Supplementary Information

Selective Small Molecule Induced Degradation of the BET Bromodomain Protein BRD4

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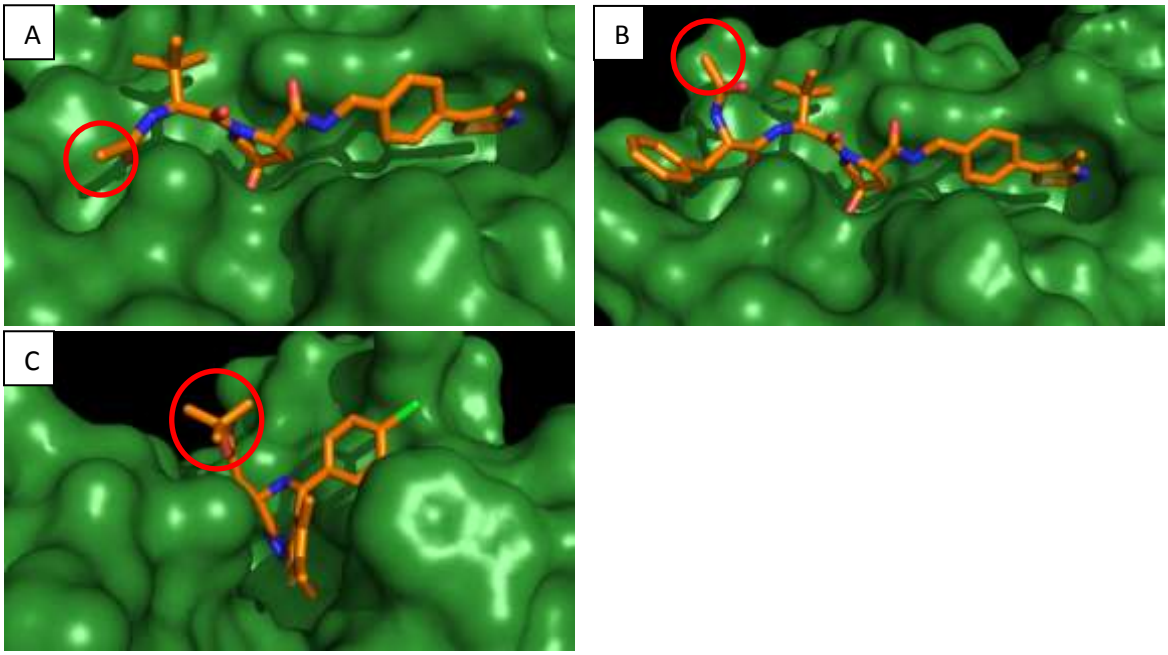


Figure S1: Structure-guided design of VHL-BET bromodomain PROTACs.

A: compound VHL-1 bound to the von-Hippel-Lindau protein (pdb-code 4W9H); B: compound VHL-2 bound to the von-Hippel-Lindau protein (pdb-code 4W9K); C: JQ1 bound to the first bromodomain of BRD4 (pdb-code 3XMF). The red circles show the solvent exposed parts of the molecules chosen for connection.

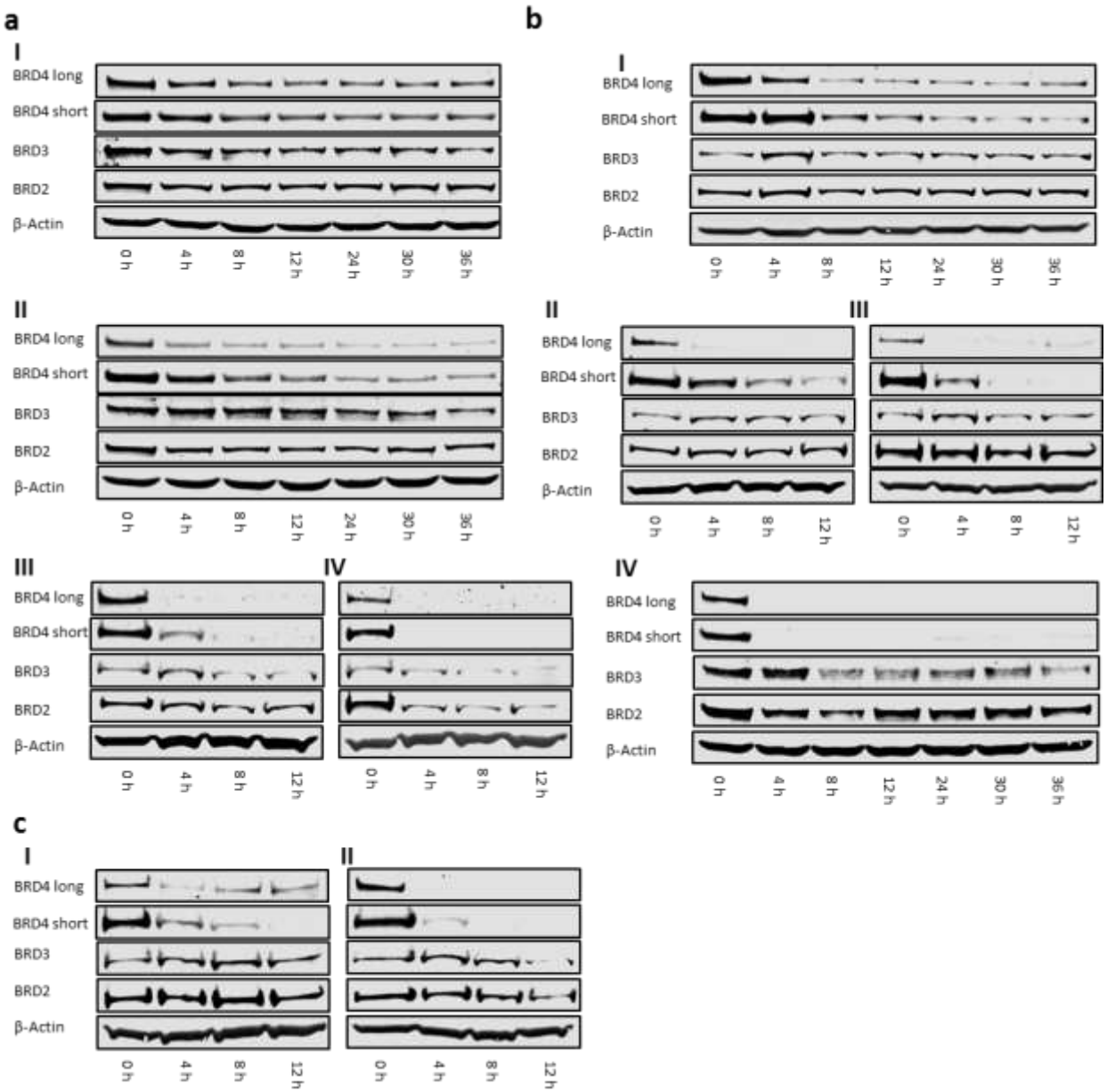


Figure S2: Time dependent treatment of HeLa cells with compounds MZ1, MZ2 and MZ3 at different concentrations.

(**a**) time dependent treatment of HeLa cells with MZ1 at concentrations of **I** 10 nM, **II** 50 nM, **III** 250 nM, **IV** 500 nM; (**b**) time dependent treatment of HeLa cells with MZ2 at concentrations of **I** 100 nM , **II** 250 nM, **III** 500 nM, **IV** 1 µM; (**c**) time dependent treatment of HeLa cells with MZ3 at concentrations of **I** 500 nM and **II** 1 µM.

