemical shift (ppm)	-124.875	-125.400	-126.178
19 μM	Δδ (ppm)	-0.015	0.005	0.233
38 μM	Δδ (ppm)	-0.031	0.009	*
75 μM	Δδ (ppm)	-0.069	-0.016	0.546
149 μM	Δδ (ppm)	-0.089	-0.032	*
294 μM	Δδ (ppm)	-0.105	-0.031	0.748

Table S10. 3FY-Brd4 ^{19}F NMR chemical shift perturbations at different dinaciclib concentrations:

Dinaciclib Concentration	Chemical Shift	Y119	Y137	Y98/139	Y98/139	Y65	Y118	Y97
0 μM	Chemical shift (ppm)	-127.884	-133.995	-136.595	-136.595	-137.411	-138.035	-140.074
24 μM	Δδ (ppm)	-0.100	0.186	-0.041	-0.041	-0.002	-0.068	-0.037
47 μM	Δδ (ppm)	-0.084	0.283	-0.024	-0.024	0.043	-0.015	-0.183
94 μM	Δδ (ppm)	-0.037	0.328	-0.031	-0.031	0.059	-0.009	-0.192
186 μM	Δδ (ppm)	-0.016	0.373	-0.048	-0.048	0.081	-0.036	-0.219

5FW-Brd4 BI2536 titration:

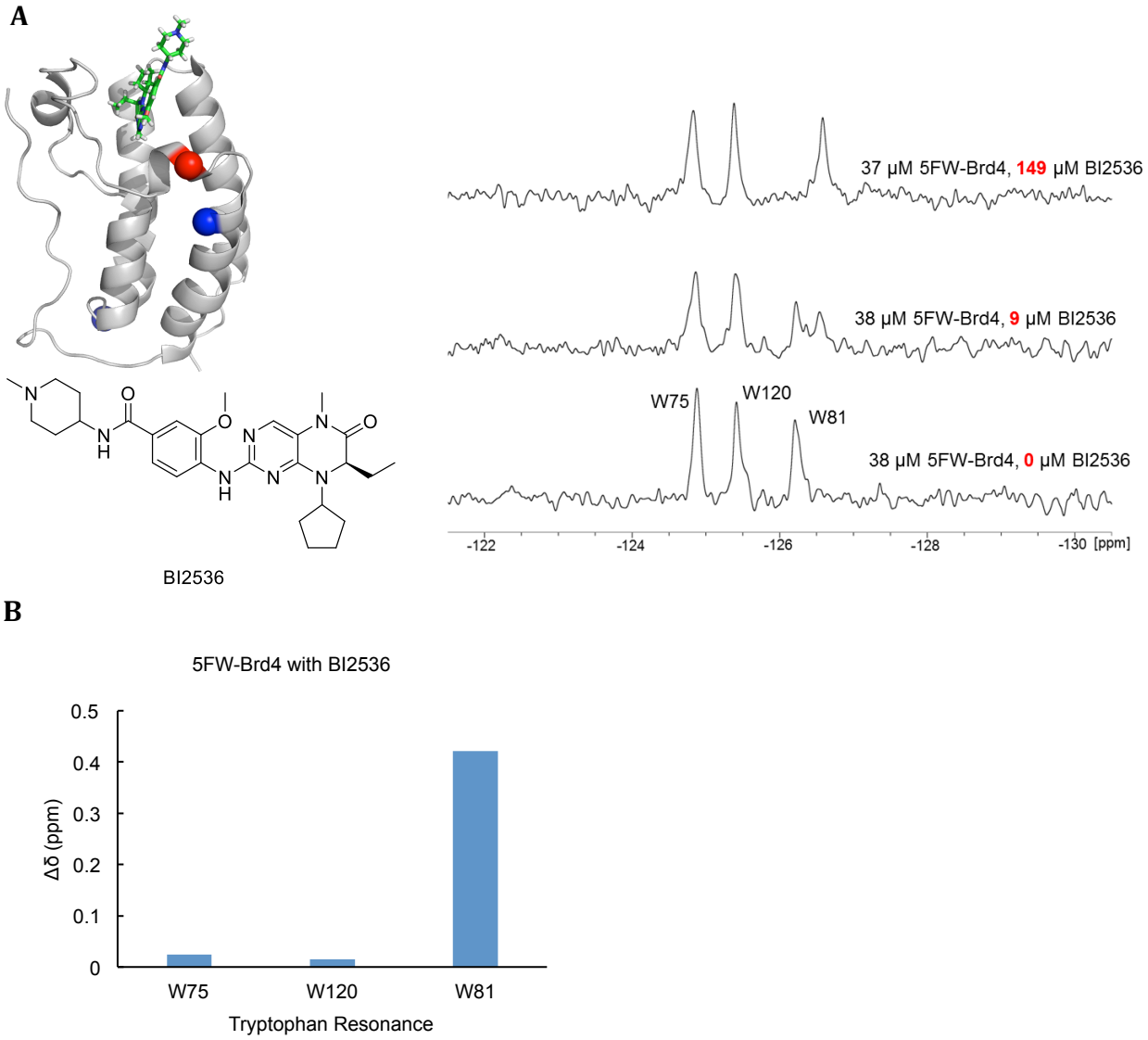


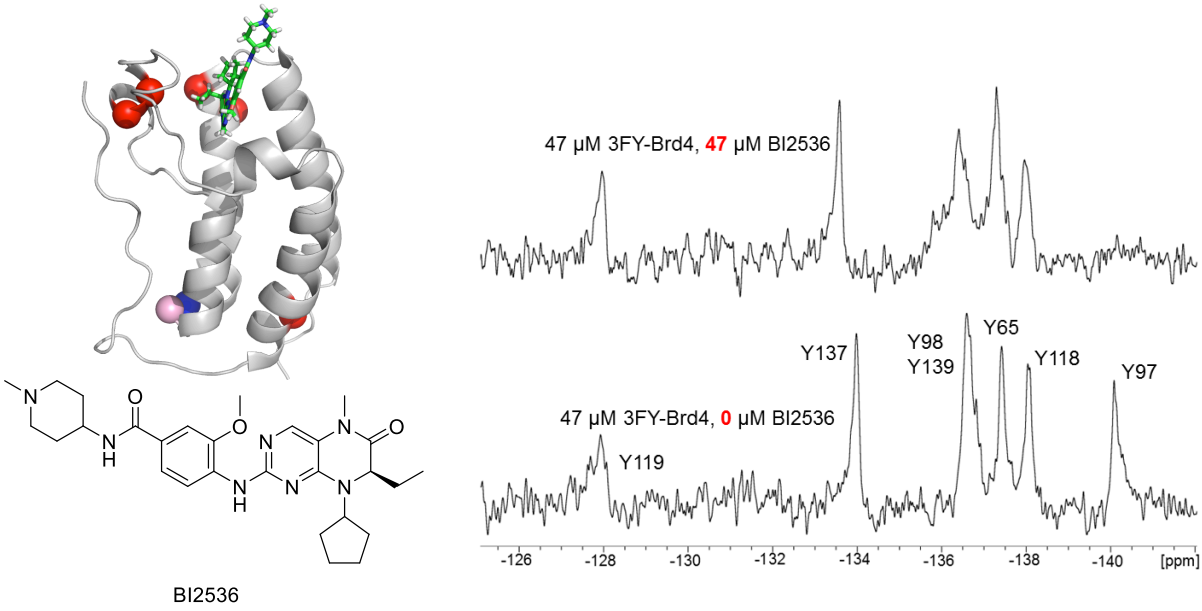
Fig. S13: ^{19}F NMR spectral analysis of 5FW-Brd4 demonstrating chemical shift perturbation of the W81 resonance in response to increasing concentration of BI2536. **A)** Representative ^{19}F NMR spectra from titration with BI2536. Ribbon diagram of BI2536 bound to Brd4(1) (PDB Code: 4O74). The α-carbon of tryptophan are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 5FW-Brd4(1) tryptophan resonances at 149 μM BI2536.

