Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Supporting Information

Estimating the cooperativity of PROTAC-induced ternary complexes using 19F NMR displacement assay

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Contents

1. Methods

1.1 Competition experiments ^{19}F NMR

A 500 MHz Bruker AVANCE NMR spectrometer equipped with a CPQCI-F cryoprobe was used for all NMR experiments described. Each sample was analysed in triplicates *via* proton-decoupled ^{19}F CPMG experiments (128 scans) at a fixed 2τ filter of 0.293 s (when [VBC] = 1.0 µM) or 0.398 s (when [VBC] = 0.5 µM). These optimal filters (d_{max}) were previously determined in our laboratory for spy molecule 19 at these concentrations of VBC. [1]

All experiments were performed at 20 °C in buffer supplied with 50 mM KH_2PO4, pH 7.5, 100 mM NaCl, 1 mM TCEP, 2% (v/v) DMSO, 20% deuterium oxide and 10 µM trifluoroacetic acid. All samples used in the competition experiments contained 50 µM of spy molecule 19 and 1 µM of VBC, with the exception of the titrations with PROTAC 15b where 0.5 µM of VBC was used. Where applicable, the final concentrations of bromodomain proteins were 10 µM to ensure saturation of bromodomain-binding portion of all PROTACs used. The concentrations of PROTACs varied between 5 µM and 390 nM (2-fold dilutions apart), with the exception of 15b where an additional concentration of 10 µM was also tested.

For every titration two controls were prepared (10 µM of the respective bromodomain added to both samples where applicable):

1) <u>100% displacement control:</u> Spy molecule 19 at 50 µM;
2) <u>0% displacement control:</u> Spy molecule 19 at 50 µM and VBC at 1.0 µM (with the exception of the titrations with PROTAC 15b where 0.5 µM of VBC was used);

At each concentration of the competitor, the displacement of the spy molecule was calculated as shown below:

$$Displacement = \frac{I_C - I_0}{I_{100} - I_0} \times 100\%$$

Where the ^{19}F CPMG peak integral of the 100% displacement control, 0% displacement control and competitor sample are respectively I_{100}, I_0 and I_C. The pIC_{50} of the competitors were determined by plotting the displacement against the respective competitor concentrations, fitting to a $\log_{10}$[Inhibitor] *versus* Normalised response model using GraphPad Prism 6.0.

1.2 Estimation of K_i and α

The derived IC_{50} values were converted to K_i using tight binding model.[2,3] In the absence of inhibitor, the equilibrium between spy molecule 19 (S) and VBC (P) is described as:

$$S + P \rightleftharpoons PS$$

$$K_d = \frac{[S][P]}{[PS]} = \frac{([S]_t - [PS])([P]_t - [PS])}{[PS]}$$

Where $[P]_t$ and $[S]_t$ are, respectively, the total concentrations of VBC and spy molecule 19 and K_d is the dissociation constant of the interaction (K_d = 145 µM, previously determined by SPR).[1] At equilibrium, the concentration of bound spy molecule (PS) can be determined as shown below:

$$[PS] = \frac{[P]_t + K_d + [S]_t - \sqrt[2]{(K_d + [S]_t + [P]_t)^2 - 4[P]_t[S]_t}}{2}$$

In a competition experiment, when the concentration of a competitor (C) is sufficient to displace 50% of [PS], *i.e.* [C] equals the IC_{50}, the total concentration of VBC bound to the competitor (PC) can be estimated.[4]

$$[PC] = [P]_t - \frac{[PS]}{2} - \frac{K_d[PS]}{2[S]_t - [PS]}$$

For MZ1, MZP-54, MZP-55, MZP-61, 15b and CMP99 the IC_{50} values could then be converted to the respective K_i using the equations below:

$$C + P \rightleftharpoons PC$$

$$K_i = \frac{[C][P]}{[PC]} = \frac{(IC_{50} - [PC])\left([P]_t - [PC] - \frac{[PS]}{2}\right)}{[PC]}$$