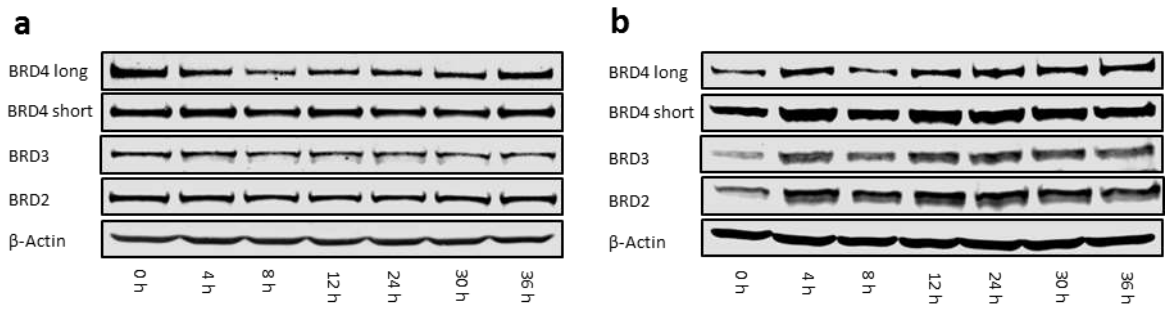


Figure S3: Time dependent treatment over 36 h of HeLa cells with (**a**) 0.01 % DMSO, (**b**) 1 µM JQ1.

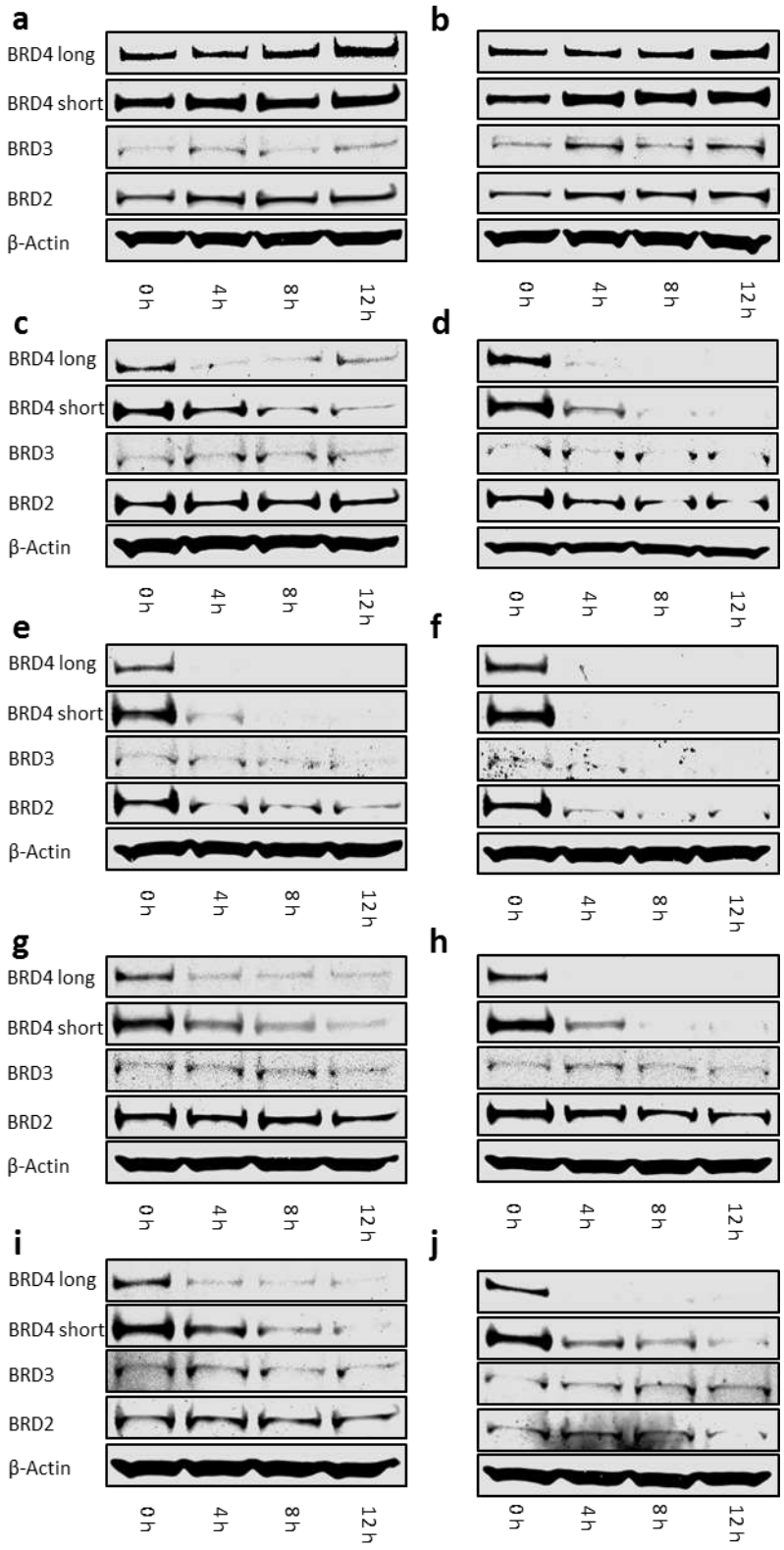


Figure S4: U2OS cells were treated over a time course of 12 h in 4 h intervals with the following compounds and concentrations: (**a**) DMSO 0.01%, (**b**) JQ1 1 µM, (**c**) MZ1 100 nM, (**d**) MZ1 250 nM, (**e**) MZ1 500 nM, (**f**) MZ1 1 µM nM, (**g**) MZ2 500 nM, (**h**) MZ2 1 µM, (**i**) MZ3 500 nM, and (**j**) MZ3 1 µM.

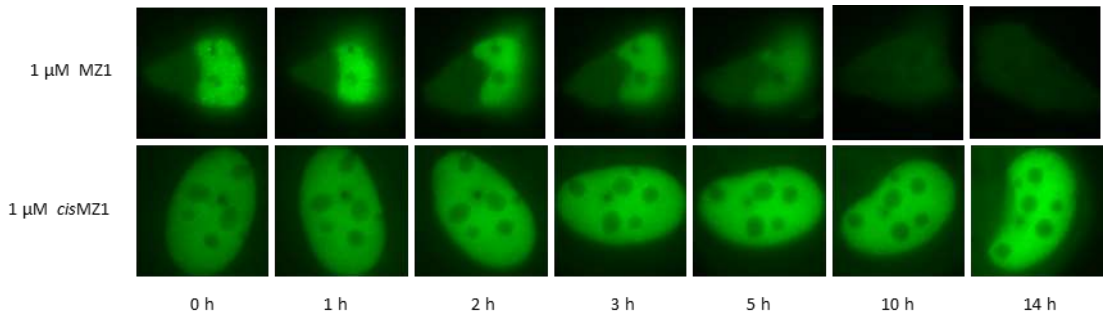


Figure S5: U2OS cells, transfected with GFP-BRD4 were treated with either 1 µM of MZ1 or *cis*MZ1 over a time course of 14 h. BRD4 degradation was followed by live fluorescence imaging.

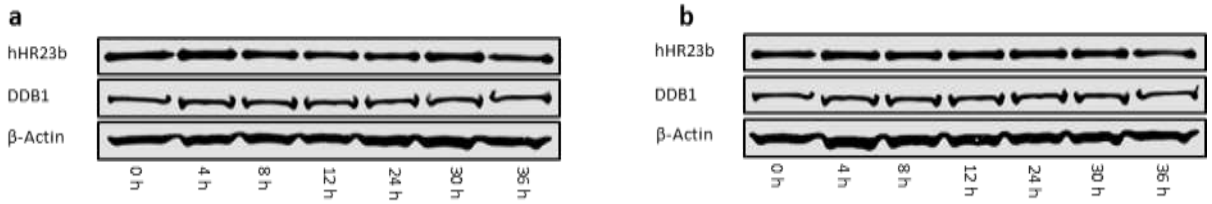


Figure S6: MZ1 does not degrade known off-targets of JQ1.

Time dependent treatment over 36 h of HeLa cells with (a) 1 µM of MZ1 or (b) 100 nM of MZ1. The abundance of DDB1 and hHR23b was analyzed by western blotting.