Table S11. 5FW-Brd4 ^{19}F NMR chemical shift perturbations at different BI2536 concentrations:

BI2536 Concentration	Chemical Shift	W75	W120	W81
0 μM	Chemical shift (ppm)	-124.884	-125.417	-126.207
149 μM	Δδ (ppm)	0.024	0.015	-0.421

3FY-Brd4 BI2536 titration:

A



B

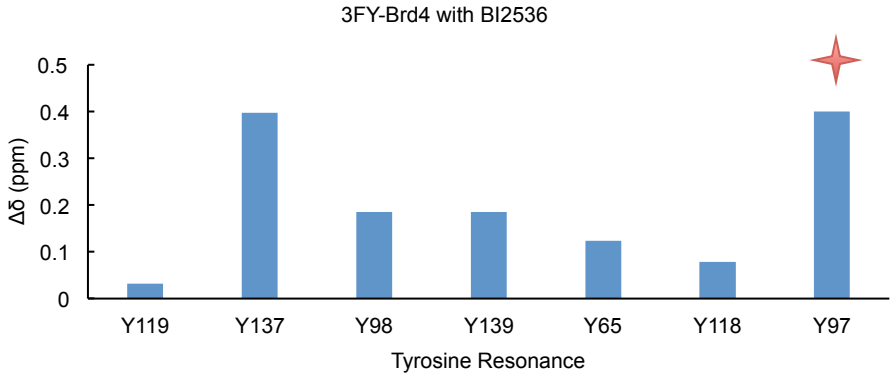


Fig. S14: ^{19}F NMR spectral analysis of 3FY-Brd4 demonstrating chemical shift perturbation of various resonances in response to increasing concentration of BI2536. **A)** Representative ^{19}F NMR spectra from titration with BI2536. Ribbon diagram of BI2536 bound to Brd4(1) (PDB Code: 4O74). The α-carbon of tyrosine are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 3FY-Brd4(1) tyrosine resonances at 47 μM BI2536. Red star indicates broadening into baseline.

Table S12. 3FY-Brd4 ^{19}F NMR chemical shift perturbations at different BI2536 concentrations:

BI2536 Concentration	Chemical Shift	Y119	Y137	Y98/139	Y98/139	Y65	Y118	Y97
0 μM	Chemical shift (ppm)	-127.9	-134	-136.6	-136.6	-137.4	-138	-140.084
47 μM	Δδ (ppm)	-0.032	0.398	0.1853	0.1853	0.1233	0.0788	*

5FW-Brd4 TG101348 titration:

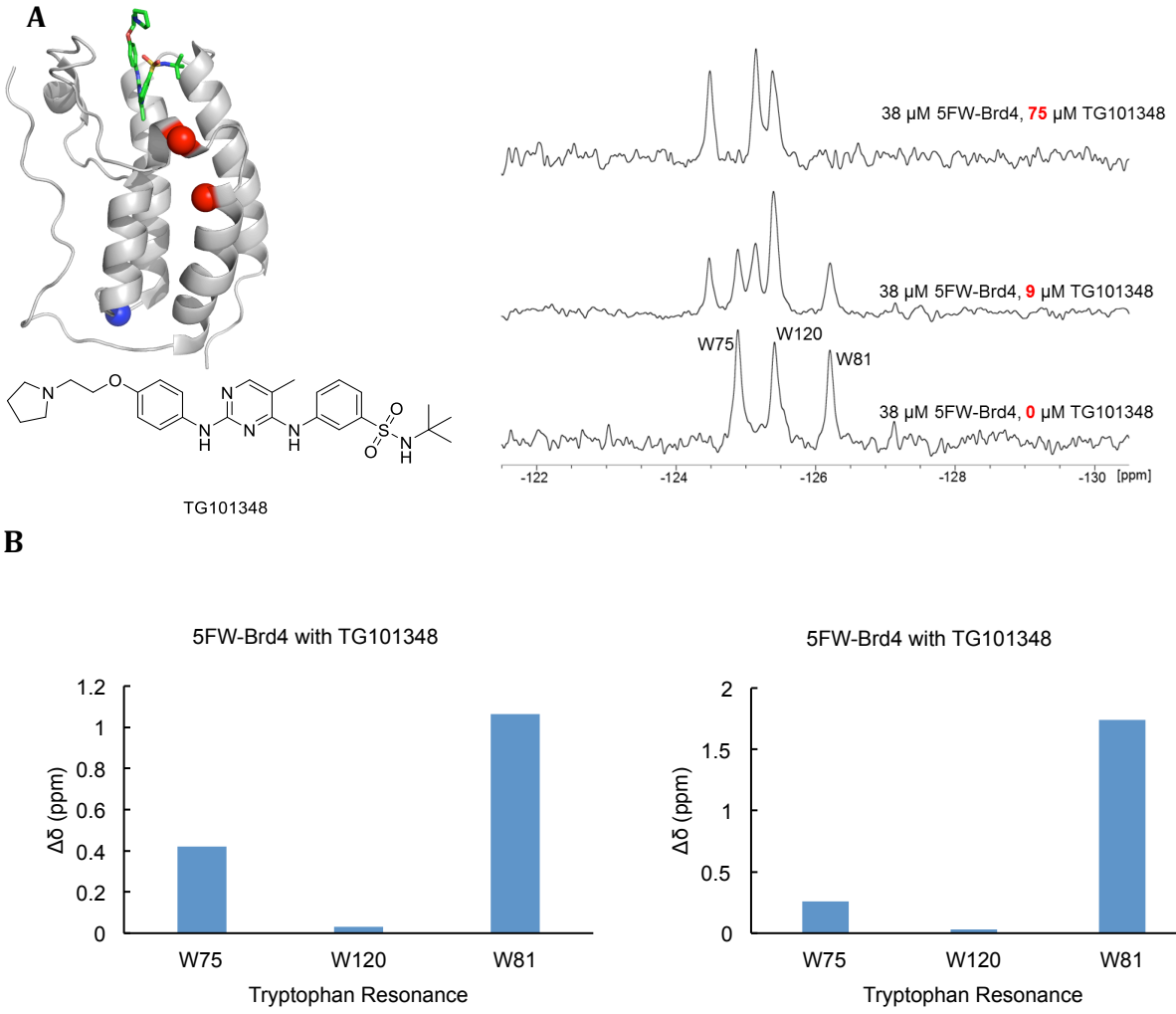


Fig. S15: ^{19}F NMR spectral analysis of 5FW-Brd4 demonstrating chemical shift perturbation of various resonances in response to increasing concentration of TG101348. **A)** Representative ^{19}F NMR spectra from titration with TG101348. Ribbon diagram of TG101348 bound to Brd4(1) (PDB Code: 4PS5). The α-carbon of tryptophan are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 5FW-Brd4(1) tryptophan resonances at 75 μM TG101348. Because the new resonances cannot be unambiguously assigned, two graphs have been generated representing the two possible tryptophan assignments of the new resonances.

Table S13. 5FW-Brd4 ^{19}F NMR chemical shift perturbations at different TG101348 concentrations with the first possible resonance assignment:

TG101348 Concentration	Chemical Shift	W75	W120	W81
0 μM	Chemical shift (ppm)	-124.887	-125.415	-126.210
75 μM	Δδ (ppm)	-0.259	0.031	1.742

Table S14. 5FW-Brd4 ^{19}F NMR chemical shift perturbations at different TG101348 concentrations with the second possible resonance assignment:

TG101348 Concentration	Chemical Shift	W75	W120	W81
0 μM	Chemical shift (ppm)	-124.887	-125.415	-126.210
75 μM	Δδ (ppm)	0.420	0.031	1.064

3FY-Brd4 TG101348 titration:

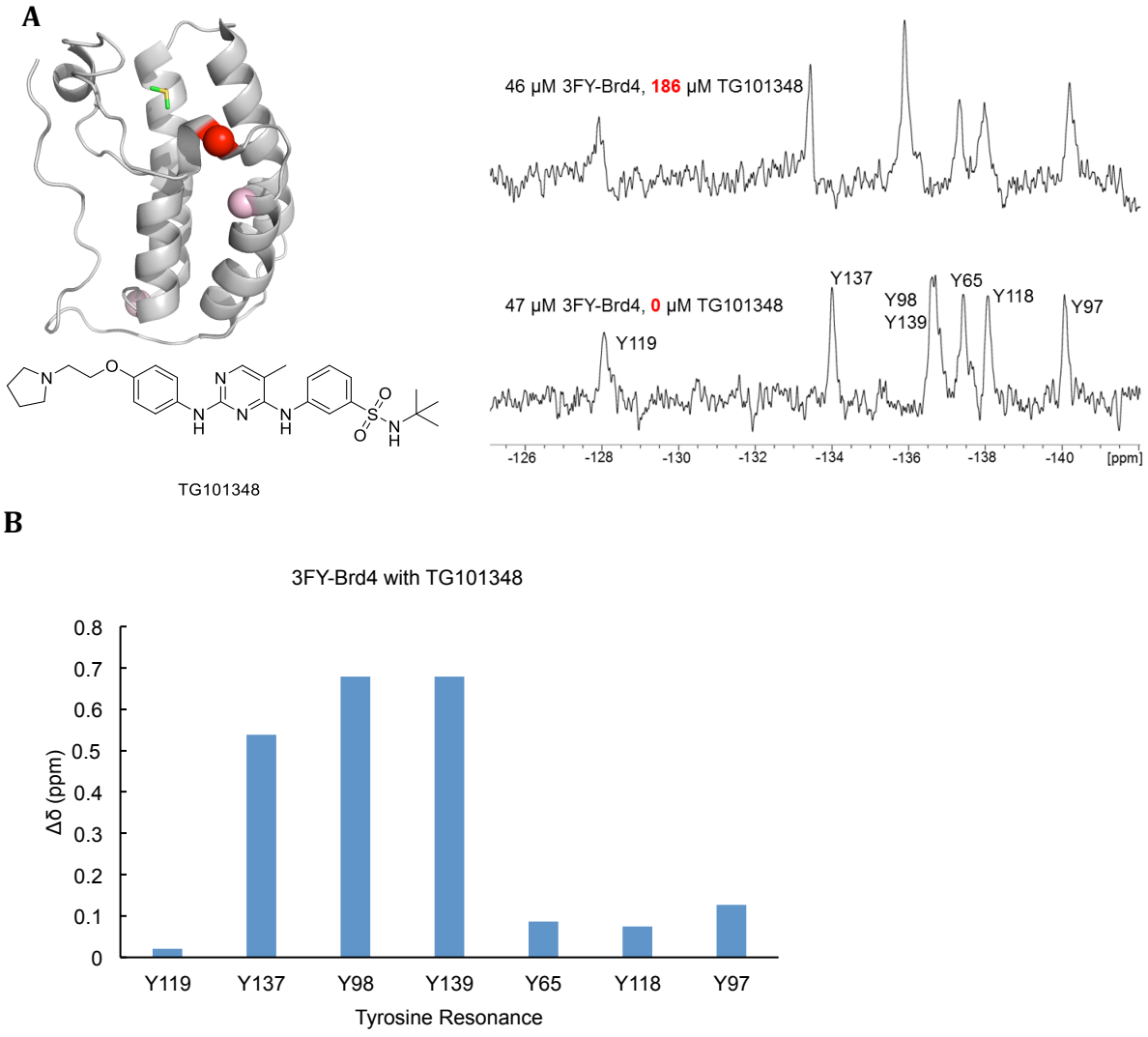


Fig. S16: ^{19}F NMR spectral analysis of 3FY-Brd4 demonstrating chemical shift perturbation of various resonances in response to increasing concentration of TG101348. **A)** Representative ^{19}F NMR spectra from titration with TG101348. Ribbon diagram of TG101348 bound to Brd4(1) (PDB Code: 4PS5). The α-carbon of tyrosine are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 3FY-Brd4(1) tyrosine resonances at 186 μM TG101348.

Table S15. 3FY-Brd4 ^{19}F NMR chemical shift perturbations at different TG101348 concentrations:

TG101348 Concentration	Chemical Shift	Y119	Y137	Y98/139	Y98/139	Y65	Y118	Y97
0 μM	Chemical shift (ppm)	-127.950	-133.980	-136.577	-136.577	-137.416	-138.055	-140.073
186 μM	Δδ (ppm)	0.020	0.538	0.679	0.679	0.087	0.074	-0.126

5FW-Brd4 DMSO titration:

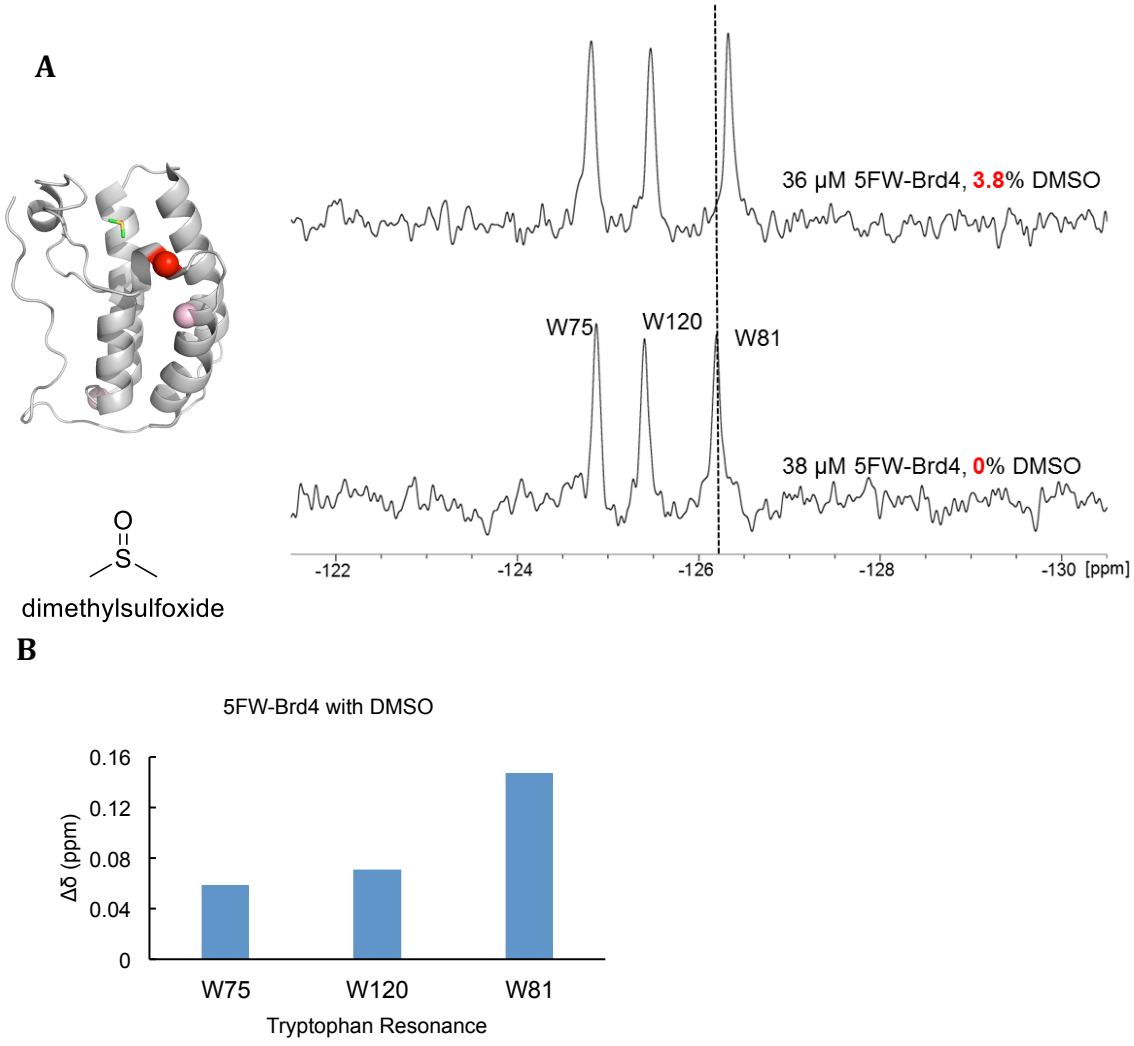


Fig. S17: ^{19}F NMR spectral analysis of 5FW-Brd4 demonstrating chemical shift perturbation of the W81 resonance in response to increasing concentration of DMSO. **A)** Representative ^{19}F NMR spectra from titration with DMSO. Ribbon diagram of DMSO bound to Brd4(1) (PDB Code: 4IOR). The α-carbon tryptophan are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 5FW-Brd4(1) tryptophan resonances at 3.8% DMSO.

