Protein-Observed Fluorine NMR is a Complementary Ligand Discovery Method to ^{1}H CPMG Ligand-Observed NMR.

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Supporting Information

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. 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 Protein Expression:

The pNIC28-BSA4 plasmid containing the first bromodomain of Brd4 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 °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 °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 °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 °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 °C.

Bromodomain Purification:

To purify fluorinated and unlabeled Brd4, the cell pellet was thawed at room temperature followed by the addition of lysis buffer (50 mM Phosphate pH 7.4, 300 mM NaCl) containing protease inhibitor PMSF (5 mM) as well as the Halt protease inhibitor. Cells were lysed by sonication and the cell lysate was centrifuged at 7500 g for 30 minutes followed by supernatant filtration over Whatman filter paper. Filtrate containing the histidine-tagged Brd4 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 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 °C overnight on a rotating carrousel. The cleaved His-tag, TEV protease and uncleaved Brd4 were removed using nickel-NTA affinity resin.

Protein Mass Spectral Analysis

Product molecular weight was confirmed by electrospray ionization mass spectrometry (ESI-MS) using a Waters Acquity UPLC/Synapt G2 QTOF LC-MS. 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 5FW-Brd4 with one fluorine substitution, 2FWBrd4 has two fluorine atoms substituted and FWBrd4 has 3 fluorine atoms substituted. Below is an example mass spectrum of 5FW-Brd4 (expected mass of triply fluorinated 5FW-Brd4, 15137 m/z).

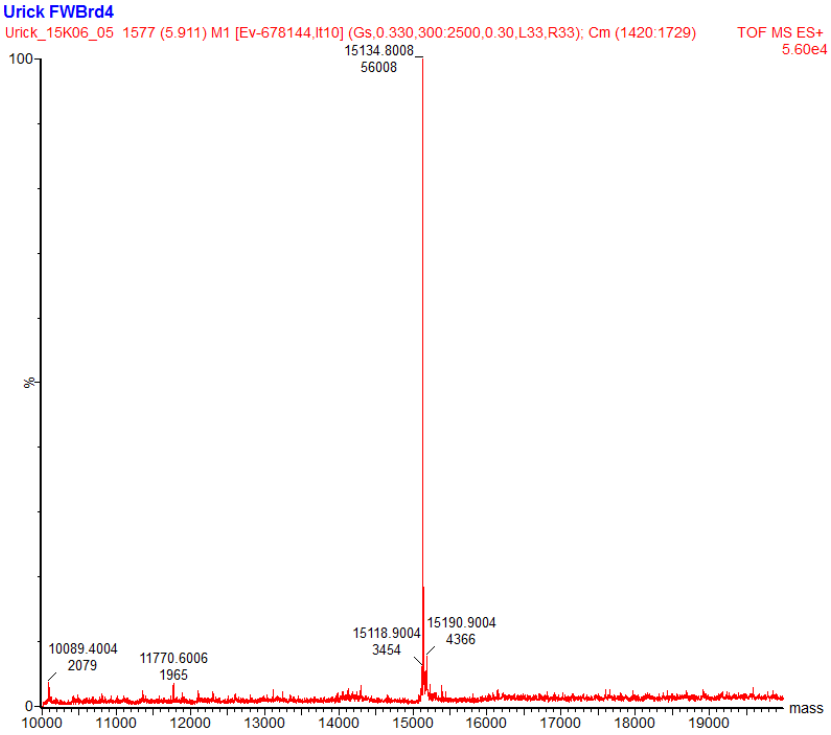


Table S1. A list of discrepancies between hits identified by PrOF NMR and ^{1}H CPMG

Compound No.	Δδ W81 (ppm)	ΔlinewidthW81 (ppm)	Δδ W75 (ppm)	% Drop (CPMG)	% recovery (CPMG)
13	0.264	0.0738	-0.0058	<10%	N/A
14	-0.2107	0.0123	-0.0021	14.4%	50.6%
15	0.0878	0.0123	-0.0772	<10%	N/A
16	-0.0763	0.0246	-0.0168	<10%	N/A
17	-0.062	0.0123	-0.0176	<10%	N/A
18	-0.0443	0	-0.0007	<10%	N/A
19	0.0389	0.0123	-0.0039	<10%	N/A
20	0.0313	0	0.0001	10.4%	258.5%
21	0.0271	0	-0.0187	100.0%	29.2%
22	0.0262	0.0123	-0.0291	90.2%	119.4%
23	0.02	0	0.0014	28.5%	-34.0%
24	0.0198	0.0123	-0.0191	41.6%	82.1%
25	0.0171	0	-0.0181	22.3%	35.5%
26	0.0168	0.0123	-0.0236	61.7%	26.6%
27	0.0159	0.0123	-0.0042	23.4%	102.9%
28	-0.0113	0	-0.0193	43.6%	160.2%
29	-0.008	0	-0.0167	20.2%	33.3%
30	0.0074	0	-0.0199	36.8%	130.0%
31	0.007	0	-0.0242	100.0%	40.0%
32	-0.0034	0	-0.0165	65.8%	119.7%
33	0.0028	0	-0.0088	41.2%	14.8%
34	-0.0014	0	-0.0111	69.0%	5.3%
35	0.0013	0	-0.0068	56.1%	-4.5%
36	-0.0011	0.0123	-0.0054	20.4%	-20.6%
37	-0.0009	0	-0.0144	43.4%	10.9%
38	0.0005	0.0123	-0.0275	28.6%	32.6%
39	-0.0005	0	0.0015	30.9%	-77.5%
40	0.0001	0	-0.0247	28.7%	134.8%

Dark Blue: Strong hit by PrOF NMR but no hit by ^{1}H CPMG
Light Blue: Weak hit by PrOF NMR but no hit by ^{1}H CPMG
Dark Orange: No hit by PrOF NMR but strong hit by ^{1}H CPMG (competition results omitted)
Light Orange: No hit by PrOF NMR but weak hit by ^{1}H CPMG (competition results omitted)
Light Green: No hit by PrOF NMR but strong and competitive hit by ^{1}H CPMG