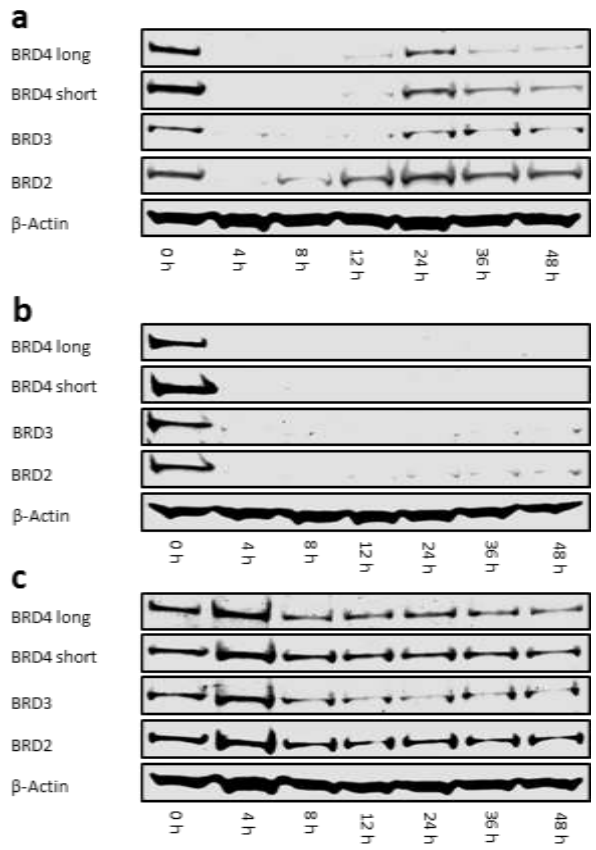


Figure S7: BET protein levels were observed (**a**) with single treatment of MZ1 for 4 h and then exchange of media, (**b**) single treatment with MZ1 at *t* = 0 but no exchange of media, (**c**) single treatment with 0.01 % DMSO for 4 h and then exchange of media.

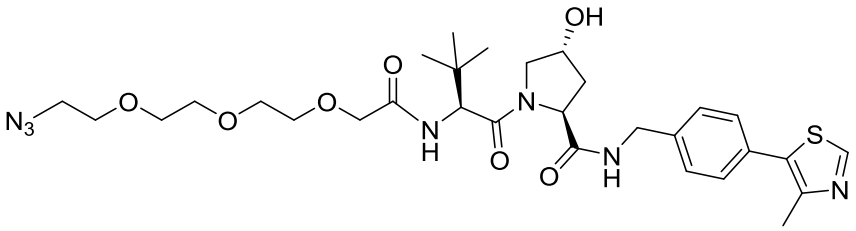


Figure S8: Chemical structure of compound VHL-1' (**13**) lacking the BET protein binding part. For synthetic information see materials and methods part.

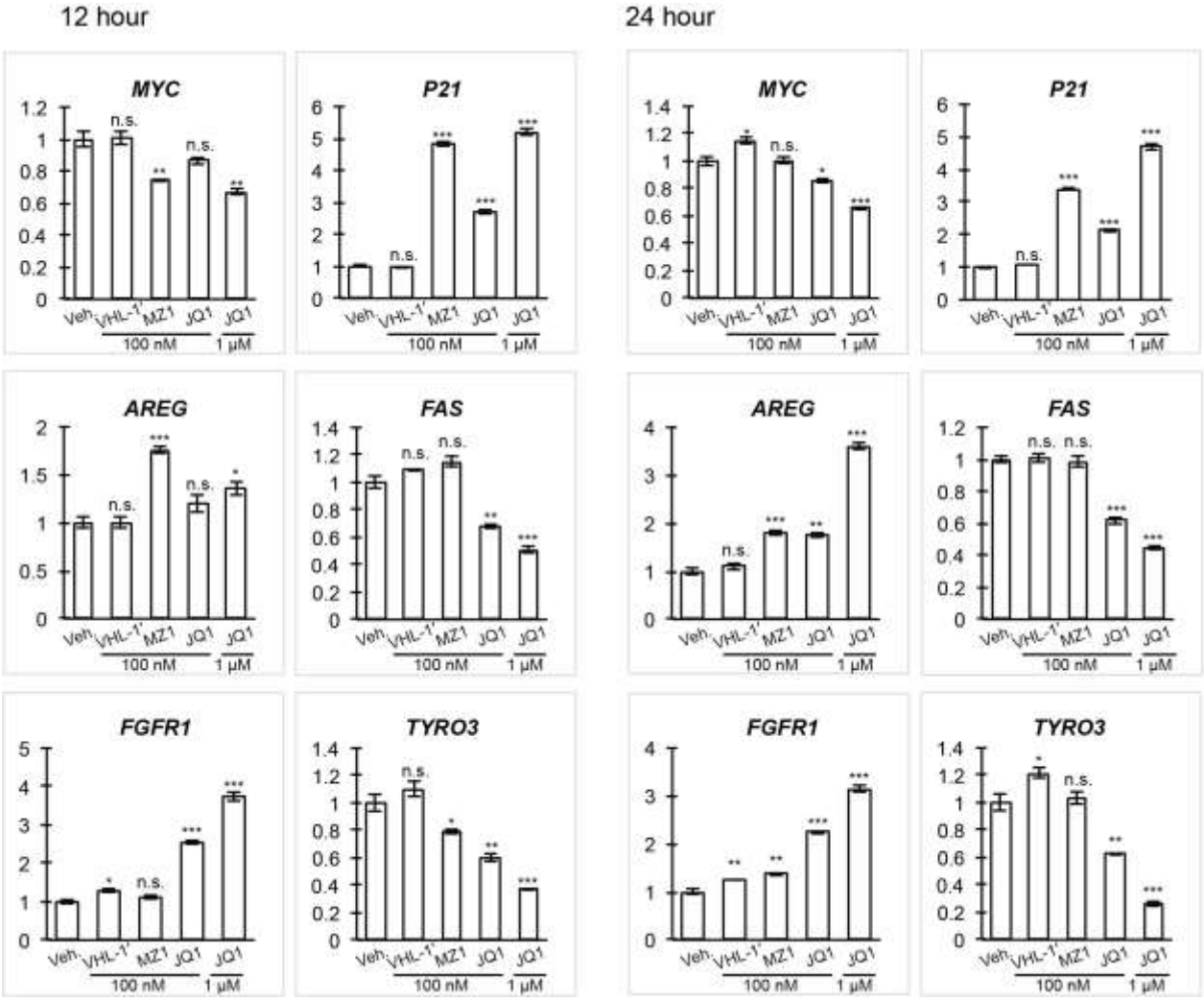


Figure S9: Comparison of mRNA expression profiles of *MYC*, *P21*, *AREG*, *FAS*, *FGFR1* and *TYRO3* upon treatment with MZ1 and JQ1.

HeLa cells were treated with 100 nM of MZ1, VHL-1', or JQ1, or 1 µM of JQ1 or 0.01% DMSO vehicle control (Veh.) for (A) 12 hours or (B) 24 hours. Quantitative PCR was performed to analyze relative gene expression level of treated HeLa cells using target specific primers. Gene expression levels relative to *GAPDH* were normalized to control treatment. The data shown represent the mean ± SEM (n= 3, technical replicates) of one experiment. Statistical significance compared to the control was determined with two-tailed t tests: *P < 0.05; **P < 0.01; ***P < 0.001; n.s. not significant.