Table S16. 5FW-Brd4 ^{19}F NMR chemical shift perturbations at different DMSO concentrations:

DMSO Amount	Chemical Shift	W75	W120	W81
0%	Chemical shift (ppm)	-124.8749	-125.3996	-126.1784
3.8%	Δδ (ppm)	0.0588	-0.0709	-0.1473

3FY-Brd4 DMSO titration:

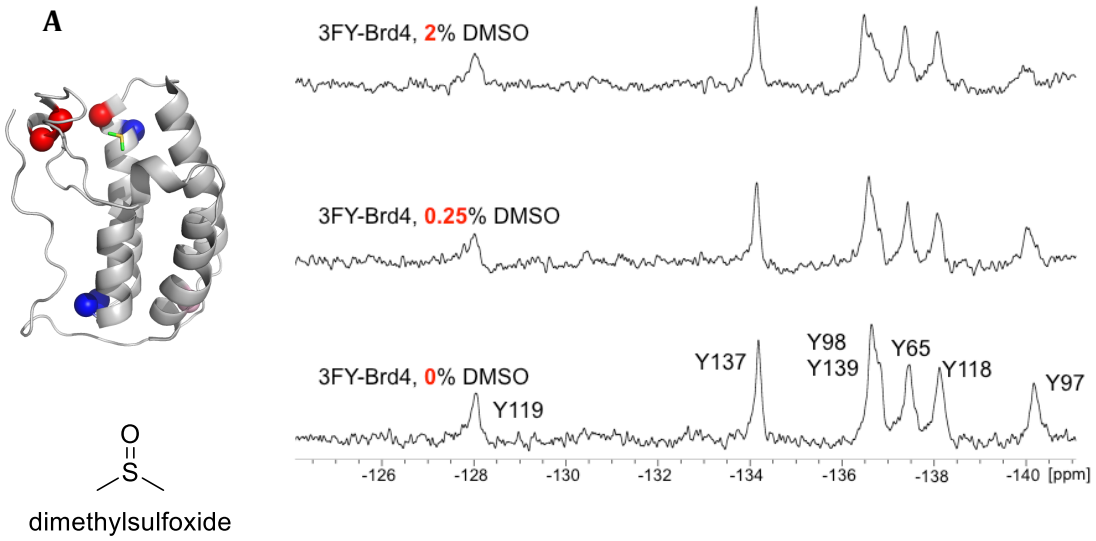


Fig. S18: ^{19}F NMR spectral analysis of 3FY-Brd4 demonstrating chemical shift perturbation of various resonances in response to increasing concentration of DMSO. **A)** Representative ^{19}F NMR spectra from titration with DMSO. Ribbon diagram of DMSO bound to Brd4(1) (PDB Code: 4IOR). The α-carbon of tyrosine are indicated as colored balls. Resonances not significantly perturbed are blue, resonances perturbed 0.05 ppm – 0.1 ppm are light pink, and resonances perturbed greater than 0.1 ppm or broadened significantly in a dose dependent fashion are red. **B)** Absolute value of chemical shift perturbation for the 3FY-Brd4(1) tyrosine resonances at 2% DMSO.

Table S17. 3FY-Brd4 ^{19}F NMR chemical shift perturbations at different DMSO concentrations:

DMSO Amount	Chemical Shift	Y119	Y137	Y98/139	Y98/139	Y65	Y118	Y97
0%	Chemical shift (ppm)	-128.0426	-134.1584	-136.6456	-136.6456	-137.4521	-138.1011	-140.1711
0.25%	Δδ (ppm)	0.0267	0.0082	0.0589	0.0589	0.022	0.0281	0.157
2%	Δδ (ppm)	0.0199	0.0178	0.159	0.159	0.0813	0.0271	0.2337

5FW-Brd4 ethylene glycol titration:

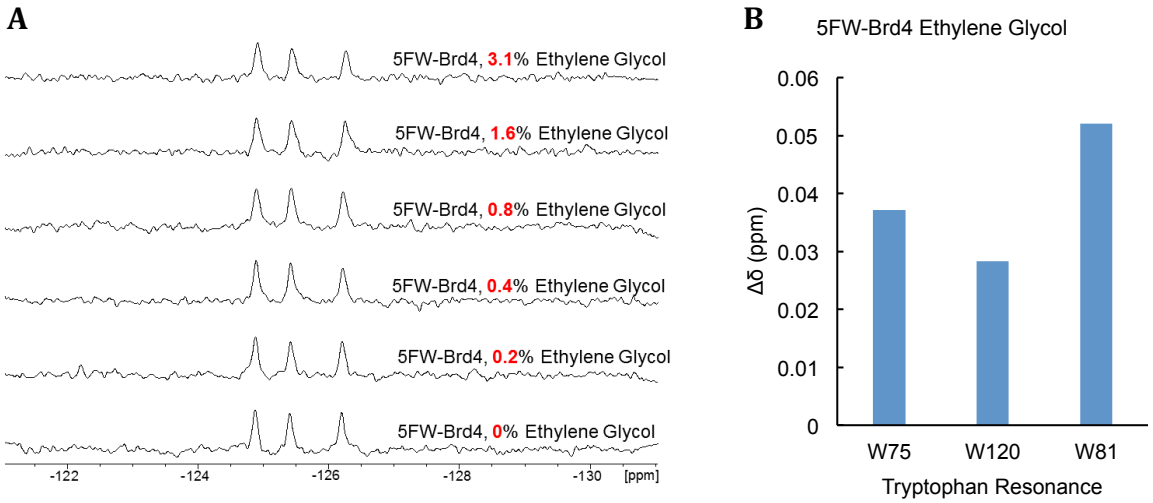


Fig. S19: ^{19}F NMR spectral analysis of 5FW-Brd4 demonstrating low chemical shift perturbation of all resonances in response to increasing concentration of ethylene glycol. **A)** Representative ^{19}F NMR spectra from titration with ethylene glycol. **B)** Absolute value of chemical shift perturbation for the 5FW-Brd4(1) tryptophan resonances at 1.6% ethylene glycol.

Table S18. 5FW-Brd4 ^{19}F NMR chemical shift perturbations at different ethylene glycol concentrations:

Ethylene Glycol Amount	Chemical Shift	W75	W120	W81
0%	Chemical shift (ppm)	-124.872	-125.403	-126.198
0.2%	Δδ (ppm)	-0.005	-0.011	-0.007
0.4%	Δδ (ppm)	-0.015	-0.012	-0.015
0.8%	Δδ (ppm)	-0.019	-0.020	-0.020
1.6%	Δδ (ppm)	-0.020	-0.024	-0.052
3.1%	Δδ (ppm)	-0.037	-0.028	-0.064

FYBrd4 with Ethylene Glycol:

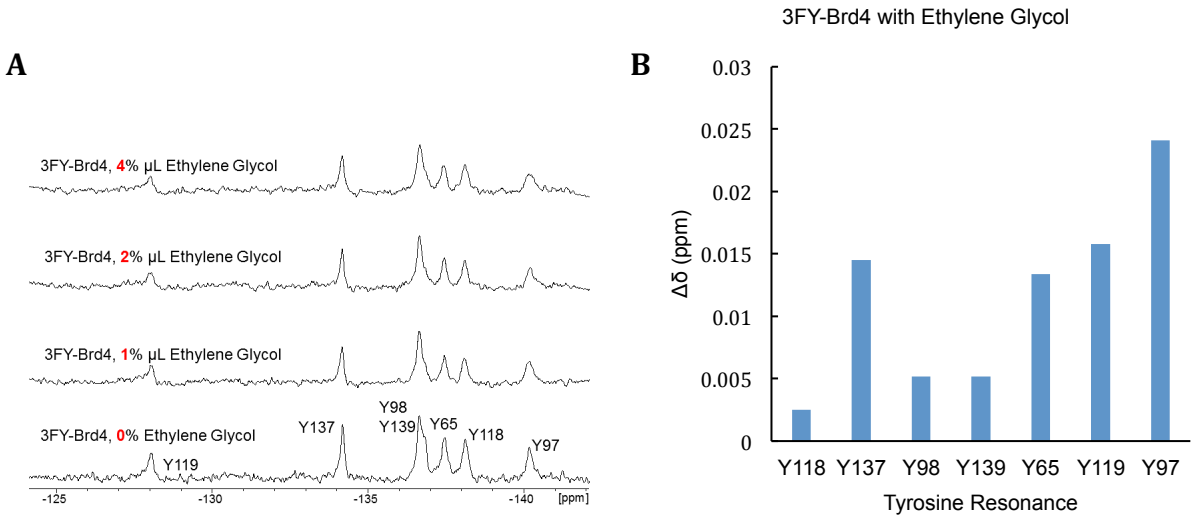


Fig. S20: ^{19}F NMR spectral analysis of 3FY-Brd4 demonstrating low chemical shift perturbation of all resonances in response to increasing concentration of ethylene glycol. **A)** Representative ^{19}F NMR spectra from titration with ethylene glycol. **B)** Absolute value of chemical shift perturbation for the 3FY-Brd4(1) tyrosine resonances at 2% ethylene glycol.