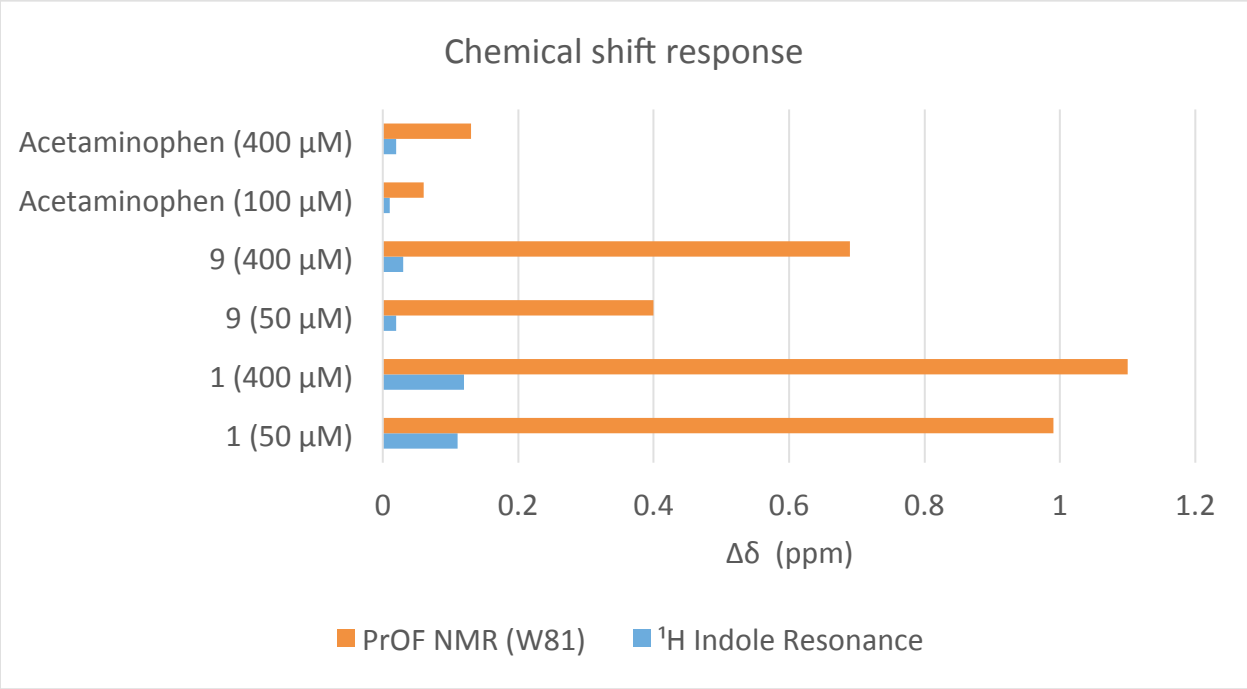


Fig. S1. A comparison of chemical shift perturbation of PrOF NMR and the most perturbed ^{1}H tryptophan resonance.

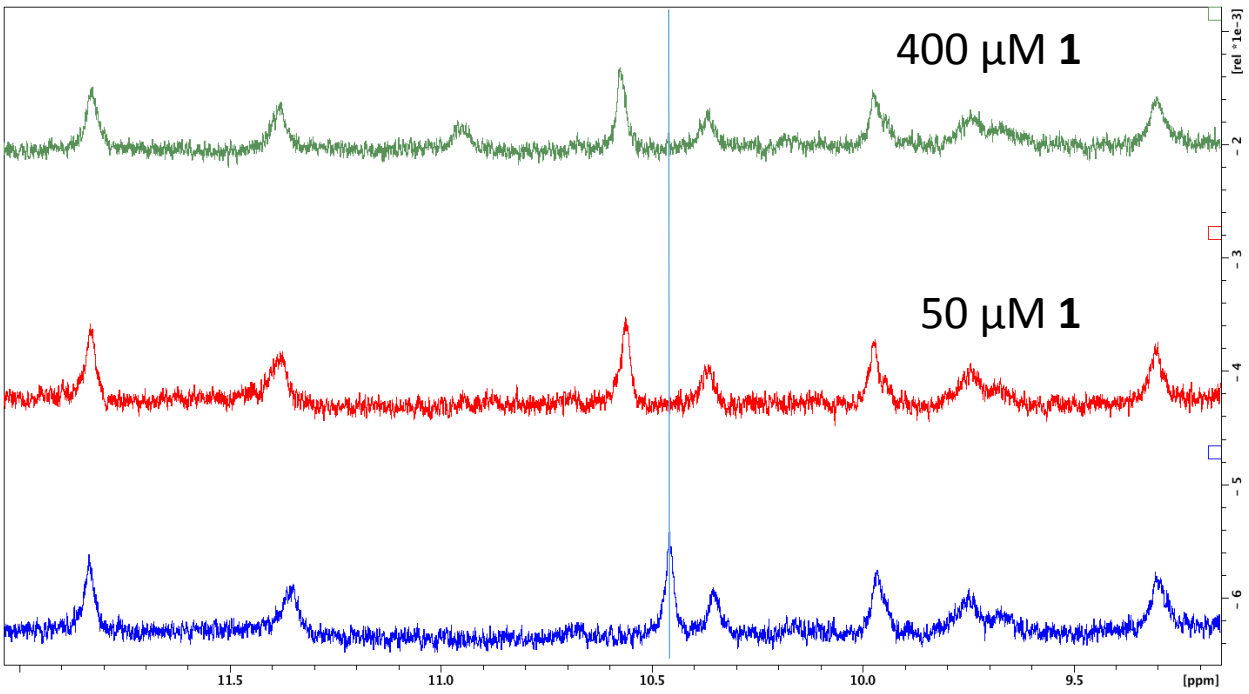


Fig. S2. The area of the ^{1}H NMR spectrum corresponding to the N-H tryptophan resonances. The most perturbed resonance is highlighted, and is perturbed in a dose dependent fashion by **1**.