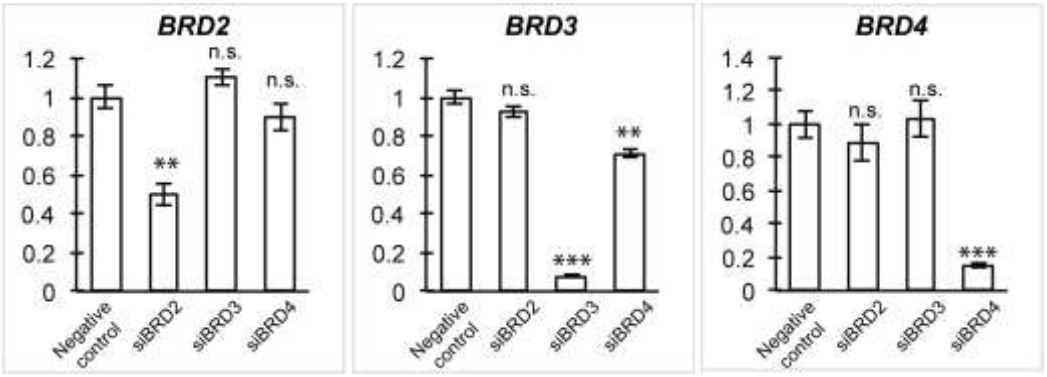


Figure S10: Verification of the effectiveness of siRNA suppression of BET genes.

mRNA expression of *BRD2*, *BRD3* and *BRD4* were selectively suppressed upon transfection of their respective siRNA. HeLa cells were transfected with siRNA targeting individual *BRD2*, *BRD3* or *BRD4* or with negative control siRNA and were harvested after 48 hours. Quantitative PCR was performed to analyze relative gene expression level of treated HeLa cells using target specific primers. Gene expression levels relative to GAPDH were normalized to control treatment. The data shown represent the mean ± SEM (n= 3, technical replicates) of one experiment. Statistical significance compared to the control was determined with two-tailed t tests: *P < 0.05; **P < 0.01; ***P < 0.001; n.s. not significant.

Materials and Methods

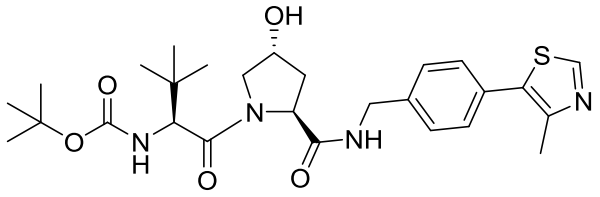
Chemistry

General information

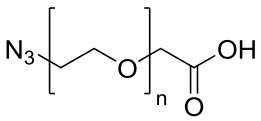
NMR spectra were recorded on a Bruker 500 Ultrashield or a Bruker Ascend 400. Chemical shifts are quoted in ppm and referenced to the residual solvent signals: ^{1}H δ = 7.26 ($CDCl_3$), ^{13}C δ = 77.16 ($CDCl_3$). High Resolution Mass Spectra (HRMS) were recorded on a Bruker micrOTOF. All chemicals, unless otherwise stated were commercially available and used without further purification. Enantiopure (+)-JQ1 was purchased from Medchemexpress LLC, Princeton, USA. Flash column chromatography was performed using a Teledyne Isco Combiflash Rf or Rf200i. As prepacked columns RediSep Rf Normal Phase Disposable Columns were used. Preparative HPLC was performed on a Gilson Preparative HPLC System with a Waters X-Bridge C18 column (100 mm x 19 mm; 5 µm particle size) and a gradient of 20 % to 95 % acetonitrile in water with 0.1 % ammonia in the aqueous phase.

Synthetic procedures

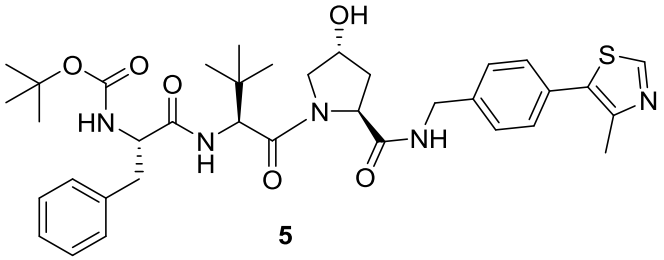
The following compounds were prepared according to literature procedures : **4** and **5**,[1] PEG linkers **6** and **7**.[2]



4

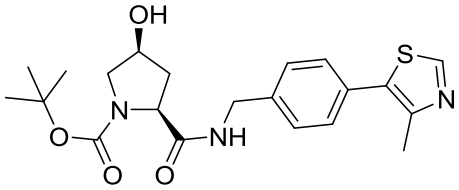


n = 3 **6**
n = 4 **7**



5

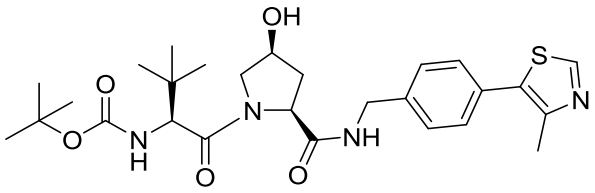
tert-butyl (2S,4S)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-carboxylate (**8**)



To a solution of (4-(4-methylthiazol-5-yl)phenyl)methanamine[1] (500 mg, 2.43 mmol, 1 eq.) in dichloromethane (DCM) was added (2S,4S)-1-(tert-butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic acid (565 mg, 2.43 mmol, 1 eq.) and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (827 mg, 2.68 mmol, 1.1 eq.). After the pH of the reaction was adjusted to >9 by addition of *N,N*-Diisopropylethyl amine (1.70 ml, 9.72 mmol, 4 eq.) the reaction was stirred for 2 h at 25 °C. The reaction mixture was washed with water and the organic phase then dried over $MgSO_4$. After removing the solvent in vacuum the residue was purified by flash column chromatography using a gradient of 10% to 70% Acetone in Hexane. Yield: 587 mg (58%); ^{1}H-NMR ($CDCl_3$, 400 MHz) 1.45 (s, 9H), 2.14-2.23 (m, 1 H), 2.34-2.39 (m, 1 H), 2.51 (s, 3H), 3.44-3.53 (m, 2H), 4.40-4.46 (m, 4H), 4.58 (dd, 1 H, *J*(*H*,*H*)= 7.1 Hz, *J*(*H*,*H*)= 14.9 Hz), 7.32-7.39 (m, 4 H), 7.51-7.54 (m, 1 H), 8.67 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 101

MHz) δ 12.7, 28.4, 35.9, 55.9, 57.2, 59.7, 70.9, 81.0, 127.8, 129.7, 131.2, 137.7, 148.7, 150.4, 155.9, 162.8, 173.5; HRMS m/z calc. for $C_{21}H_{28}N_3O_4S$ [M+H$^+$] 418.1795, found 418.1786.

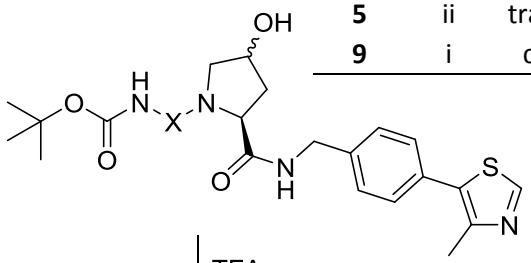
tert-butyl ((S)-1-((2S,4S)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (**9**).



8 was boc-deprotected as described below to obtain **8***TFA. **8***TFA (604 mg, 1.40 mmol, 1 eq.) and (S)-2-((tert-butoxycarbonyl)amino)-3,3-dimethylbutanoic acid (324 mg, 1.40 mmol, 1 eq.) were dissolved in DCM (100 ml). After addition of HATU (798 mg, 2.10 mmol, 1.5 eq.) the pH was adjusted to >9 by addition of *N*,*N*-Diisopropylethyl amine (978 µl, 5.60 mmol, 4 eq.)and the reaction stirred at 25 °C for 2 h. The reaction mixture was then washed with water and the remaining organic phase dried over magnesium sulfate. After removing the solvent in vacuum the residue was purified by flash column chromatography using a gradient of 10% to 60% Acetone in Hexane. Yield: 300 mg (40 %); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.90 (s, 9 H), 1.41 (s, 9 H), 2.16-2.23 (m, 1 H), 2.36-2.40 (m, 1 H), 2.52 (s, 3H), 3.78-3.91 (m, 2 H), 4.18 (d, 1 H, *J*(*H*,*H*)= 8.4 Hz), 4.29 (dd, 1 H, *J*(*H*,*H*)= 5.1 Hz, *J*(*H*,*H*)= 14.9 Hz), 4.48 (s, 1 H), 4.64 (dd, 1 H, *J*(*H*,*H*)= 7.1 Hz, *J*(*H*,*H*)= 14.9 Hz), 4.77 (d, 1 H, *J*(*H*,*H*)= 8.8 Hz), 5.12 (d, 1H, *J*(*H*,*H*)= 9.0 Hz), 5.56 (s, 1 H), 7.33-7.39 (m, 4 H), 7.52-7.56 (m, 1 H), 8.69 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ 14.3, 16.1, 22.8, 26.4, 28.5, 32.0, 35.1, 58.5, 58.7, 60.0, 71.2, 80.0, 128.3, 129.8, 131.4, 131.7, 137.4, 148.6, 150.6, 155.8, 172.7, 173.0; HRMS m/z calc. for $C_{27}H_{39}N_4O_5S$ [M+H$^+$] 531.2636, found 531.2660.