For CM11, to facilitate comparisons with values reported in the literature, each molecule of CM11 was treated as two VBC-binding competitors. Therefore, the observed IC_{50} was multiplied by two in the equation above in order to obtain an overall K_i for the two binding events:

$$K_i = \frac{[C][P]}{[PC]} = \frac{(2 \times IC_{50} - [PC])\left([P]_t - [PC] - \frac{[PS]}{2}\right)}{[PC]}$$

The cooperativity (α) of the PROTAC towards its target was then determined by the following equation:[5]

$$\alpha = \frac{K_{i}^{Binary\ complex}}{K_{i}^{Ternary\ complex}}$$

1.3 Measurement of the F^{19} transverse relaxation rates

For each sample multiple decoupled ^{19}F CPMG experiments were performed varying the total 2τ filter. The same buffer composition, temperature and number of scans described is section 1.1 were employed. The peaks from each spectra were integrated and plotted against the respective filter lengths. The data was fitted using GraphPad Prism 6 with the equation below:[6]

$$I_{(t)} = I_{(0)} \times e^{-R_2 t}$$

Where $I_{(t)}$ is the ^{19}F peak integral, t is the length of the respective filter in seconds, $I_{(0)}$ is the signal intensity when t = 0 (extrapolated from the fitting) and R_2 is the observed transverse relaxation rate. The transverse relaxation times (T_2) were derived using the following equation:[6]

$$T_2 = \frac{1}{R_2}$$

1.4 Resources

<u>Recombinant proteins</u>

The VBC complex was expressed and purified as previously reported.[7] Bromodomains Brd2(1), Brd2(2), Brd3(1), Brd3(2), Brd4(1) and Brd4(2) were expressed and purified as previously reported.[5]

<u>Small molecules</u>

All small molecules used were synthesised previously in our laboratory following the procedures described in the references below:

- Spy molecules 7, 18 and 19;[1]
- PROTAC MZ1;[8]
- PROTAC MZP-54, MZP-55 and MZP-61;[9]
- PROTAC 15b;[10]
- PROTACs CMP98, CMP99 and CM11.[11]