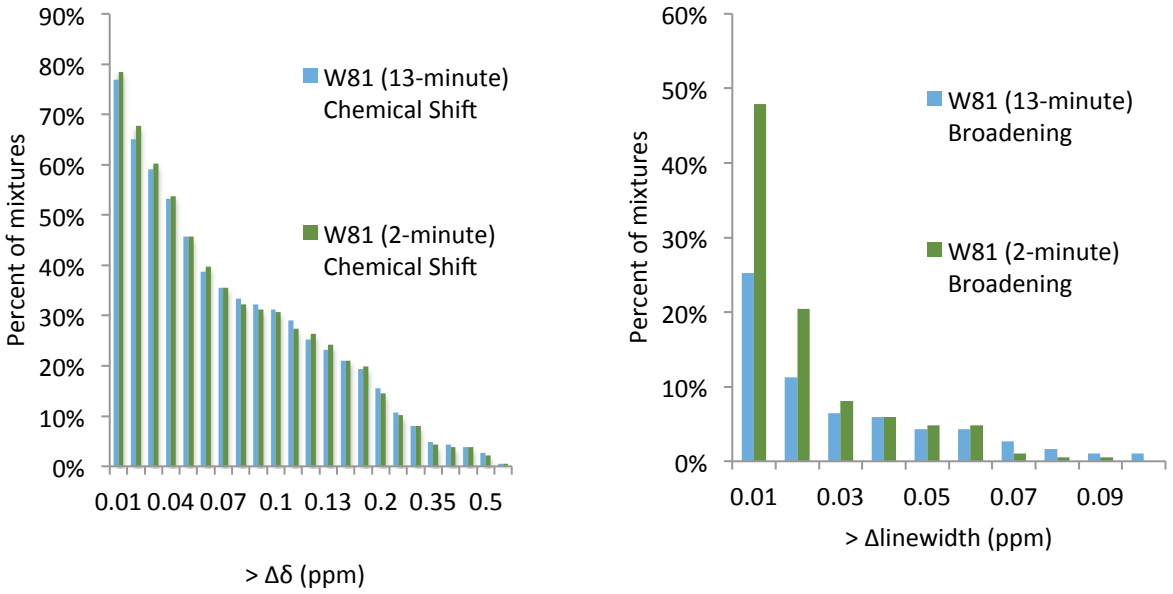


Fig. S3. A comparison of the data in the 13-minute PrOF NMR experiment and the 2-minute PrOF NMR experiment, comparing the percentage of mixtures perturbing W81 greater than a specific chemical shift (left) or linewidth (right). The average difference in perturbation of chemical shift of W81 between the 13-minute experiment and the 2-minute experiment was 0.0049 ppm, with a standard deviation in chemical shift perturbation average of 0.0055 ppm. Chemical shift perturbation is very robust even with low signal to noise, while linewidth is comparable after the widths are greater than 0.03 ppm. The average difference in linewidth between the 13-minute and 2-minute experiment is 0.0116 ppm, with a standard deviation of 0.0111 ppm.

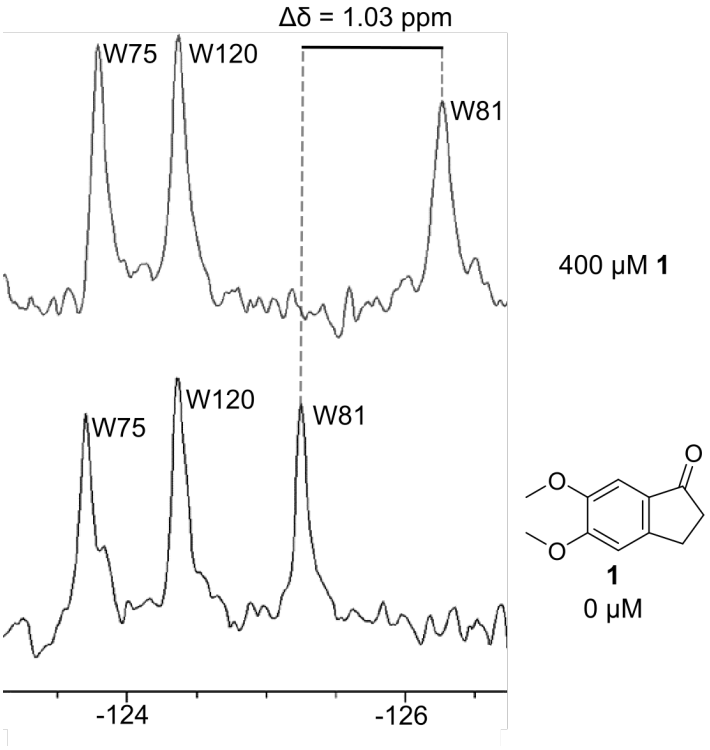


Fig. S4. A 2-minute PrOF NMR experiment (S/N: 7:1). The perturbation due to the binding interaction of **1** is clearly visible in this spectra as it was in Fig. 1 with the 13-minute PrOF NMR experiments.

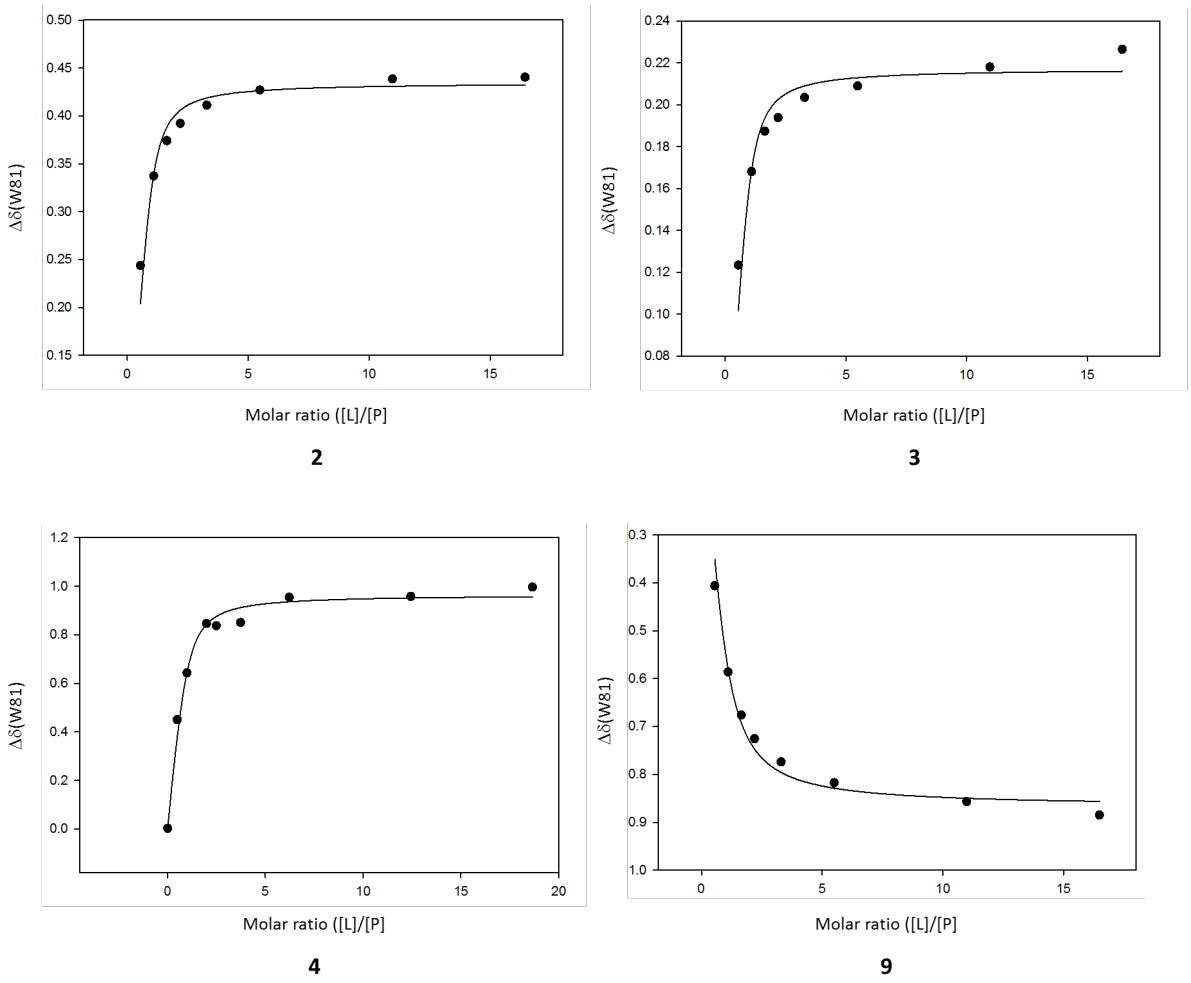
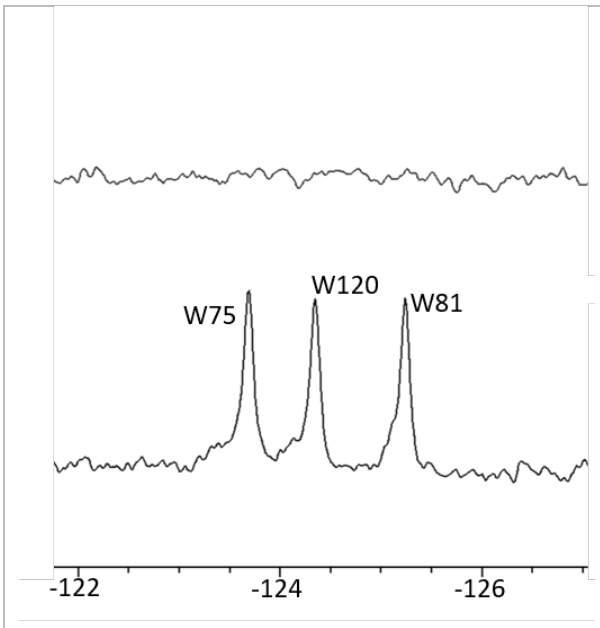
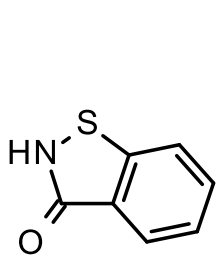
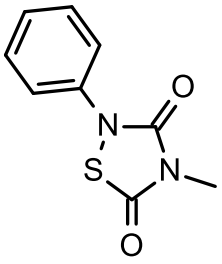