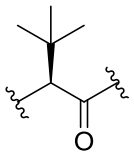
General procedure for Boc-deprotection:

	x	Hyp
4	i	trans
5	ii	trans
9	i	cis

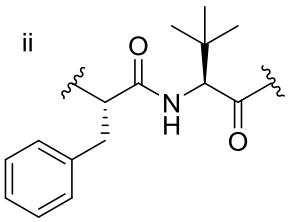


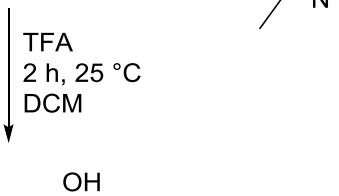
X =

i

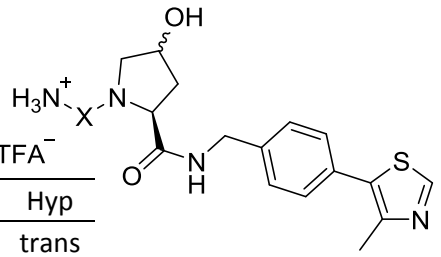


ii





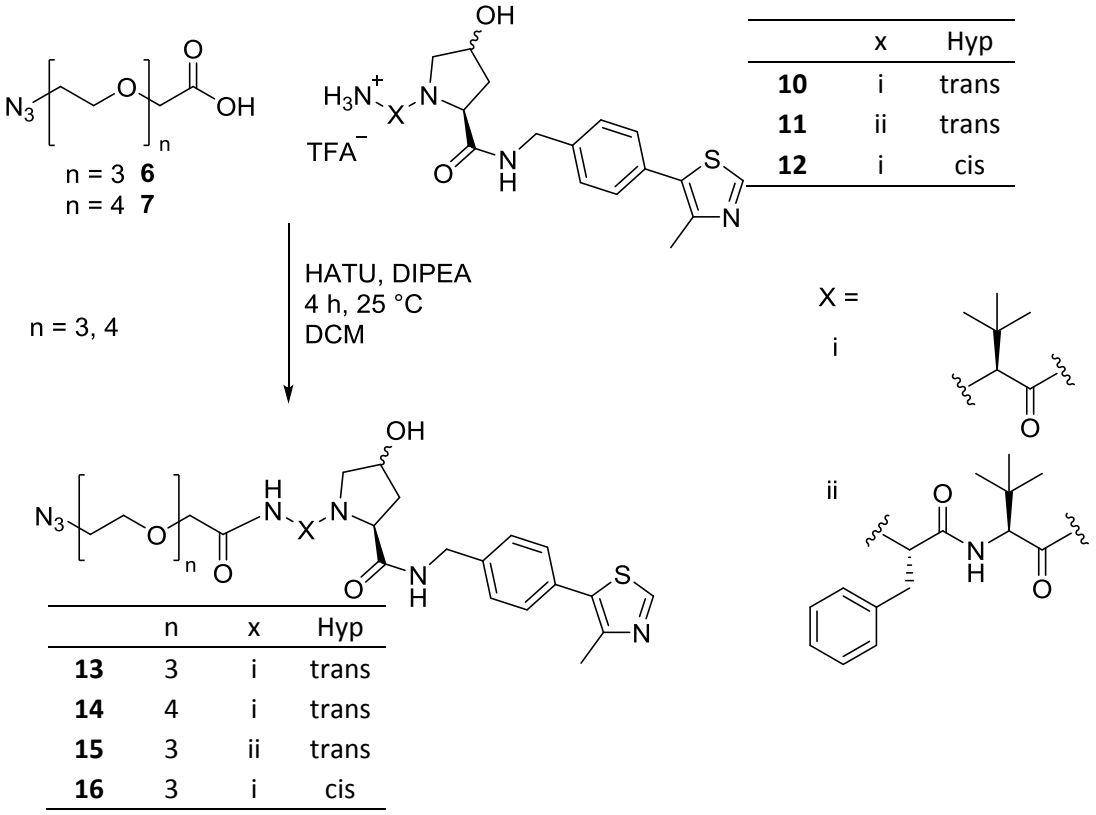
TFA
2 h, 25 °C
DCM



	x	Hyp
10	i	trans
11	ii	trans
12	i	cis

The *N*-Boc-protected compound was dissolved in dichloromethane (10 ml/1 mmol). Trifluoroacetic acid (10 ml/1 mmol) was added and the reaction mixture stirred at room temperature for 2 h. The solvent was removed under reduced pressure. For three times dichloromethane (5 ml /1 mmol) was added and then the solvent again removed in vacuum to remove residual trifluoroacetic acid.

General procedure for linker coupling:

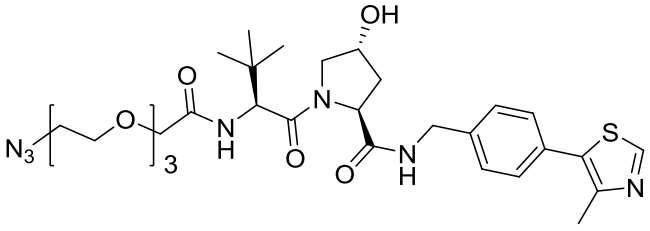


	x	Hyp
10	i	trans
11	ii	trans
12	i	cis

	n	x	Hyp
13	3	i	trans
14	4	i	trans
15	3	ii	trans
16	3	i	cis

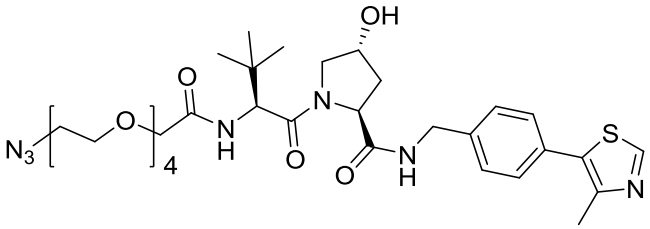
The amine (**10** - **12**) (1 mmol, 1 eq.) was added to a solution of PEG-linker (**6**, **7**) (1.2 mmol, 1.2 eq.) in DCM (40 ml). HATU (570 mg, 1.5 mmol, 1.5 eq.) was added and the pH adjusted to >9 by addition of DIPEA (700 µl, 4 mmol, 4 eq.). After stirring for 4 h at 25 °C the reaction mixture was extracted with water. The organic phase was dried over Magnesium sulfate and evaporated to dryness. The crude product was purified by flash column chromatography using a gradient of 0%-6% of Methanol in Dichloromethane.

(2S,4R)-1-((S)-14-azido-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**13**).