Table S19. 3FY-Brd4 ^{19}F NMR chemical shift perturbations at different ethylene glycol concentrations:

Ethylene Glycol Amount	Chemical Shift	Y119	Y137	Y98/139	Y98/139	Y65	Y118	Y97
0%	Chemical shift (ppm)	-128.044	-134.186	-136.643	-136.643	-137.469	-138.120	-140.172
1%	Δδ (ppm)	0.025	0.015	0.004	0.004	0.015	0.034	-0.018
2%	Δδ (ppm)	-0.002	0.014	-0.005	-0.005	0.013	0.016	-0.024
4%	Δδ (ppm)	0.017	0.008	-0.020	-0.020	0.056	0.007	0.002

Fast acquisition of 5FW-Brd4 ^{19}F NMR spectra:

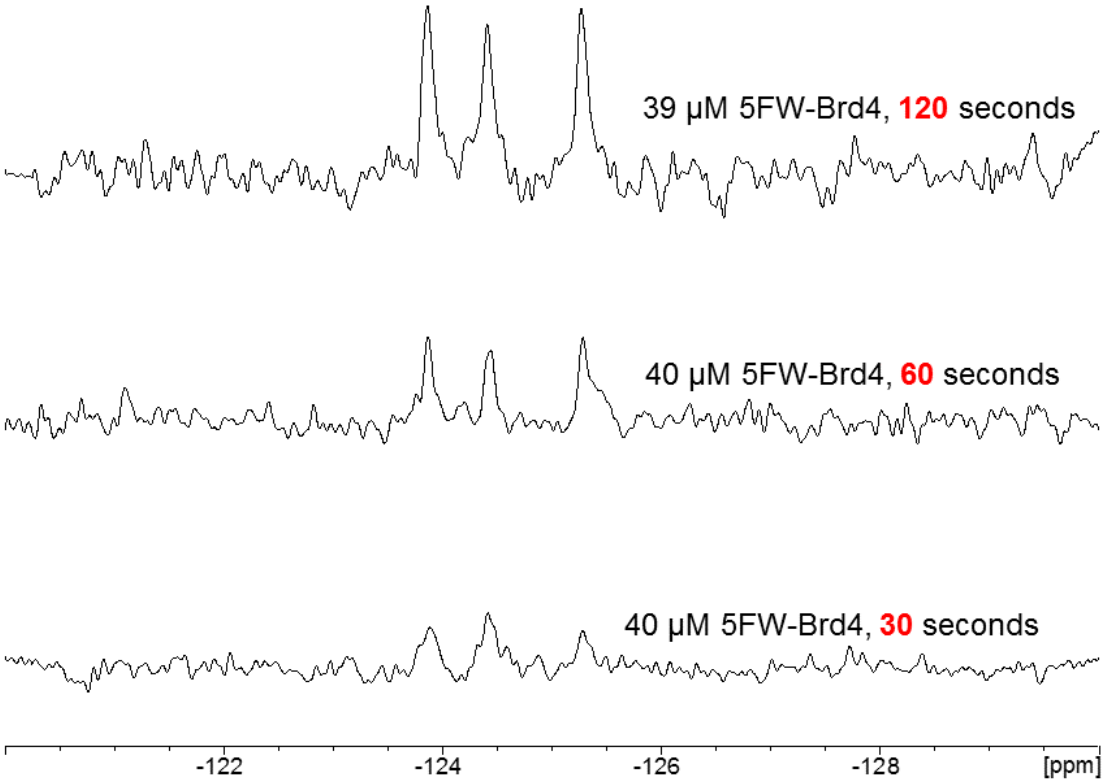


Fig. S21: ^{19}F NMR spectra of 5FW-Brd4 taken at various experiment times.

5FW-BPTF with BI2536:

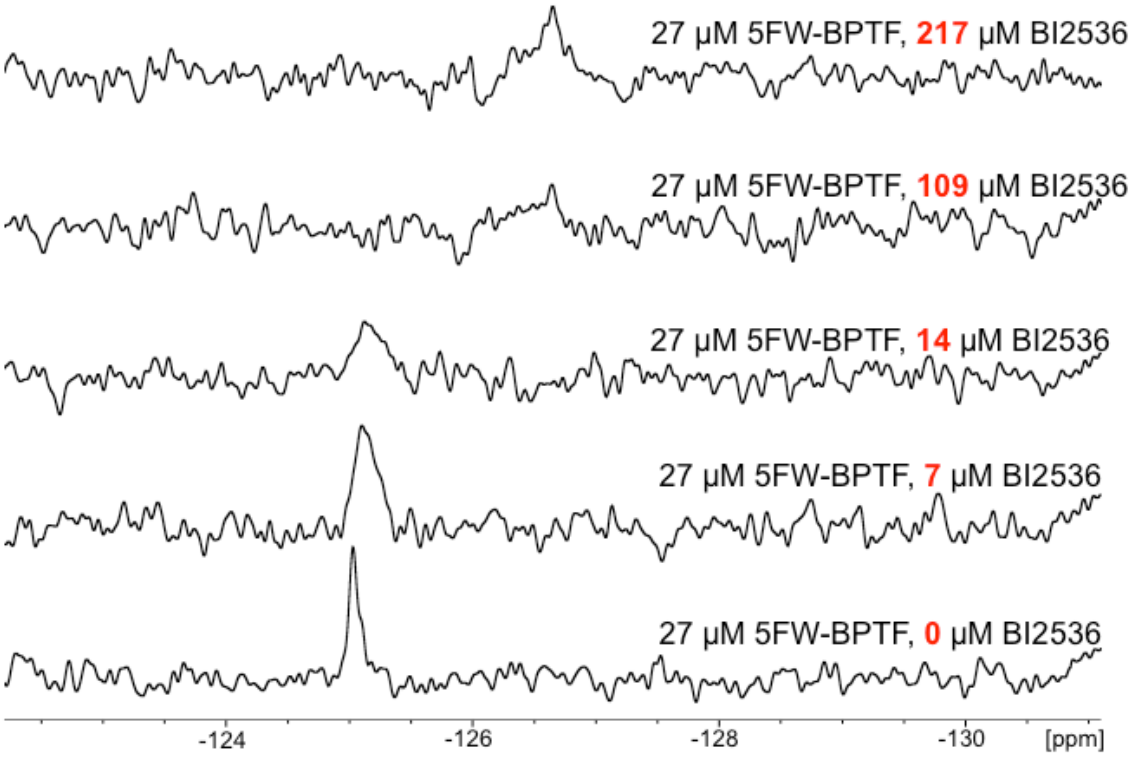


Fig. S22: ^{19}F NMR spectral analysis of 5FW-BPTF demonstrating chemical shift perturbation (intermediate exchange) of resonance W2824 in response to increasing concentration of BI2536. Above are representative ^{19}F NMR spectra from titration with BI2536.

Table S20. 5FW-BPTF ^{19}F NMR chemical shift perturbations at different BI2536 concentrations:

BI2536 Concentration	Chemical Shift	W2824
0 μM	Chemical shift (ppm)	-125.031
7 μM	Δδ (ppm)	-0.072
14 μM	Δδ (ppm)	-0.092
109 μM	Δδ (ppm)	*
217 μM	Δδ (ppm)	-1.621

5FW-BPTF and 5FW-Brd4 with BI2536:

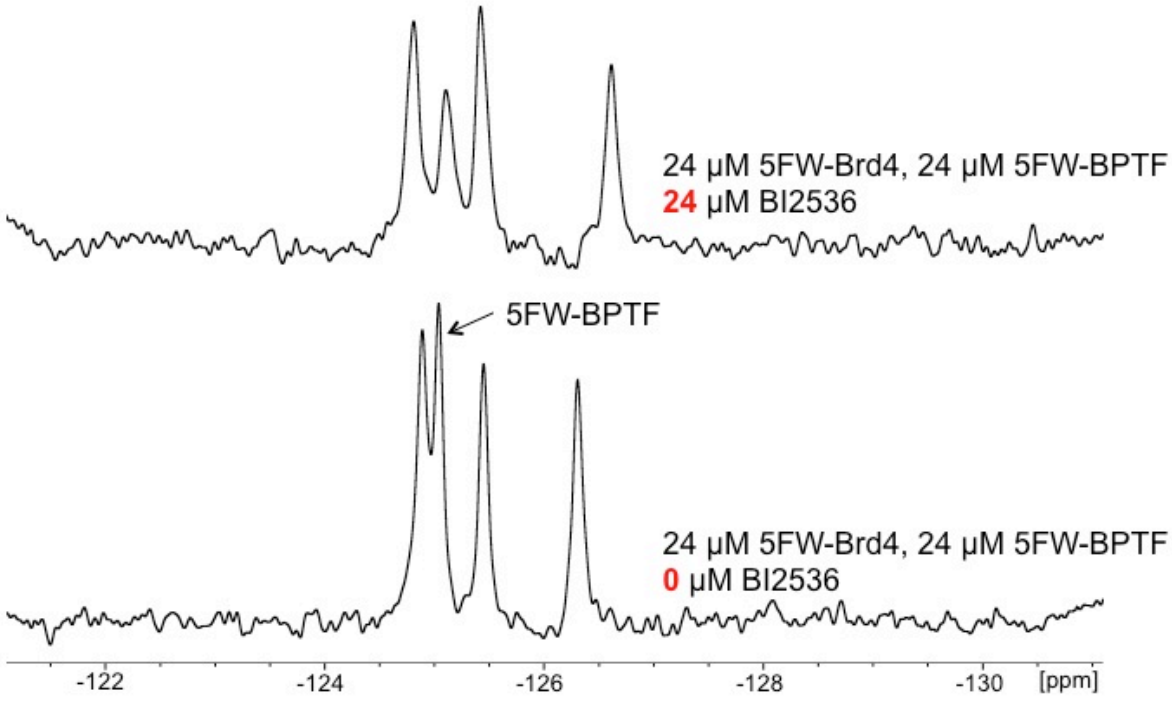


Fig. S23: ^{19}F NMR spectral analysis of 5FW-Brd4 and 5FW-BPTF demonstrating chemical shift perturbation of resonances of both proteins in response to presence of BI2536. The 5FW-Brd4 W81 resonance is perturbed as it was previously in Fig. S13, and the 5FW-BPTF W2824 resonance broadens and shifts upfield, as shown in Fig. S22. This is indicative of intermediate exchange of BI2536 with 5FW-BPTF in the presence of 5FW-Brd4.

5FW-Brd4 and wtBPTF and wtBrdT with BI2536:

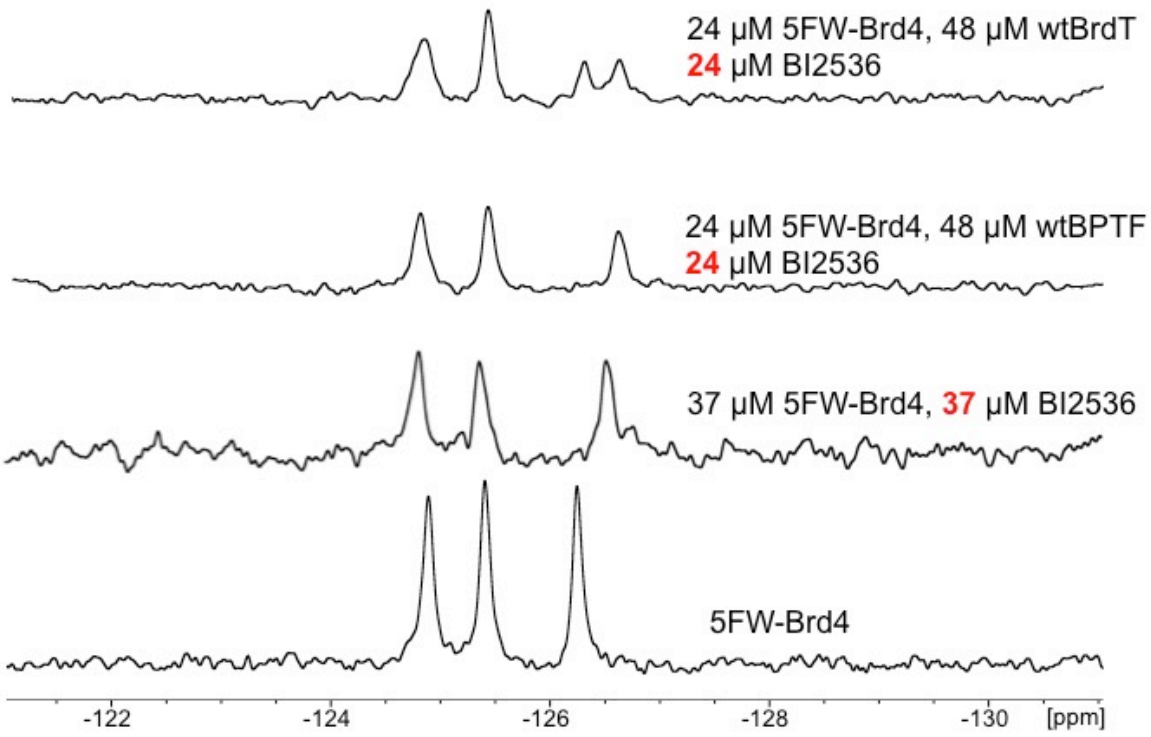


Fig. S24: ^{19}F NMR spectral analysis of 5FW-Brd4 demonstrating chemical shift perturbation of various resonances in response to increasing concentration of BI2536 in the presence of other bromodomains. Above, wtBrdT is shown to partially compete off BI2536 from Brd4 as there are two populations of 5FW-Brd4 present in the NMR spectrum. Protein wtBPTF is shown to not compete off BI2536 from Brd4, as there is only one population present in the NMR spectrum indicative of the fully bound state obtained from using only 5FW-Brd4 and BI2536.

5FW-BPTF with (+)-JQ1:

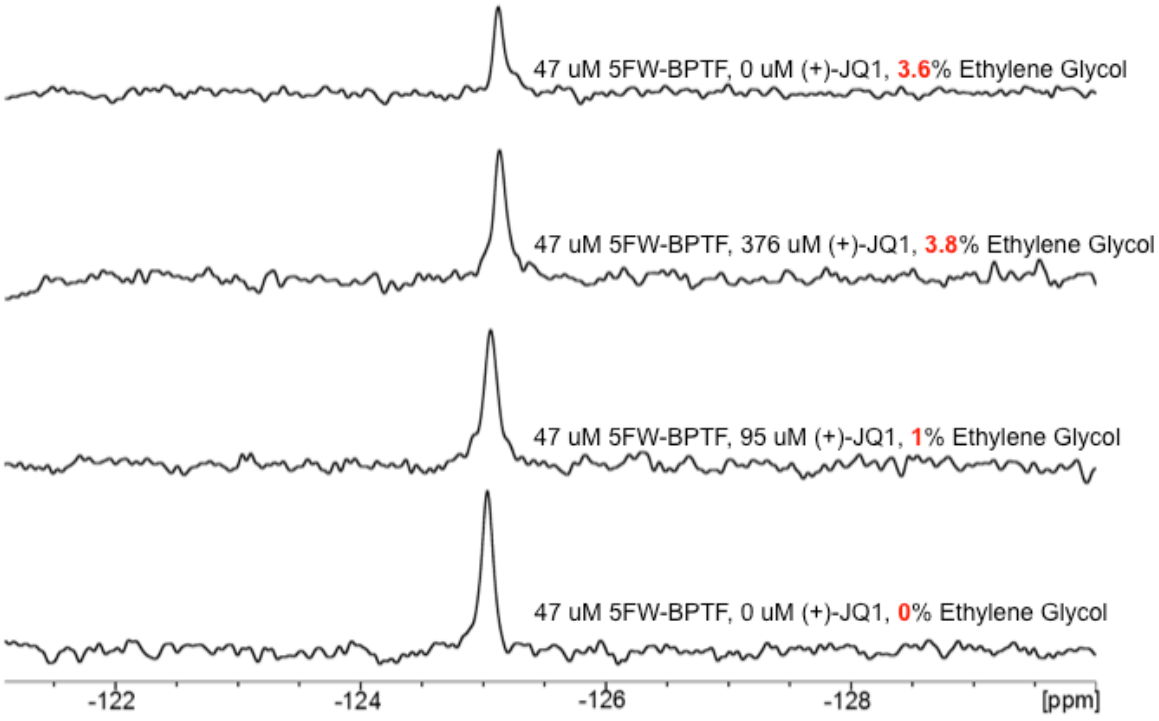


Fig. S25: ^{19}F NMR spectral analysis of 5FW-BPTF demonstrating chemical shift perturbation of W2824 in response to increasing concentration of (+)-JQ1. Above are ^{19}F NMR spectra from titration with (+)-JQ1. Small chemical shift perturbations in fast exchange at high micromolar concentrations of (+)-JQ1 could not be separated from solvent effects with just ethylene glycol, supporting selectivity of (+)-JQ1 for BET bromodomains.