Fig. S5. Fitting graphs for selected compounds for $\Delta\delta_{50}$ determination using PrOF NMR titrations. The data were fitted to the following equation using SigmaPlot (Systat Software Inc., San Jose, California, USA):

$$\Delta\delta_{obs} = \Delta\delta_{max} - \frac{1}{2}\Delta\delta_{max}\left\{\sqrt{\left(\frac{[L]_{tot}}{[E]_0} + \frac{k_D}{[E]_0} - 1\right)^2 + 4\frac{k_D}{[E]_0}} - \left(\frac{[L]_{tot}}{[E]_0} + \frac{k_D}{[E]_0} - 1\right)\right\}$$







Aggregator 1 **Aggregator 2**

Fig. S6. An example of potential protein aggregators found by PrOF NMR. In the bottom spectrum is the protein in the absence of the small molecule mixture. In the top spectrum all resonances for the protein disappear indicating that the small molecule mixture induces the formation of a large protein aggregate, or the protein has crashed out of solution. Two such compounds that resulted in this spectrum are shown on the right, and are similar to known PAINs compound categories (Baell, J. & Walters, M., *Nature,* **2014**, 481-483.)

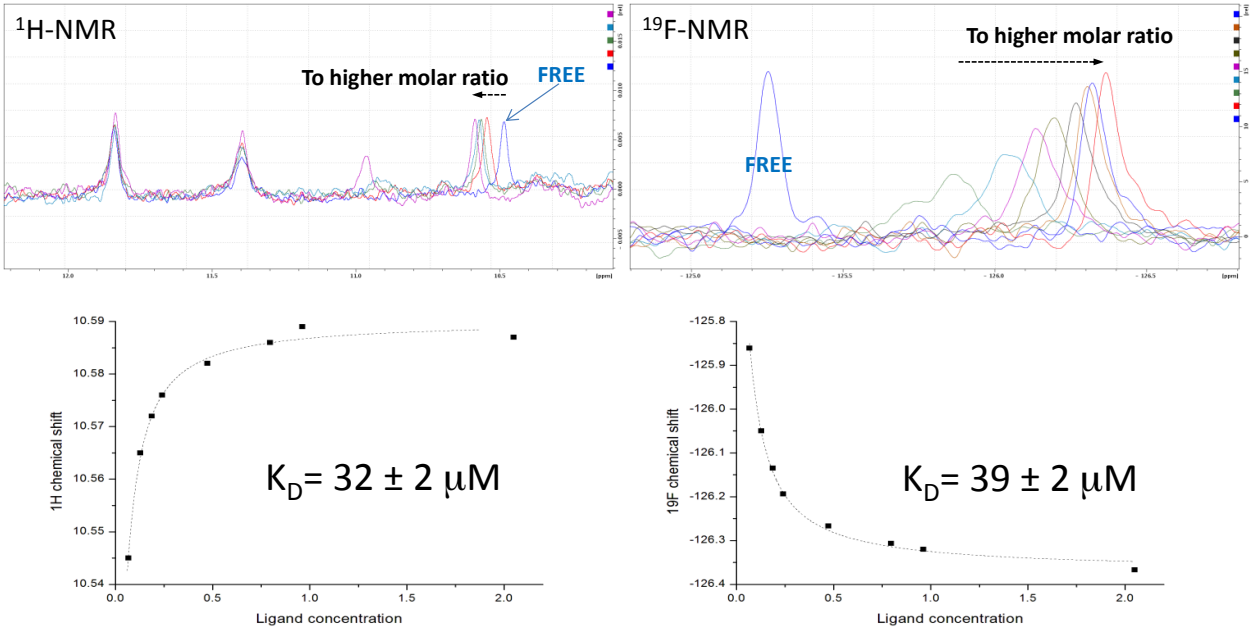


Fig. S7. A titration of 5FW-Brd4 (40 µM active protein) with **1** monitored by both PrOF NMR and ^{1}H NMR of the N-H tryptophan resonance (2% DMSO).