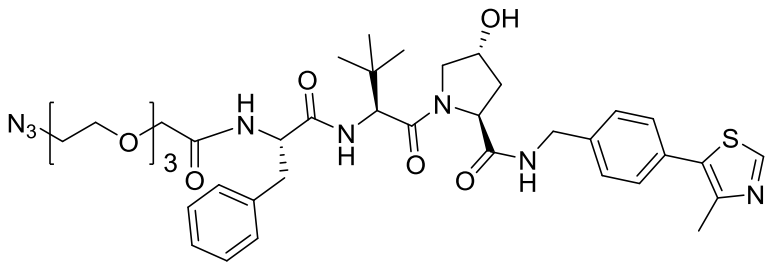
Yield: 491 mg (76 %); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.95 (s, 9 H), 2.09-2.14 (m, 1 H), 2.52 (s, 3 H), 2.58-2.63 (m, 1 H), 2.85 (s, 1 H), 3.37 (t, 2 H, *J*(*H*,*H*)= 10.1 Hz), 3.60 (dd, 1 H, *J*(*H*,*H*)= 3.6 Hz, *J*(*H*,*H*)= 11.4 Hz), 3.64-3.69 (m, 10 H), 3.96-4.05 (m, 2 H), 4.12-4.14 (m, 1 H), 4.34 (dd, 1 H, *J*(*H*,*H*)= 5.2 Hz, *J*(*H*,*H*)= 14.9 Hz), 4.46 (d, 1 H, *J*(*H*,*H*)= 8.4 Hz), 4.53-4.59 (m, 2 H), 4.75 (t, 1 H, *J*(*H*,*H*)= 7.9 Hz), 7.27 (s, 1 H), 7.33-7.38 (m, 5 H), 8.67 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 16.2, 26.6, 34.8, 35.7, 43.5, 50.9, 56.7, 57.4, 58.4, 70.2, 70.3, 70.5, 70.7, 70.8, 70.9, 71.3, 128.4, 129.7, 131.2, 131.7, 138.2, 148.7, 150.4, 170.6, 170.8, 171.8; HRMS m/z calc. for $C_{30}H_{44}N_7O_7S$ [M+H$^+$] 646.3017, found 646.3023.

(2S,4R)-1-((S)-17-azido-2-(tert-butyl)-4-oxo-6,9,12,15-tetraoxa-3-azaheptadecanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**14**).



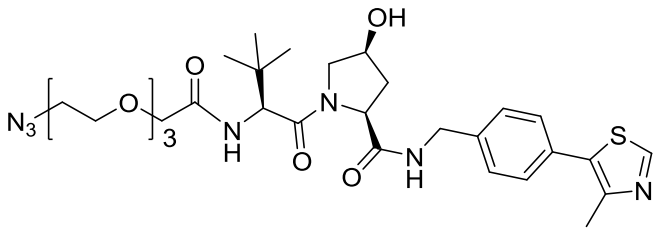
Yield: 607 mg (88 %); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.95 (s, 9 H), 2.10-2.15 (m, 1 H), 2.51-2.58 (m, 4 H), 2.96 (d, 1 H, *J*(*H*,*H*)= 3.0 Hz), 3.39-3.41 (m, 2 H), 3.60-3.67 (m, 15 H), 3.97-4.05 (m, 2 H), 4.07-4.09 (m, 1 H), 4.35 (dd, 1 H, *J*(*H*,*H*)= 5.3 Hz, *J*(*H*,*H*)= 14.9 Hz), 4.49 (d, 1 H, *J*(*H*,*H*)= 8.5 Hz), 4.53-4.57 (m, 2 H), 4.73 (t, 1 H, *J*(*H*,*H*)= 7.9 Hz), 7.15 (d, 1 H, *J*(*H*,*H*)= 8.5 Hz), 7.30-7.38 (m, 5 H), 8.68 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 16.2, 26.5, 26.6, 35.0, 35.9, 43.4, 50.8, 56.8, 57.3, 58.5, 70.2-71.3, 128.3, 129.7, 131.1, 131.8, 138.3, 148.6, 150.5, 170.7, 170.8, 171.6; HRMS m/z calc. for $C_{32}H_{48}N_7O_8S$ [M+H$^+$] 690.3280, found 690.3308.

(2S,4R)-1-((2S,5S)-17-azido-5-benzyl-2-(tert-butyl)-4,7-dioxo-9,12,15-trioxa-3,6-diazaheptadecanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**15**).



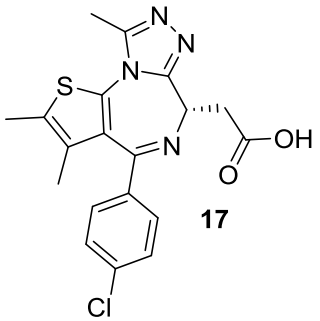
Yield 642 mg (81 %); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.89 (s, 9 H), 2.12-2.16 (m, 1 H), 2.51-2.56 (m, 4 H), 2.97-3.01 (m, 2 H), 3.09-3.14 (m, 1 H), 3.33-3.37 (m, 2 H), 3.51-3.65 (m, 12 H), 3.89-3.92 (m, 2 H), 3.99-4.01 (m, 1 H), 4.34 (dd, 1 H, *J*(*H*,*H*)= 5.2 Hz, *J*(*H*,*H*)= 14.9 Hz), 4.42-4.47 (m, 1 H), 4.50-4.54 (m, 2 H), 4.64-4.70 (m, 1 H), 4.74 (t, 1 H, *J*(*H*,*H*)= 7.8 Hz), 7.01-7.09 (m, 1 H), 7.15-7.24 (m, 4 H), 7.31-7.37 (m, 5 H), 7.43-7.48 (m, 1 H), 8.67 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 16.2, 26.6, 35.6, 36.5, 37.5, 43.3, 50.7, 54.0, 56.9, 58.0, 58.7, 70.1-71.1, 127.0, 128.2, 128.7, 129.4, 129.6, 131.0, 131.7, 136.4, 138.2, 148.5, 150.4, 170.6, 171.1, 171.1, 171.3; HRMS m/z calc. for $C_{39}H_{52}N_8O_8S$ [M+H$^+$] 793.3702, found 793.3707.

(2S,4S)-1-((S)-14-azido-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**16**).



Yield 194 mg (30 %); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.93 (s, 9 H), 2.14-2.21 (m, 1 H), 2.35-2.39 (s, 1 H), 2.52 (s, 3 H), 3.36 (t, 2 H, *J*(*H*,*H*)= 5.1 Hz), 3.63-3.68 (m, 10 H), 3.79-3.82 (m, 1 H), 3.91-3.95 (m, 1 H), 3.95-4.05 (m, 2 H), 4.30 (dd, 1 H, *J*(*H*,*H*)= 5.1 Hz, *J*(*H*,*H*)= 14.9 Hz), 4.45-4.50 (m, 1 H), 4.53 (d, 1 H, *J*(*H*,*H*)= 9.2 Hz), 4.64 (dd, 1 H, *J*(*H*,*H*)= 7.1 Hz, *J*(*H*,*H*)= 14.9 Hz), 4.74 (d, 1 H, *J*(*H*,*H*)= 9.0 Hz), 5.53 (d, 1 H, *J*(*H*,*H*)= 9.9 Hz), 7.18 (d, 1 H, *J*(*H*,*H*)= 9.1 Hz), 7.33-7.39 (m, 4 H), 7.50-7.53 (m, 1 H), 8.68 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ 16.2, 26.4, 35.1, 35.2, 43.7, 50.8, 56.6, 58.8, 60.0, 70.2-71.3, 128.3, 129.8, 131.4, 131.6, 137.5, 148.7, 150.5, 169.9, 172.0, 172.7; HRMS m/z calc. for $C_{30}H_{44}N_7O_7S$ [M+H$^+$] 646.3017, found 646.3040.