2. Supplementary figures

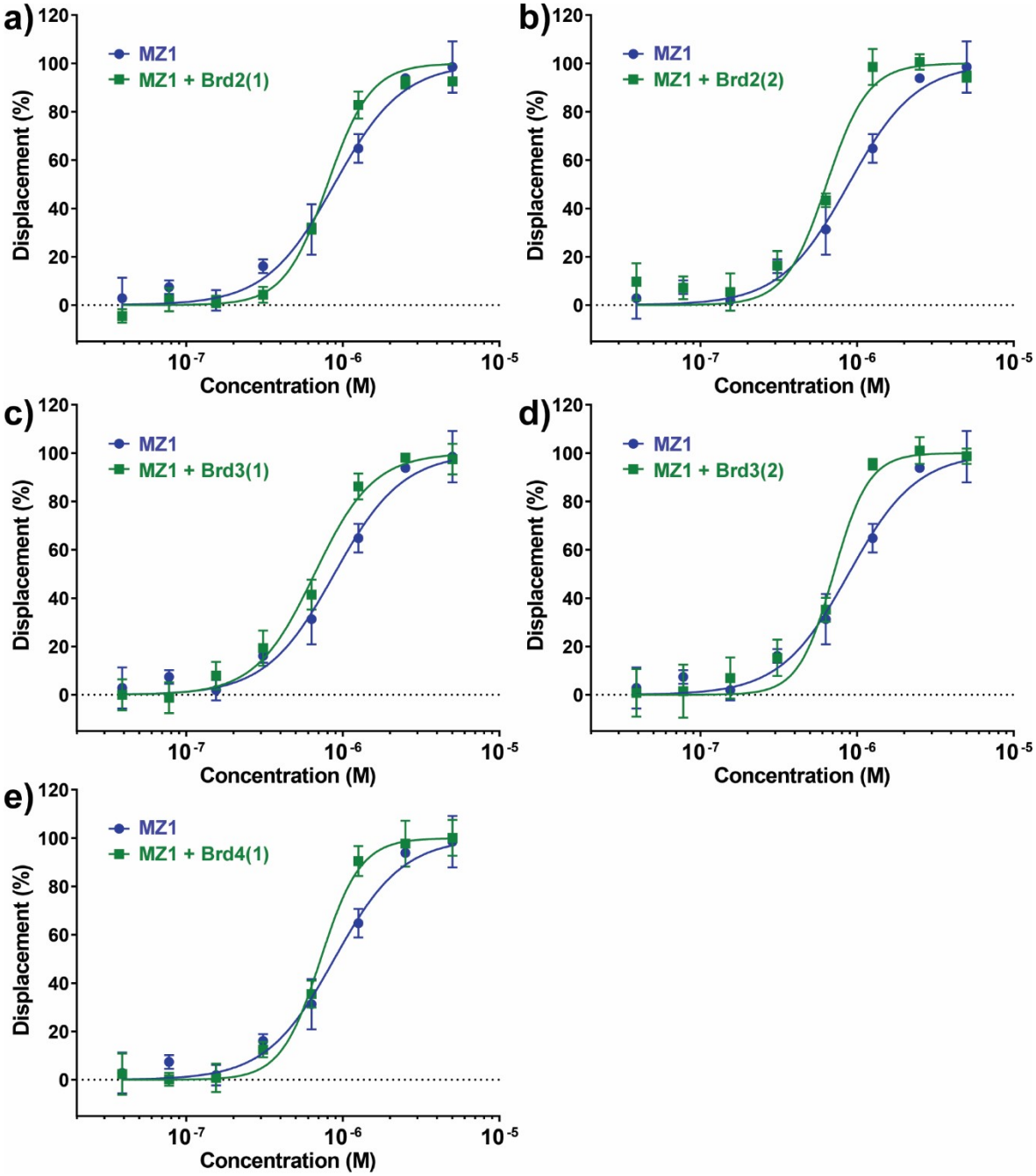

Figure S1. Displacement curves of spy molecule 19 by increasing concentrations of MZ1 in the presence of bromodomains. Measurements were performed in the absence (blue) and in the presence (green) of 10 µM of Brd2(1) **(a)**, Brd2(2) **(b)**, Brd3(1) **(c)**, Brd3(2) **(d)** and Brd4(1) **(e)**. All measurements were performed in triplicates and the concentrations of spy molecule 19 and VBC were 50 µM and 1 µM, respectively.