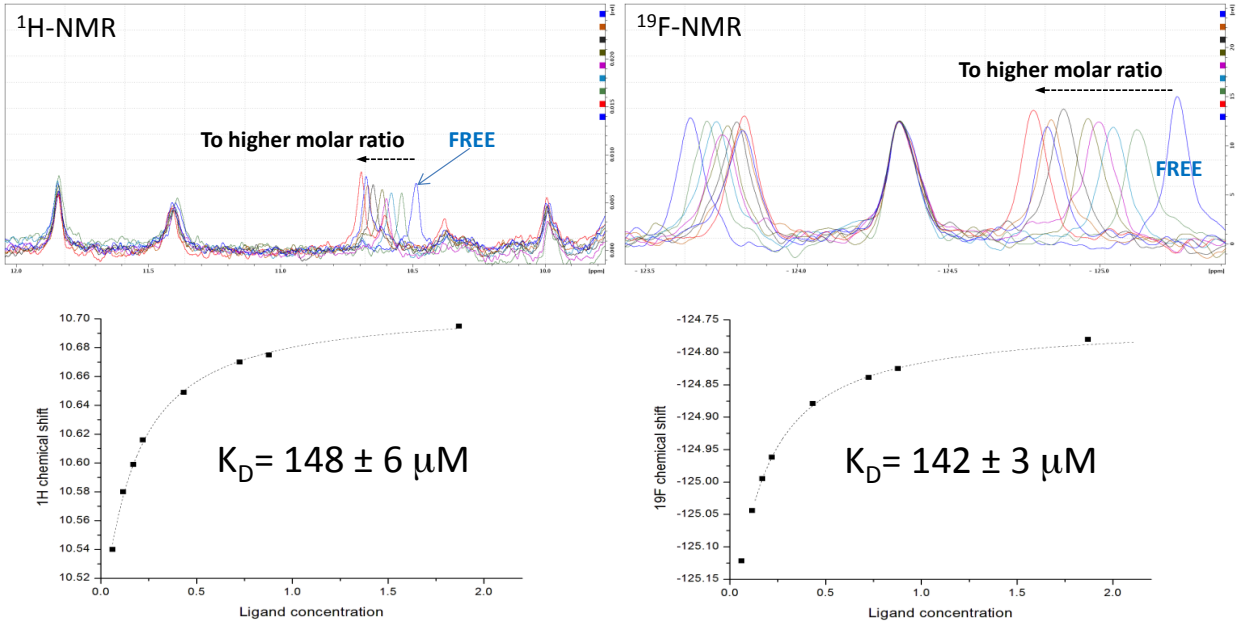
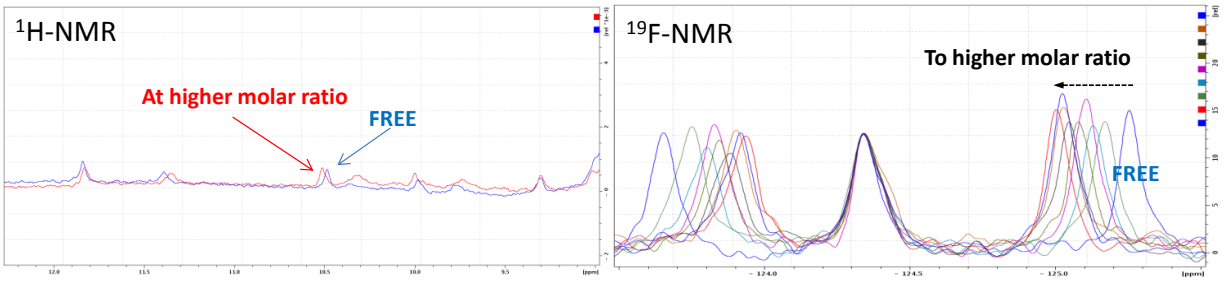


Fig. S8. A titration of 5FW-Brd4 (40 µM active protein) with **2** monitored by both PrOF NMR and ^{1}H NMR of the N-H tryptophan resonance (2% DMSO).



Monitoring ^{1}H-NMR of the N-H tryptophan resonances no significant chemical shift changes were recorded even at the highest molar ratio.

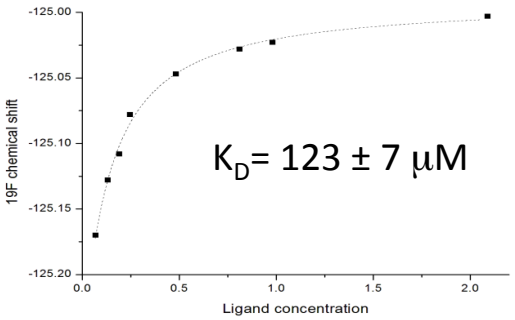
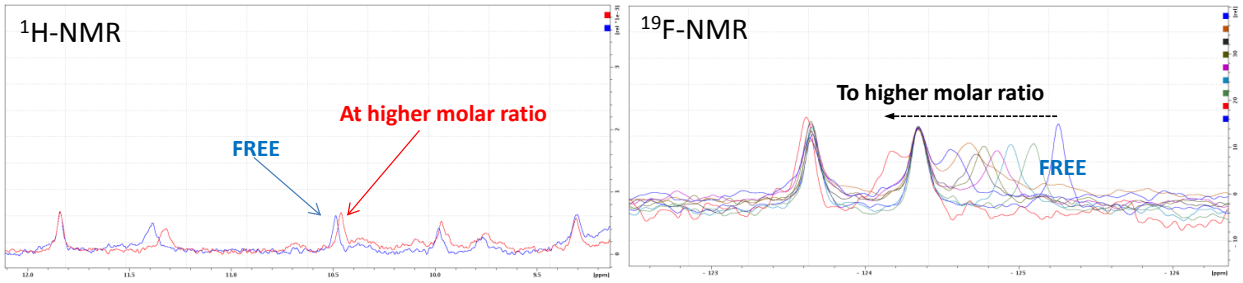


Fig. S9. A titration of 5FW-Brd4 (40 µM active protein) with **3** monitored by both PrOF NMR and ^{1}H NMR of the N-H tryptophan resonance (2% DMSO).



Monitoring ^{1}H-NMR of the N-H tryptophan resonances no significant chemical shift changes were recorded even at the highest molar ratio.

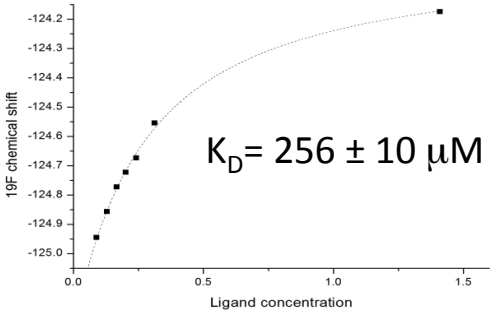
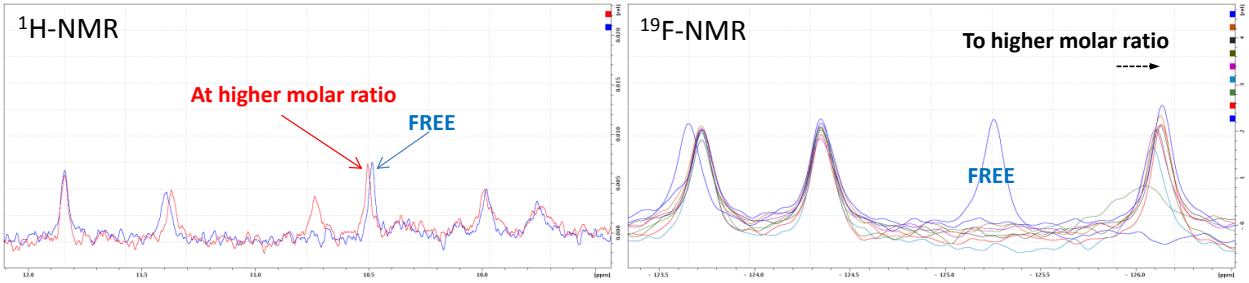


Fig. S10. A titration of 5FW-Brd4 (40 µM active protein) with **4** monitored by both PrOF NMR and ^{1}H NMR of the N-H tryptophan resonance (2% DMSO).