Table S21. 5FW-BPTF ^{19}F NMR chemical shift perturbations at different (+)-JQ1 concentrations:

(+)-JQ1 Concentration	Chemical Shift	W2824
0 μM	Chemical shift (ppm)	-125.036
95 μM	Δδ (ppm)	-0.026
376 μM	Δδ (ppm)	-0.099
3.6% Ethylene Glycol	Δδ (ppm)	-0.088

Titration of 5FW-BrdT with (+)-JQ1:

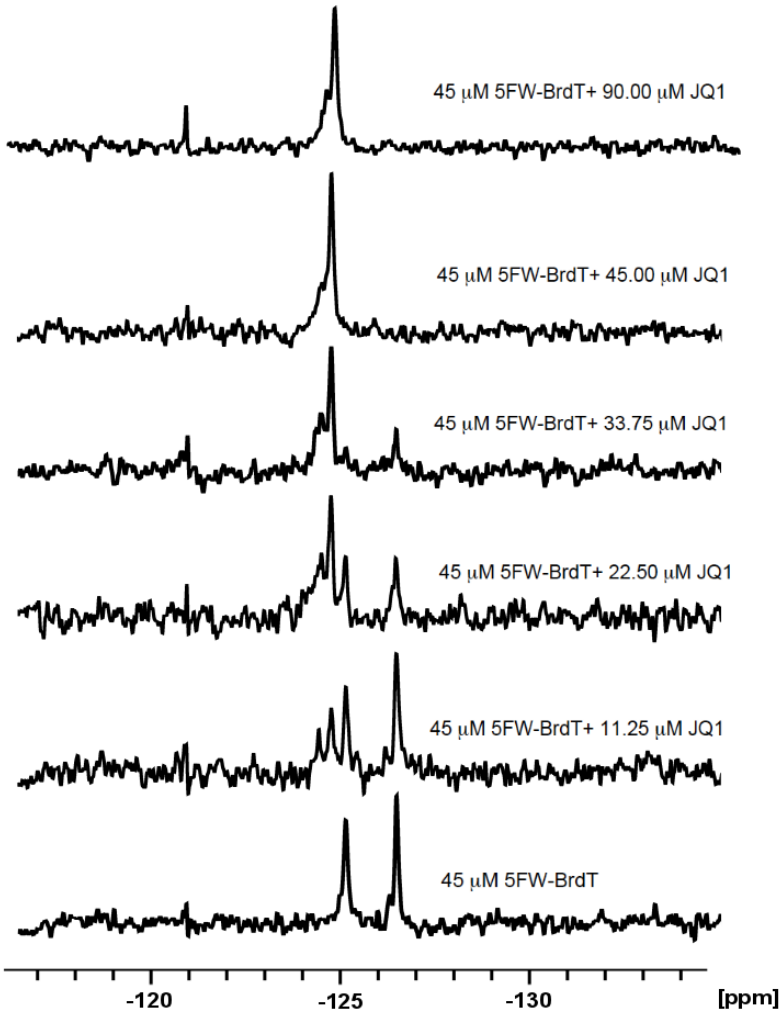


Fig. S26: ^{19}F NMR spectral analysis of 5FW-BrdT(1) demonstrating chemical shift perturbation of both fluorinated tryptophan residues (analogous to residues W81 and W75 in Brd4(1)) in response to increasing concentration of (+)-JQ1. Above are ^{19}F NMR spectra from titration with (+)-JQ1. Chemical shift perturbation in slow exchange regime supports strong binding with BET bromodomain BrdT(1).

Titration of 5FW-BrdT with BI2536:

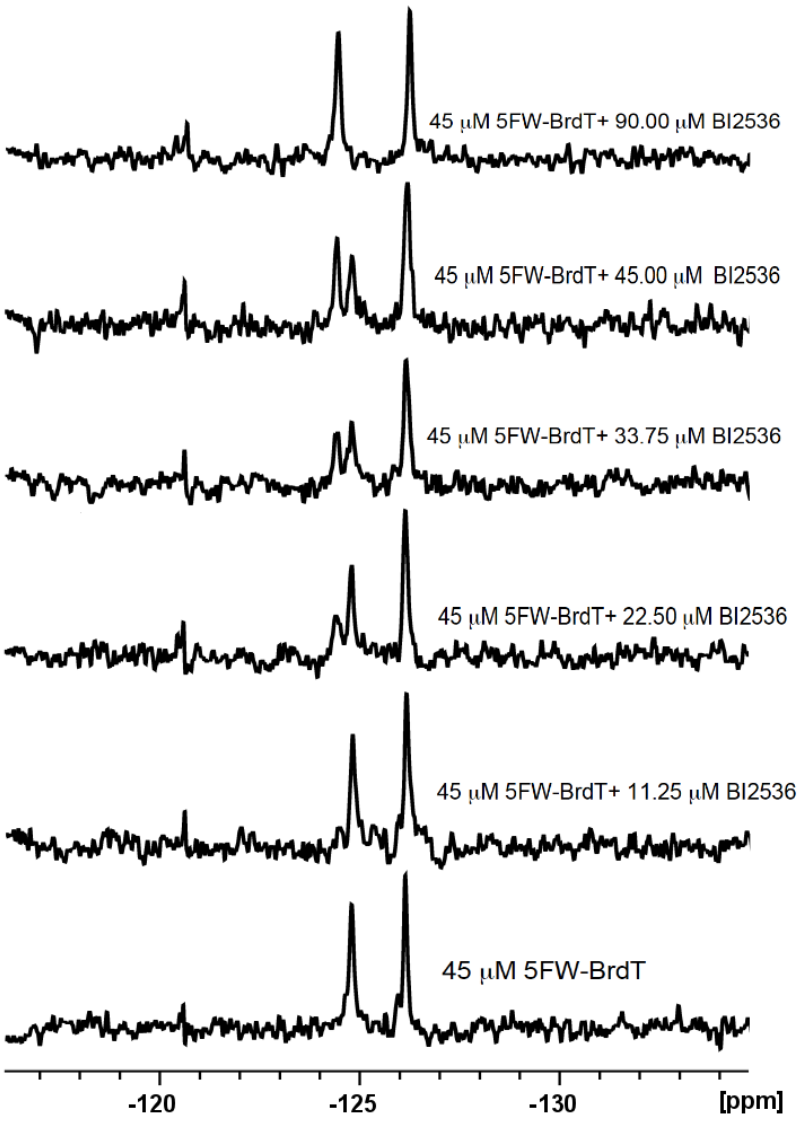


Fig. S27: ^{19}F NMR spectral analysis of 5FW-BrdT(1) demonstrating chemical shift perturbation of fluorinated tryptophan residues (analogous to residues W81 and W75 in Brd4(1)) in response to increasing concentration of BI2536. Chemical shift perturbation in slow exchange regime supports strong binding with BET bromodomain BrdT(1).