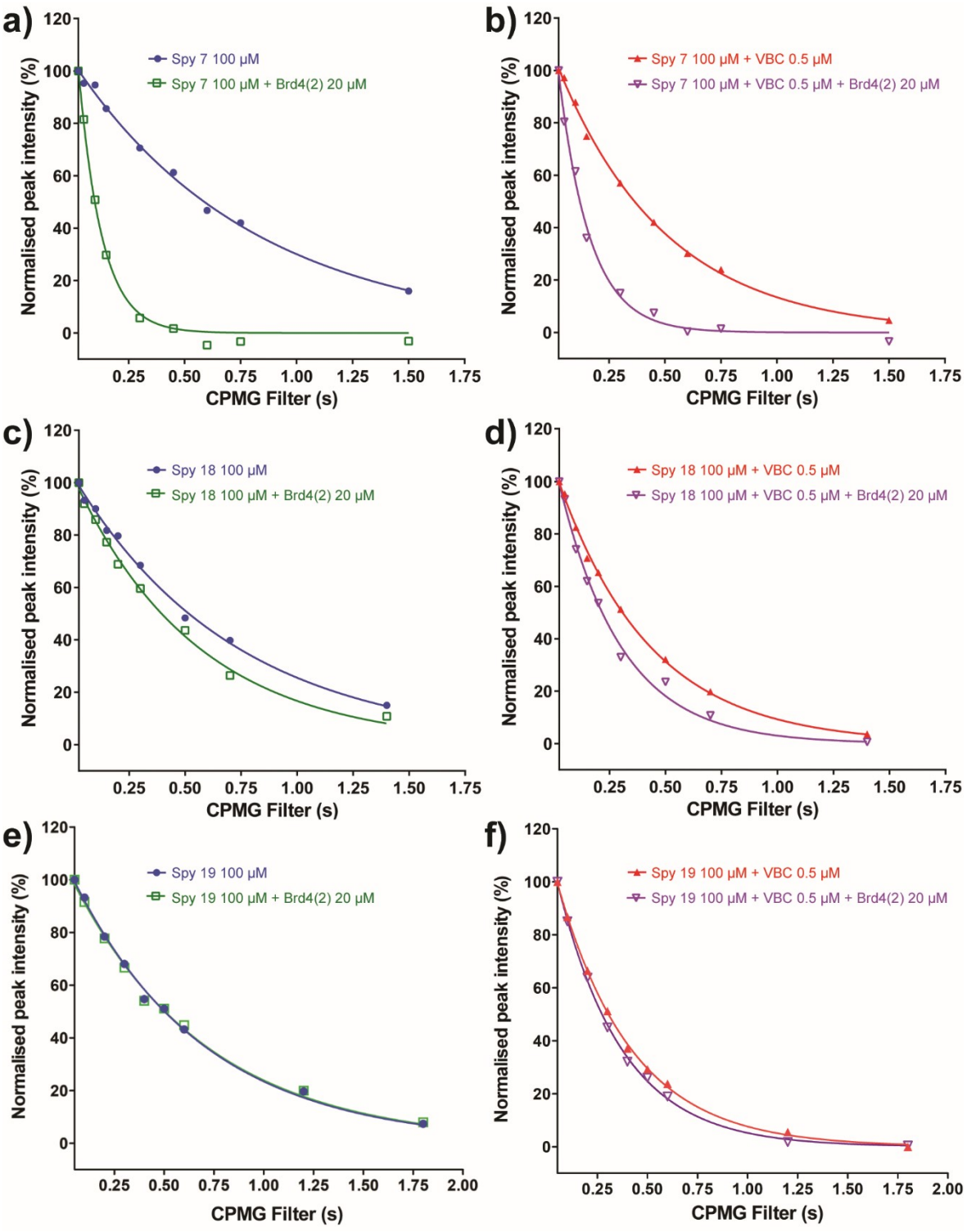

Figure S2. Measurement of transverse relaxation rate of spy molecules 7, 18 and 19 at multiple conditions. (a) Solution of spy molecule 7 at 100 µM in the absence (blue) and in the presence (green) of Brd4(2) 20 µM; **(b)** Solution of spy molecule 7 at 100 µM and VBC 0.5 µM in the absence (red) and in the presence (violet) of Brd4(2) 20 µM; **(c)** Solution of spy molecule 18 at 100 µM in the absence (blue) and in the presence (green) of Brd4(2) 20 µM; **(d)** Solution of spy molecule 18 at 100 µM and VBC 0.5 µM in the absence (red) and in the presence (violet) of Brd4(2) 20 µM; **(e)** Solution of spy molecule 19 at 100 µM in the absence (blue) and in the presence (green) of Brd4(2) 20 µM; **(d)** Solution of spy molecule 19 at 100 µM and VBC 0.5 µM in the absence (red) and in the presence (violet) of Brd4(2) 20 µM. Fitting parameters of all curves can be found at the ESI Table S2.