Monitoring ^{1}H-NMR of the N-H tryptophan resonances no significant chemical shift changes were recorded even at the highest molar ratio.

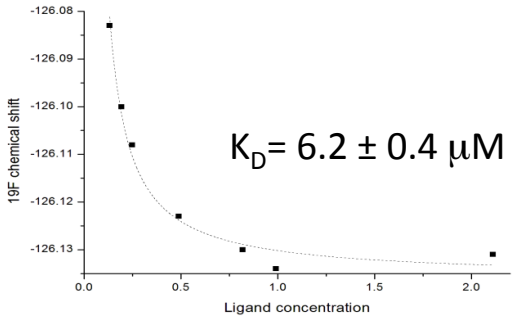


Fig. S11. A titration of 5FW-Brd4 (40 µM active protein) with **12** monitored by both PrOF NMR and ^{1}H NMR of the N-H tryptophan resonance (2% DMSO).

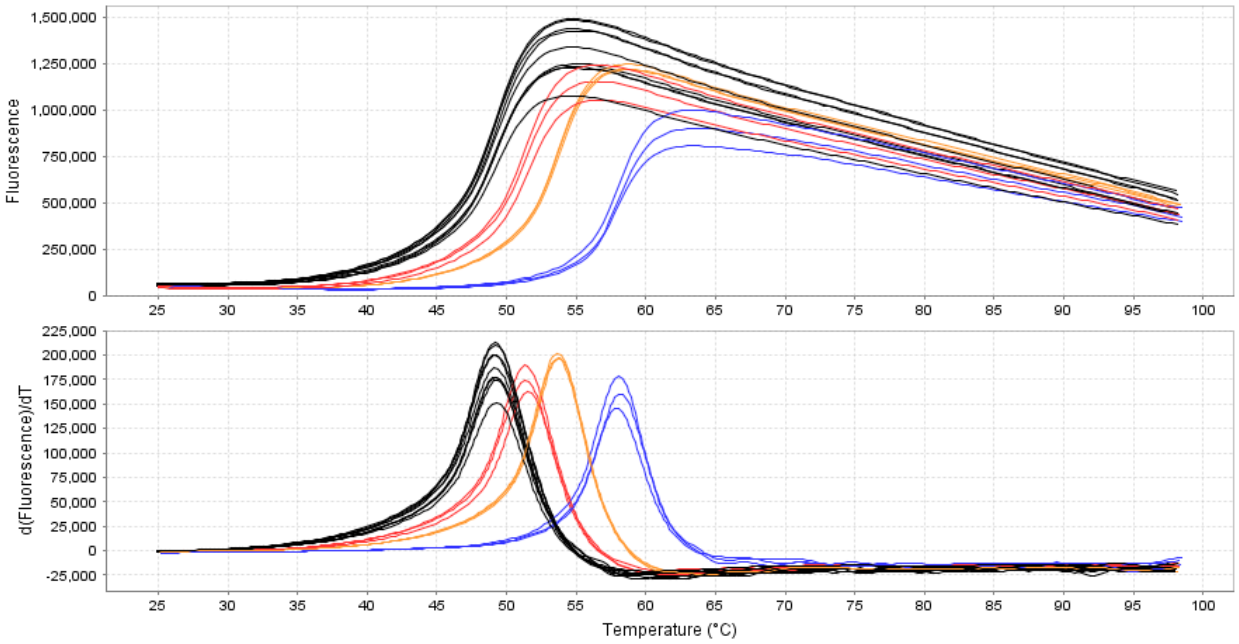


Fig. S12. Differential scanning fluorimetry of apo-form of Brd4 and three different binders. *Black curves* show thermal shift assays of apo-form of Brd4 (2.5% DMSO), *blue curves* show thermal shift assays of Brd4 in the presence of known binder bromosporine (at 250 μM, 2.5% DMSO), *orange curves* show thermal shift assays of Brd4 in the presence of **12** (at 250 μM, 2.5% DMSO), and *red curves* show thermal shift assays of Brd4 in the presence of **1** (at 250 μM, 2.5% DMSO).

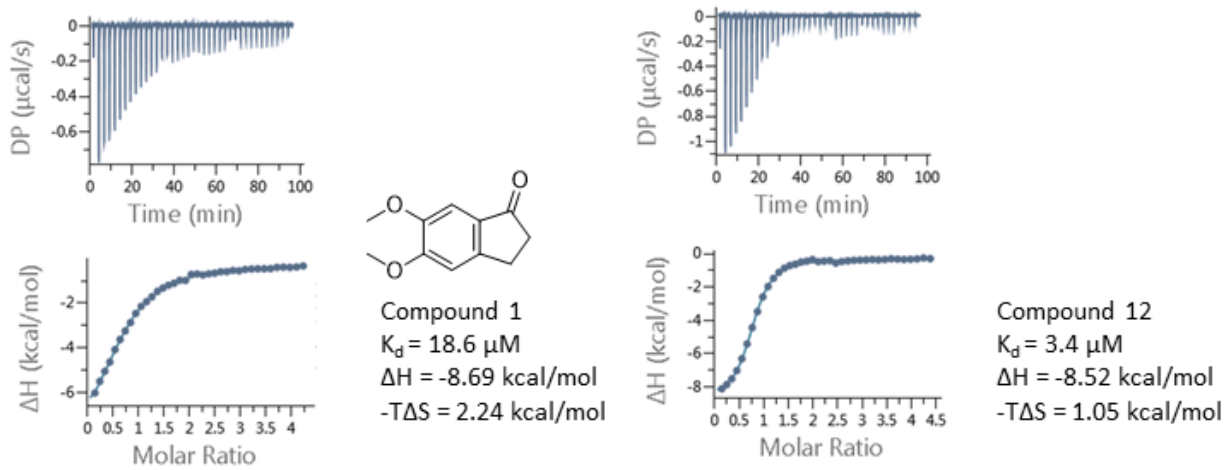


Fig. S13. ITC results of the highest affinity fragments which were identified by PrOF NMR experiments. These ITC experiments were performed with Brd4 wild-type in the presence of DMSO (0.5% DMSO).