BI2536 Binding in the presence of DMSO:

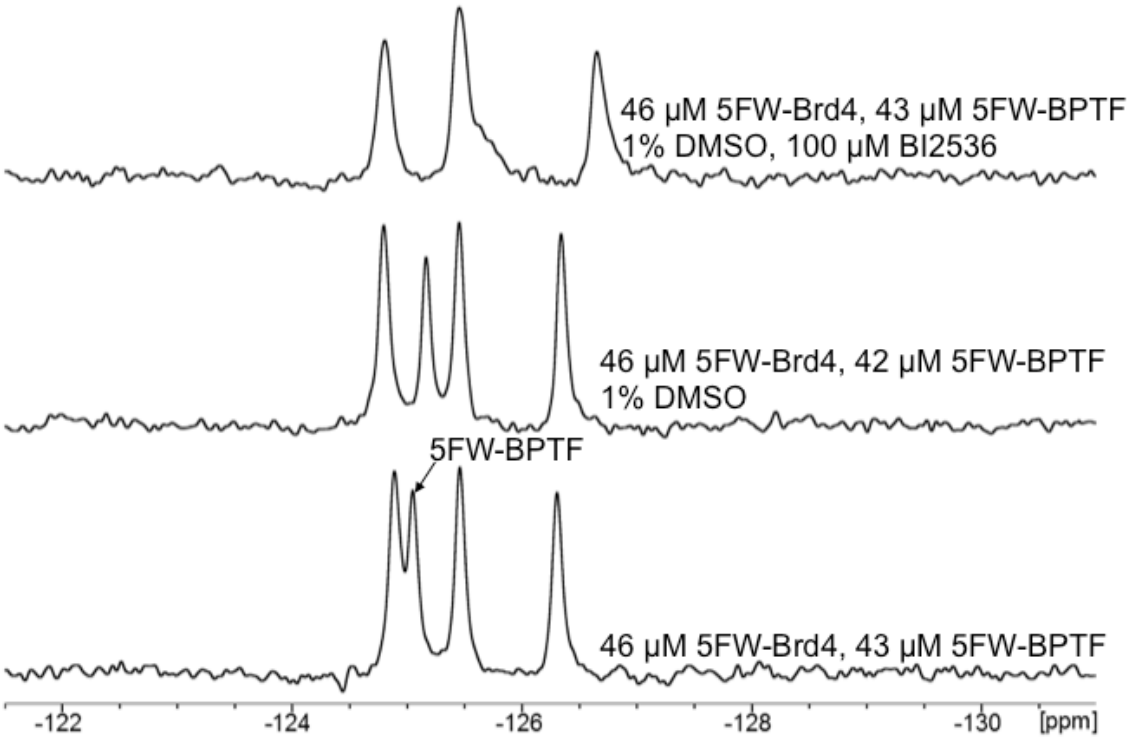


Fig. S28: ^{19}F NMR spectral analysis of 5FW-Brd4 and 5FW-BPTF demonstrating chemical shift perturbation of resonances of both proteins in response to presence of BI2536 while in a solution containing 1% DMSO. The 5FW-Brd4 W81 resonance is perturbed shifting upfield as it was previously in Fig. S13, and the 5FW-BPTF W2824 resonance broadens and shifts upfield, as shown in Fig. S22. This is indicative of intermediate exchange of BI2536 with 5FW-BPTF in the presence of 5FW-Brd4. While the 1% DMSO solution perturbs both resonances slightly, the effects of BI2536 are clearly visible for both 5FW-Brd4 and 5FW-BPTF.

ITC Binding isotherm of (+)-JQ1 with unlabeled Brd4(1) and 5FW Brd4(1)

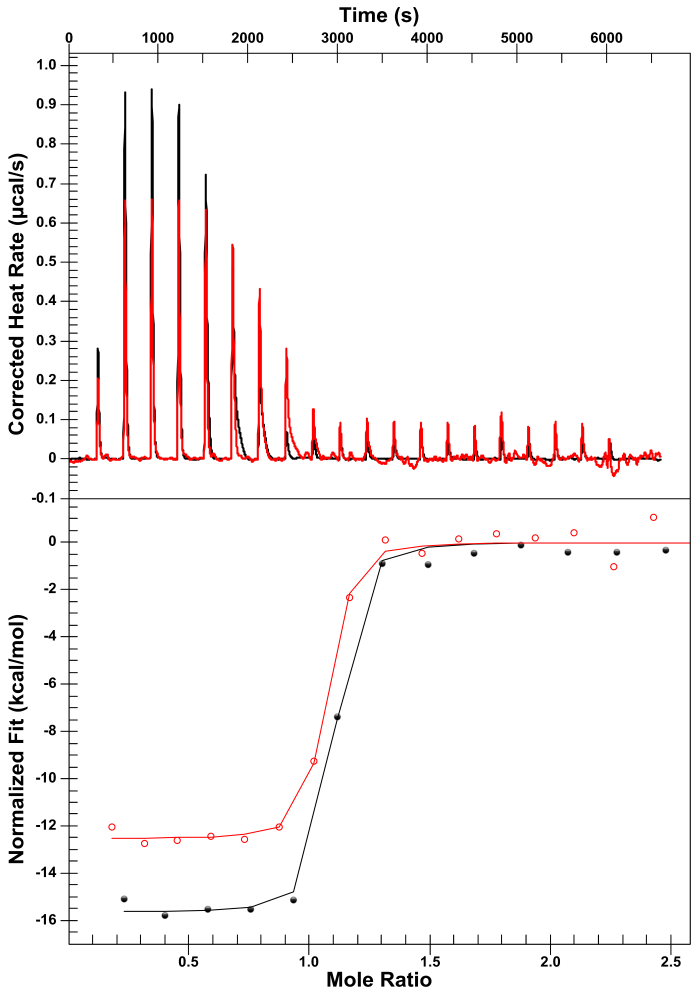


Fig. S29: Isothermal titration calorimetric analysis of binding of (+)-JQ1 to unlabeled (black filled circle) and 5FW labeled (open red circle) Brd4(1) at 25 ^{o}C. The study was performed by using Nano-ITC equipment (TA Instruments). In the following experiments, 550 µM unlabeled in 50 mM Tris pH 7.4, 100 mM NaCl and 4 mM DTT and 500 µM 5FW labeled proteins in 50 mM HEPES pH 7.5, 150 mM NaCl and 4 mM DTT were used as a ligand and loaded to the microsyringe (50 µL). 50 µM (+)-JQ1 solutions were made in the same buffers used for proteins. Titrations were carried out with first injection of 1.0 µL followed by 20 identical injections of 2.5 µL with a releasing period of 8 seconds per injection with 300 seconds spacing time between injections. Control heat of dilution was measured by independent protein titration into buffer and was subtracted from the protein-ligand binding experimental data. Data was analyzed by NanoAnalyze software to calculate enthalpy of binding (ΔH) and dissociation constants (K_d). In all cases an independent binding model was used for data fitting. The pre and post transition baselines show saturation behavior, which indicates no further protein ligand interaction event.

Table S22: ITC experimental conditions and obtained values:

Exp.	Protein	Condition	Kd nM	n value
1	Brd4	50 mM Tris pH 7.4, 100mM NaCl, 4 mM DTT	76.0	1.02
2	Brd4	50 mM Tris pH 7.4, 100mM NaCl	75.1 ± 4.5	1.51
3	5FW-Brd4	50mM HEPES pH 7.5 150mM NaCl+ 4 mM DTT	77.6 ± 2.0	0.95
4	3FY-Brd4	50 mM Tris pH 7.4, 100mM NaCl	88.7 ± 4.7	0.96

Table S23: Data collection and refinement statistics for the crystal structure determination of 3FY-Brd4(1)

Space group and unit cell dimensions	$P2_12_12_1$; a = 36.8, b = 44.9, c = 78.6; α = β = γ = 90°
Resolution (Å)	1.45 - 20.0 (1.45-1.47)
Unique reflections	22,274 (1,016)
	7.6
Rsym[a] (%)	(33.0)
Completeness (%)	93.5 (86.8)
	21.6
I/σI	(2.9)
Rcryst[b] (%)	14.0
Rfree[c] (%)	18.5
Average B all[d] (Å²)	12.54
Average B protein[d] (Å²)	11
Average B ligand[d] (Å²)	8.36
Average B solvent[d] (Å²)	22.76
Wilson B (Å)	9.78
rmsd[e] bonds (Å)	0.009
rmsd angles (deg)	1.46
Coordinate Error (Å)	0.13
Ramachadran favored (%)	97.74
Ramachadran allowed (%)	2.26

Values in paranthesis are for the highest resolution shell.

[a] Rsym = 100 xΣhΣi|Ihi-Ih|/ΣhiI where h are unique reflection indices.

[b] Rcryst = 100 xΣ|Fobs-Fmodel|/F obs where Fobs and Fmodel are observed and calculated structure factor amplitudes respectively.

[c] Rfree is Rcryst calculated for 1112 randomly chosen unique reflections.

[d] Excluding hydrogen atoms

[e] rmsd = root-mean-square deviation from ideal values.

References:

1. (a) Crowley, P. B.; Kyne, C.; Monteith, W. B., *Chem. Commun.* **2012**, *48,* 10681-10683; (b) Pomerantz, W. C.; Wang, N. K.; Lipinski, A. K.; Wang, E. W.; Cierpicki, T.; Mapp, A. K., *ACS Chem. Biol.* **2012**, *7,* 1345-50.

2. Muchmore, D. C.; McIntosh, L. P.; Russell, C. B.; Anderson, D. E.; Dahlquist, F. W., *Methods in Enzymology* **1989**, *177,* 44-73.