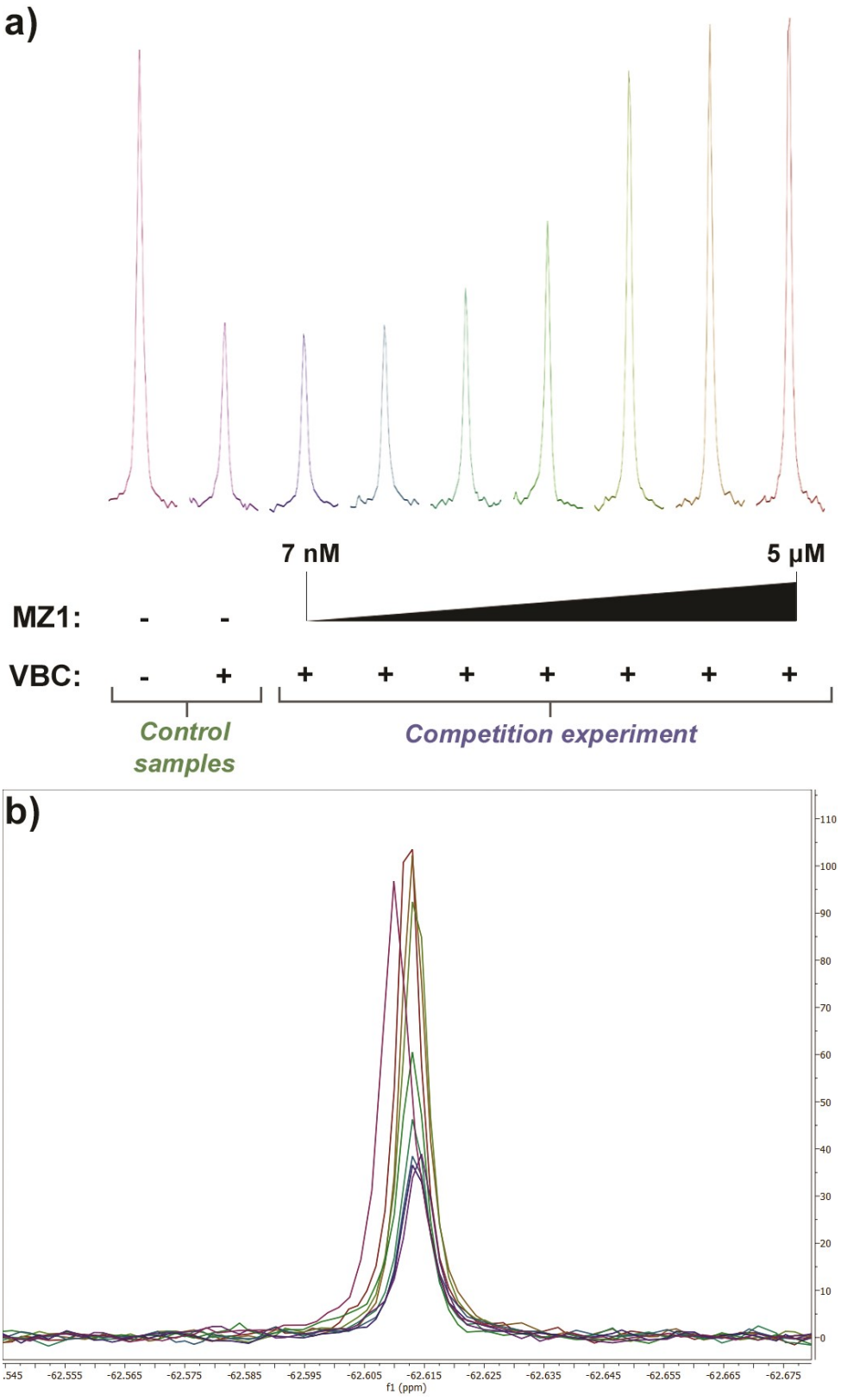

Figure S3. Representative ^{19}F NMR CPMG spectra of spy molecule 19 in the displacement assay. (a) Changes in the intensity of the ^{19}F CPMG peak of the trifluoromethyl group of spy molecule 19 at varied concentrations of VBC and MZ1. When present, the total concentration of VBC was 0.5 µM while MZ1 was titrated at multiple concentrations (3-fold dilutions from 5 µM down to approximately 7 nM). In all cases, samples contained spy molecule 19 at 100 µM and Brd4(2) at 20 µM. **(b)** Superimposition and integration window (-62.68 ppm to -62.54 ppm) of the spectra shown in **(a)**.

3. Supplementary tables

Table S1. Fitted pIC_{50} and R-squared of the PROTAC titrations performed with spy molecule 19 (Figures 2, 3 and S1). All titrations were performed in the presence of VBC at 1.0 µM and spy molecule 19 at 50 µM. Uncertainties are expressed as the standard deviation of the mean.