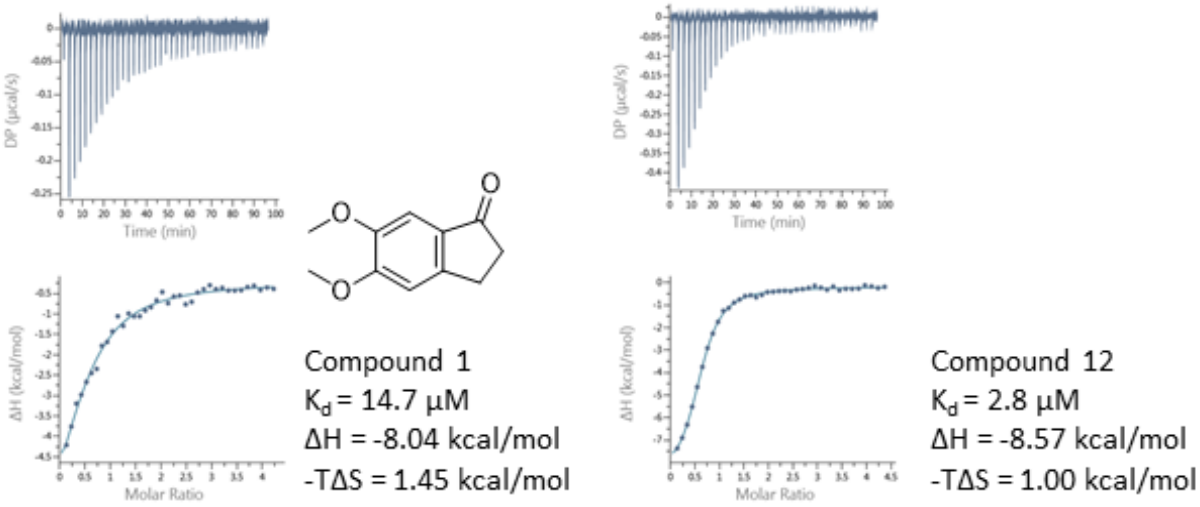
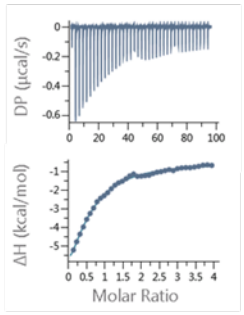
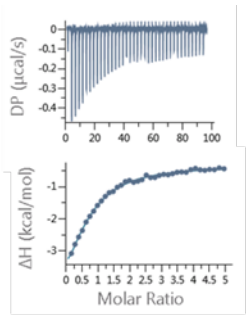


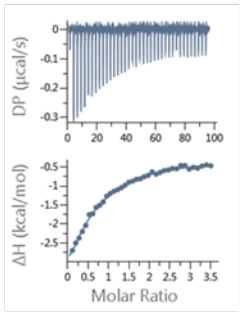
Fig. S14. ITC results of the highest affinity fragments which were identified by PrOF NMR experiments. These ITC experiments were performed with 5FW-Brd4 in the presence of DMSO (0.5% DMSO).



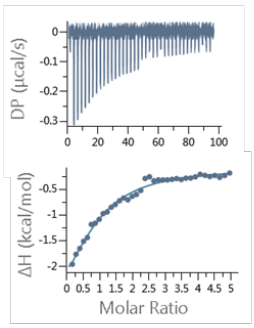
2
Kd = 37.4 µM



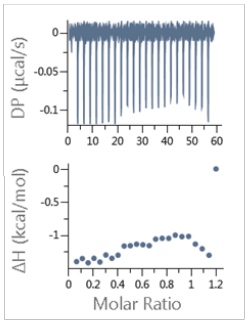
3
Kd = 42.1 µM



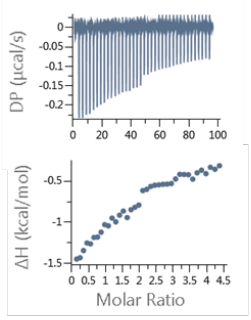
4
Kd = 54.6 µM



6
Unclear by ITC



8
Negative by ITC



9
Very low heat evolved
Unclear by ITC

Fig. S15. ITC results of the other fragments which were identified by PrOF NMR experiments. These ITC experiments were performed with Brd4 in the presence of DMSO (0.5% DMSO).

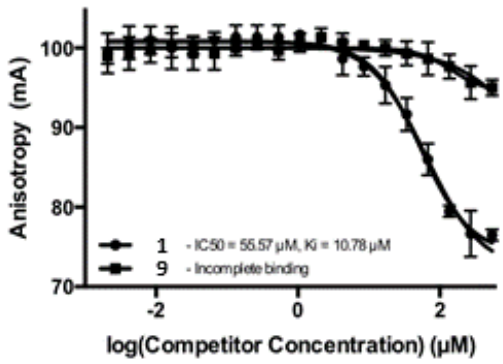
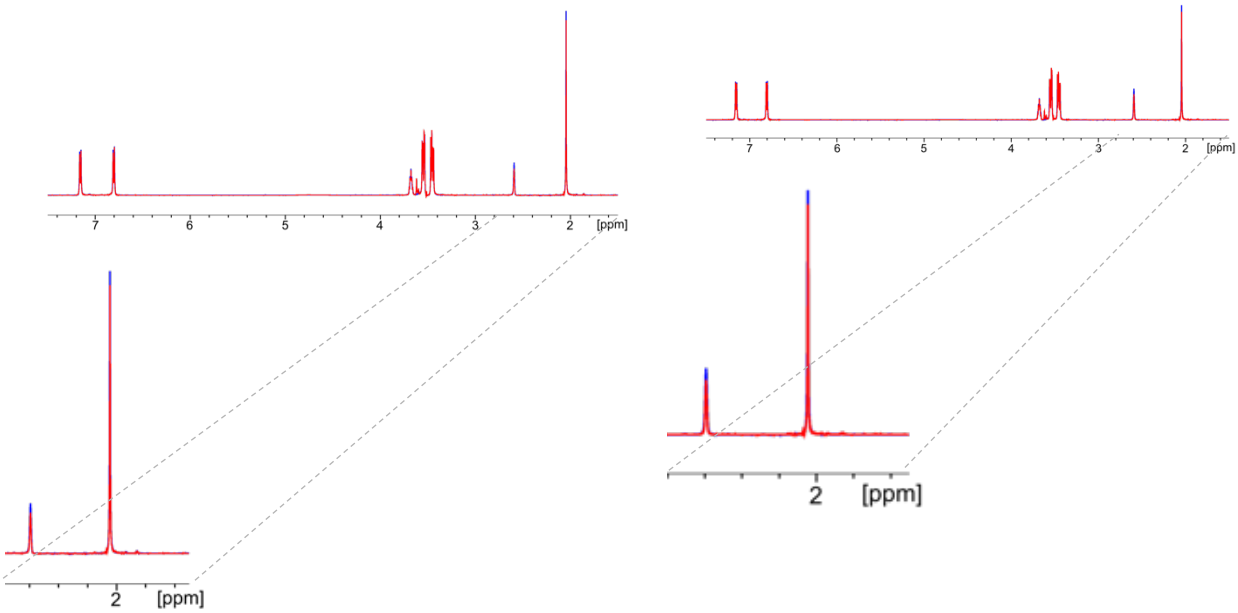


Fig. S16. Fluorescence anisotropy competitive inhibition measurement of **1** and **9**, displacing a fluorescently labeled ligand, BI-BODIPY. The isotherm for **9** shows incomplete binding, consistent with a low affinity ligand.