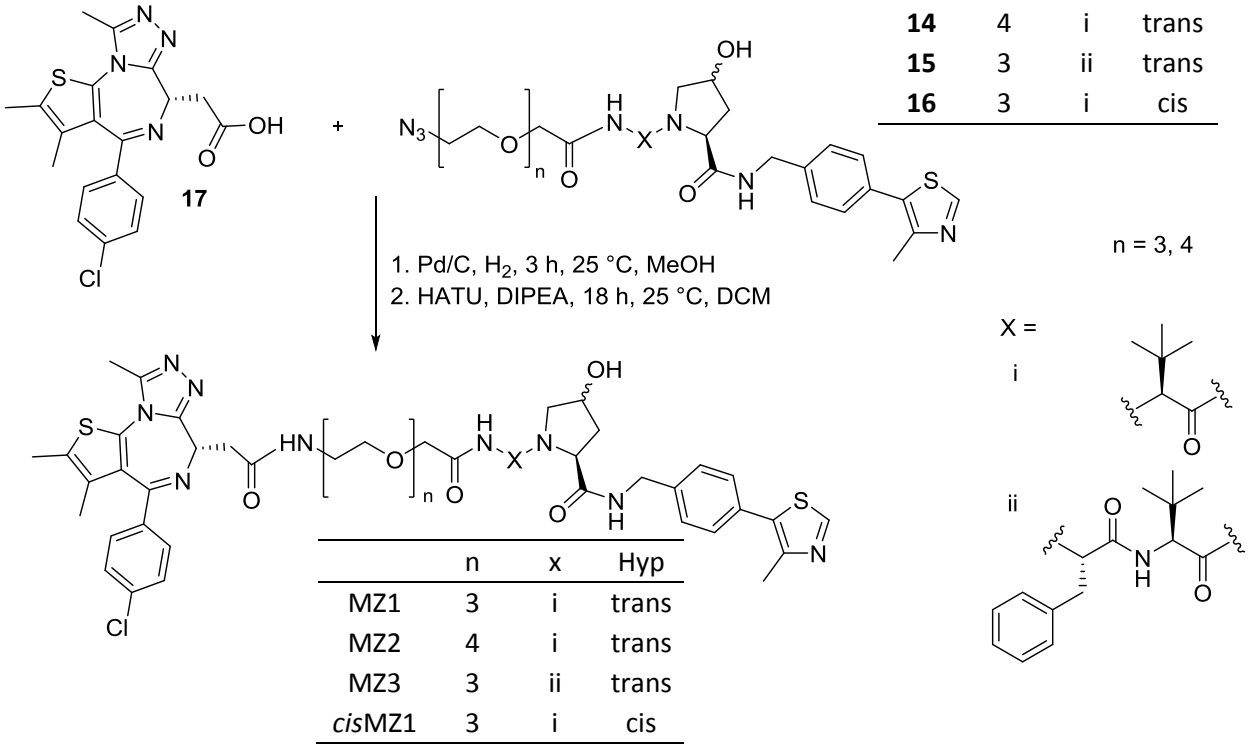
(S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetic acid (**17**).



17

(+)-JQ1 (50 mg, 109 µmol) was dissolved in formic acid (3 ml) and stirred for 18 h at 25 °C. After addition of water the reaction mixture was extracted three times with dichloromethane. The combined organic layers were dried over magnesium sulfate and evaporated to dryness to obtain the title compound which was directly used for the next reaction step. Yield 42.1 mg (96 %).

General procedure for PROTAC formation:



	n	x	Hyp
13	3	i	trans
14	4	i	trans
15	3	ii	trans
16	3	i	cis

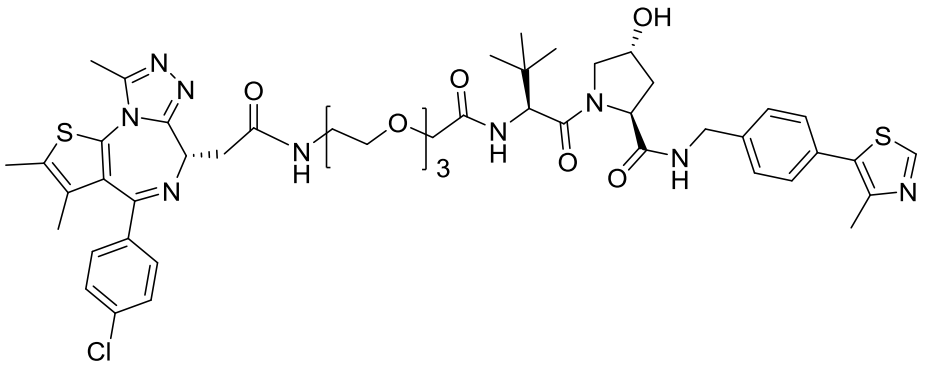
	n	x	Hyp
MZ1	3	i	trans
MZ2	4	i	trans
MZ3	3	ii	trans
*cis*MZ1	3	i	cis

Azides **13** – **16** (50 µmol) were dissolved in methanol (5 ml). Catalytic amount of palladium on Charcoal (10 wt%) was added and the reaction mixture stirred under an atmosphere of hydrogen for 3 h at 25 °C. The reaction mixture was filtered through a plug of celite and the resulting solution evaporated to dryness to obtain the desired amine.

The resulting amines (45 µmol, 1.1 eq.) and **17** (16.0 mg, 40 µmol, 1 eq.) were dissolved in DCM (2 ml). HATU (22.8 mg, 60.0 µmol, 1.5 eq.) was added and the pH adjusted to >9 by adding DIPEA (41.9 µl, 240 µmol, 4 eq.). After stirring the reaction mixture at 25 °C for 18 h the solvent

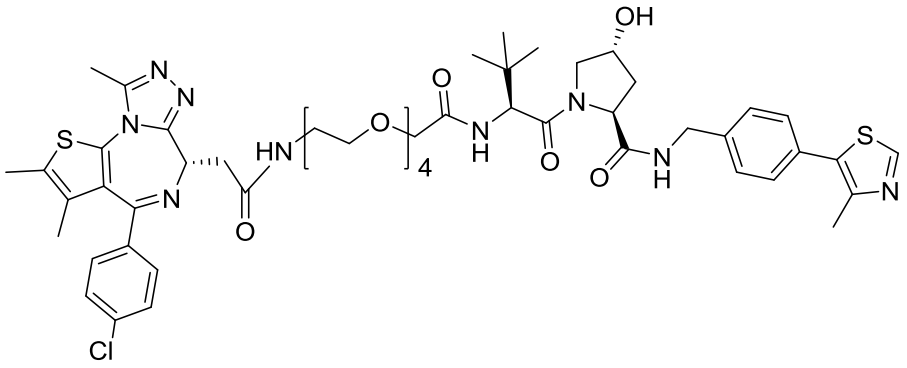
was removed in vacuum. Purification of the crude was achieved by preparative HPLC as described in the general information.

(2S,4R)-1-((S)-2-(tert-butyl)-17-((S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,16-dioxo-6,9,12-trioxa-3,15-diazaheptadecanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**MZ1**).



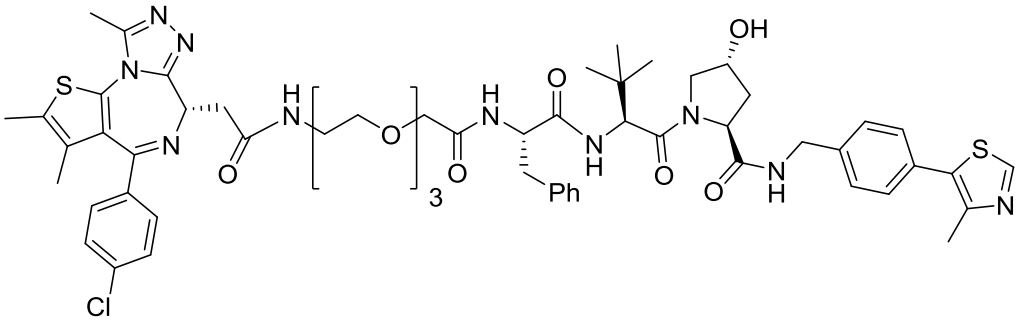
Yield: 9.51 mg (22 %); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.97 (s, 9 H), 1.66 (s, 3 H), 2.12-2.17 (m, 1 H), 2.39 (s, 3 H), 2.43-2.49 (m, 1 H), 2.51 (s, 3 H), 2.61 (s, 3 H), 3.31-3.35 (m, 2 H), 3.47-3.74 (m, 13 H), 4.11-4.14 (m, 2 H), 4.27-4.33 (m, 2 H), 4.49-4.55 (m, 2 H), 4.65-4.69 (m, 3 H), 4.84 (t, 1 H, *J*(*H*,*H*)= 7.9 Hz), 7.23-7.25 (m, 1 H), 7.29-7.39 (m, 9 H), 7.91-7.94 (m, 1 H), 8.67 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ 11.9, 13.2, 14.6, 16.2, 26.6, 35.6, 36.4, 38.2, 39.9, 43.3, 54.3, 56.9, 57.3, 59.0, 70.1-70.9, 71.7, 128.2, 128.9, 129.6, 130.1, 130.9, 131.0, 131.2, 131.8, 132.0, 136.7, 136.8, 138.4, 148.6, 149.9, 150.4, 156.0, 164.0, 171.0, 171.3, 171.4; HRMS m/z calc. for $C_{49}H_{61}ClN_9O_8S_2$ [M+H$^+$] 1002.3768, found 1002.3786.