PROTAC	Target protein	Fitted values	
		pIC_{50} [a]	R-squared
MZ1	-	6.055 ± 0.025	0.969
	Brd2(1)	6.098 ± 0.014	0.987
	Brd2(2)	6.195 ± 0.023	0.961
	Brd3(1)	6.181 ± 0.021	0.978
	Brd3(2)	6.150 ± 0.021	0.967
	Brd4(1)	6.141 ± 0.018	0.979
	Brd4(2)	6.205 ± 0.024	0.971
MZP-54	-	5.985 ± 0.037	0.936
	Brd4(2)	5.888 ± 0.021	0.977
MZP-55	-	5.944 ± 0.028	0.962
	Brd4(2)	5.884 ± 0.024	0.970
MZP-61	-	5.916 ± 0.035	0.945
	Brd4(2)	5.676 ± 0.024	0.969
15b	-	5.520 ± 0.046	0.900
	Brd4(2)	6.460 ± 0.027	0.968
CMP99	-	6.051 ± 0.025	0.970
CM11	-	6.558 ± 0.031	0.951

[a] $pIC_{50} = -\log_{10}[IC_{50}]$

Table S2. Fitted ^{19}F transverse relaxation rates (R_2), R-square of the fitting and derived ^{19}F transverse relaxation rates (T_2) of spies molecules 7, 18 and 19. Uncertainties are expressed as the standard deviation of the mean.

Spy molecule	[VBC] µM	[Brd4(2)] µM	R_2 (s^{-1})	R-square	T_2 (s)
7	-	-	1.236 ± 0.041	0.996	0.809
	0.5	-	2.067 ± 0.052	0.998	0.484
	-	20	9.570 ± 0.557	0.996	0.104
	0.5	20	7.183 ± 0.440	0.995	0.139
18	-	-	1.388 ± 0.047	0.996	0.720
	0.5	-	2.435 ± 0.056	0.999	0.411
	-	20	1.812 ± 0.071	0.996	0.552
	0.5	20	3.578 ± 0.189	0.994	0.279
19	-	-	1.520 ± 0.048	0.997	0.658
	0.5	-	2.702 ± 0.043	0.999	0.370
	-	20	1.487 ± 0.056	0.996	0.672
	0.5	20	3.103 ± 0.055	0.999	0.322

4. Supplementary references

1 G. V. de Castro and A. Ciulli, *Chem. Commun.*, 2019, **55**, 1482–1485.
2 C. H. Kenny, W. Ding, K. Kelleher, S. Benard, E. G. Dushin, A. G. Sutherland, L. Mosyak, R. Kriz and G. Ellestad, *Analytical Biochemistry*, 2003, **323**, 224–233.
3 R. Z. Cer, U. Mudunuri, R. Stephens and F. J. Lebeda, *Nucleic Acids Research*, 2009, **37**, W441–W445.
4 Z. Nikolovska-Coleska, R. Wang, X. Fang, H. Pan, Y. Tomita, P. Li, P. P. Roller, K. Krajewski, N. G. Saito, J. A. Stuckey and S. Wang, *Analytical Biochemistry*, 2004, **332**, 261–273.
5 M. S. Gadd, A. Testa, X. Lucas, K.-H. Chan, W. Chen, D. J. Lamont, M. Zengerle and A. Ciulli, *Nature Chemical Biology*, 2017, **13**, 514–521.
6 C. Dalvit and A. Vulpetti, *J. Med. Chem.*, 2019, **62**, 2218–2244.
7 C. Galdeano, M. S. Gadd, P. Soares, S. Scaffidi, I. Van Molle, I. Birced, S. Hewitt, D. M. Dias and A. Ciulli, *J. Med. Chem.*, 2014, **57**, 8657–8663.
8 M. Zengerle, K.-H. Chan and A. Ciulli, *ACS Chem. Biol.*, 2015, **10**, 1770–1777.
9 K.-H. Chan, M. Zengerle, A. Testa and A. Ciulli, *J. Med. Chem.*, 2018, **61**, 504–513.
10 A. Testa, X. Lucas, G. V. Castro, K.-H. Chan, J. E. Wright, A. C. Runcie, M. S. Gadd, W. T. A. Harrison, E.-J. Ko, D. Fletcher and A. Ciulli, *J. Am. Chem. Soc.*, 2018, **140**, 9299–9313.
11 C. Maniaci, S. J. Hughes, A. Testa, W. Chen, D. J. Lamont, S. Rocha, D. R. Alessi, R. Romeo and A. Ciulli, *Nature Communications*, 2017, **8**, 830.