400 µM Acetaminophen, 10 µM Brd4, 400 ms

400 uM Acetaminophen, 10 µM Brd4, 800 ms



100 µM Acetaminophen, 10 µM Brd4, 400 ms

100 µM Acetaminophen, 10 µM Brd4, 800 ms

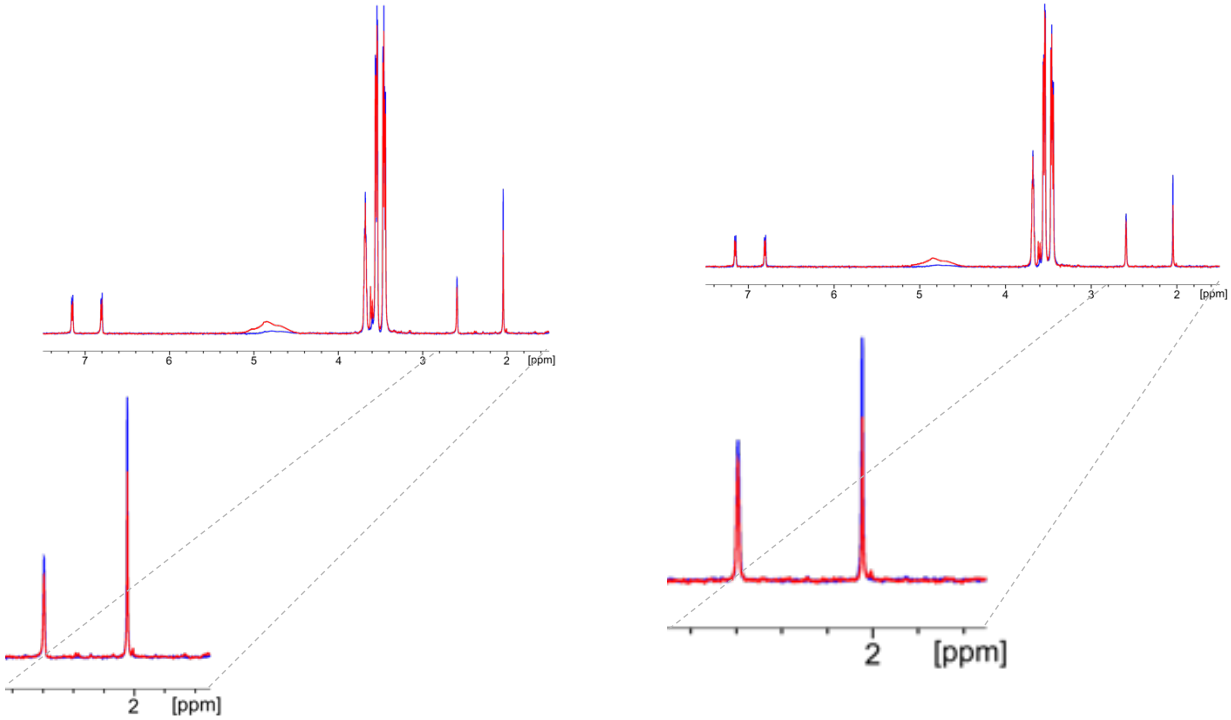


Fig. S17. A comparison of the different CPMG parameters. Blue NMR spectra are just ligand, while red NMR spectra are ligand + protein. Both concentration of ligand and CPMG filter length are varied, with 100 µM acetaminophen and an 800 ms filter length resulting in a greater signal drop upon protein addition than 400 µM acetaminophen with a 400 ms filter length.

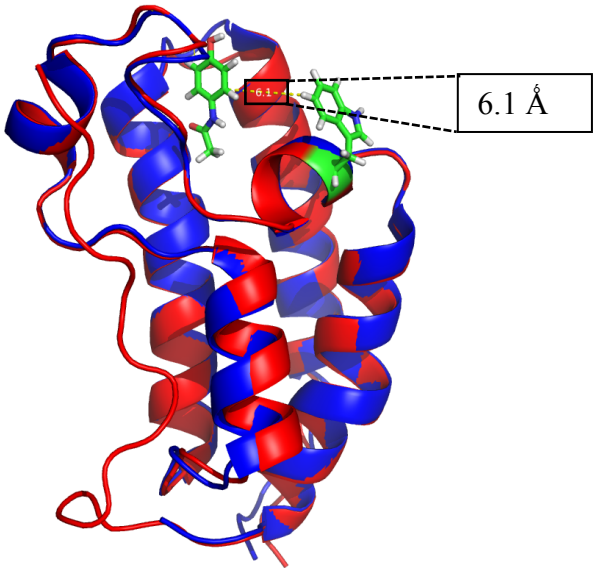


Fig. S18. An overlay of Brd4 (red) and Brd2 (blue) where Brd2 is crystallized with acetaminophen. Assuming a similar binding mode, this places the nearest heavy atom of acetaminophen 6.1 Å away from the fluorine of W81 in 5FW-Brd4.

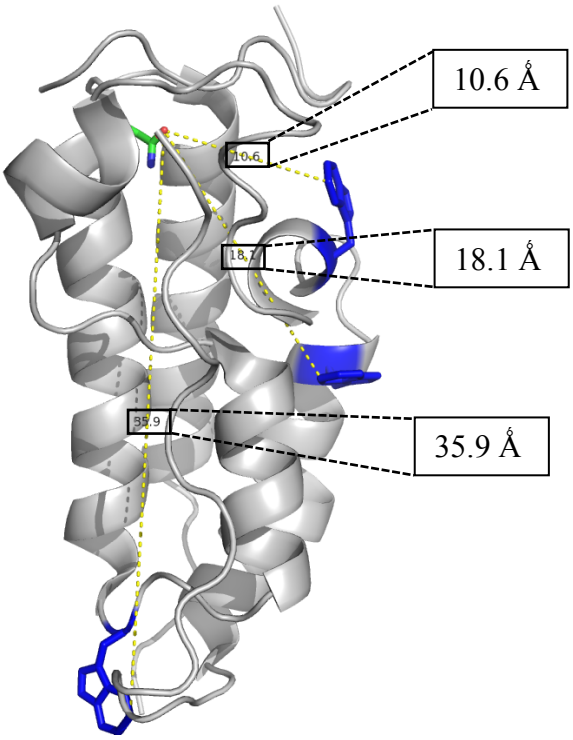


Fig. S19. Distance measurements of the three tryptophan residues from conserved asparagine 140.

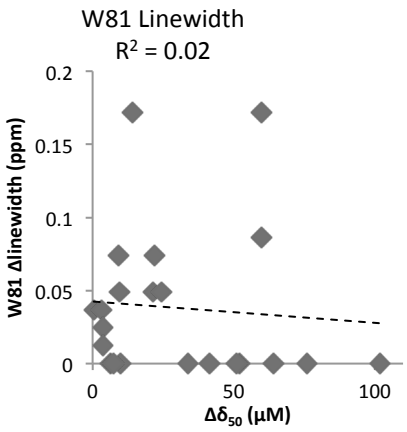


Fig. S20. A comparison of 20 different hits ranked by $\Delta\delta_{50}$ by PrOF and change in linewidth of W81.