(2S,4R)-1-((S)-2-(tert-butyl)-20-((S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,19-dioxo-6,9,12,15-tetraoxa-3,18-diazaicosanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**MZ2**).



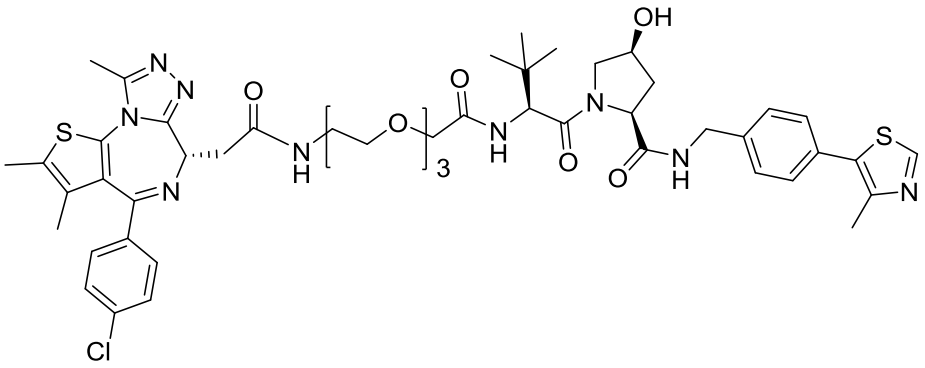
Yield: 10.1 mg (23 %); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.96 (s, 9 H), 1.65 (s, 3 H), 2.12-2.17 (m, 1 H), 2.39 (s, 3 H), 2.44-2.48 (m, 1 H), 2.51 (s, 3 H), 2.62 (s, 3 H), 3.37-3.45 (m, 2 H), 3.53-3.69 (m, 16 H), 4.01 (d, 1 H, *J*(*H*,*H*)= 15.7 Hz), 4.06-4.09 (m, 1 H), 4.15-4.18 (m, 1 H), 4.33 (dd, 1 H, *J*(*H*,*H*)= 5.4 Hz, *J*(*H*,*H*)= 15.0 Hz), 4.41-4.47 (m, 2 H), 4.54 (dd, 2 H, *J*(*H*,*H*)= 6.5 Hz, *J*(*H*,*H*)= 15.1 Hz), 4.62-4.67 (m, 2 H), 4.80 (t, 1 H, *J*(*H*,*H*)= 8.0 Hz), 7.29-7.39 (m, 9 H), 7.44 (t, 1 H, *J*(*H*,*H*)= 6.1 Hz), 7.56-7.58 (m, 1 H), 8.67 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ 11.9, 13.2, 14.6, 16.2, 26.6, 35.6, 36.4, 38.2, 39.9, 43.3, 54.3, 56.9, 57.2, 59.0, 70.1-70.9, 71.7, 128.2, 128.9, 129.6, 130.1, 130.9, 131.0, 131.2, 131.8, 132.0, 136.7, 136.8, 138.4, 148.6, 149.9, 150.4, 156.0, 164.0, 171.0, 171.3, 171.4; HRMS m/z calc. for $C_{51}H_{65}ClN_9O_9S_2$ [M+H$^+$] 1046.4030, found 1046.4067.

(2S,4R)-1-((2S,5S)-5-benzyl-2-(tert-butyl)-20-((S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,7,19-trioxo-9,12,15-trioxa-3,6,18-triazaicosanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**MZ3**).



Yield: 33.0 mg (58 %); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 0.90 (s, 9 H), 1.64 (s, 3 H), 2.09-2.14 (m, 1 H), 2.39 (s, 3 H), 2.48-2.54 (m, 4 H), 2.64 (s, 3 H), 3.06-3.12 (m, 1 H), 3.14-3.20 (m, 2 H), 3.41-3.69 (m, 15 H), 3.94 (q, 2 H, *J*(*H*,*H*)= 16.3 Hz), 4.03 (d, 1 H, *J*(*H*,*H*)= 11.1 Hz), 4.29 (dd, 1 H, *J*(*H*,*H*)= 5.4 Hz, *J*(*H*,*H*)= 15.0 Hz), 4.47 (s, 1 H), 4.52 (dd, 1 H, *J*(*H*,*H*)= 6.5 Hz, *J*(*H*,*H*)= 15.0 Hz), 4.60 (d, 1 H, *J*(*H*,*H*)= 9.1 Hz), 4.62-4.66 (m, 1 H), 4.70 (t, 1 H, *J*(*H*,*H*)= 7.0 Hz), 4.75 (t, 1 H, *J*(*H*,*H*)= 7.7 Hz), 6.99 (d, 1 H, *J*(*H*,*H*)= 8.8 Hz), 7.15-7.24 (m, 7 H), 7.31-7.36 (m, 7 H), 7.66 (d, 1 H, *J*(*H*,*H*)= 8.0 Hz), 7.82-7.84 (m, 1 H), 8.67 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 126 MHz) δ 11.9, 13.2, 14.5, 16.2, 17.5, 18.8, 26.5, 35.8, 36.3, 36.8, 38.3, 39.6, 42.0, 43.3, 53.8, 54.2, 54.7, 57.2, 57.7, 58.9, 69.9-70.8, 126.8, 128.2, 128.6, 128.8, 129.4, 129.5, 130.0, 130.8, 131.1, 131.2, 131.8, 132.0, 136.9, 137.0, 138.3, 148.5, 150.0, 150.4, 155.9, 170.8, 170.9, 171.0, 171.3, 171.4; HRMS m/z calc. for $C_{58}H_{70}ClN_{10}O_9S_2$ [M+H$^+$] 1149.4452, found 1149.4473.

(2S,4S)-1-((S)-2-(tert-butyl)-17-((S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,16-dioxo-6,9,12-trioxa-3,15-diazaheptadecanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (***cis*MZ1**).



Yield: 19.7 mg (37 %); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.99 (s, 9 H), 1.65 (s, 3 H), 2.18-2.21 (m, 2 H), 2.38 (s, 3 H), 2.49 (s, 3 H), 2.60 (s, 3 H), 3.35-3.69 (m, 14 H), 3.85-3.88 (m, 1 H), 3.93-3.96 (m, 1 H), 4.08 (q, 2 H, *J*(*H*,*H*)= 15.5 Hz), 4.25 (dd, 1 H, *J*(*H*,*H*)= 5.2 Hz, *J*(*H*,*H*)= 15.1 Hz), 4.40-4.44 (m, 1 H), 4.53-4.64 (m, 3 H), 4.80-4.83 (m, 1 H), 5.75 (d, 1 H, *J*(*H*,*H*)= 10.3 Hz), 7.24 (d, 1 H, *J*(*H*,*H*)= 9.7 Hz), 7.27-7.39 (m, 8 H), 7.43 (t, 1 H, *J*(*H*,*H*)= 5.4 Hz), 8.08 (t, 1 H, *J*(*H*,*H*)= 6.2 Hz), 8.67 (s, 1 H); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ 11.9, 13.2, 14.5, 16.2, 26.5, 35.2, 35.8, 38.7, 39.7, 43.4, 54.4, 56.6, 58.6, 60.0, 70.1-71.3, 128.0, 128.8, 129.5, 130.0, 130.9, 131.1, 131.7, 132.1, 136.7, 136.8, 137.8, 148.5, 149.9, 150.4, 155.8, 163.8, 170.2, 170.8, 171.4, 173.3; HRMS m/z calc. for $C_{49}H_{61}ClN_9O_8S_2$ [M+H$^+$] 1002.3768, found 1002.3791.

Biophysics

Protein expression and purification:

Plasmids pNIC28-Bsa4 containing the single BET bromodomain constructs BRD2 BD1, BRD2 BD2, BRD3 BD1, BRD3 BD2, BRD4 BD1 and BRD4 BD2 for protein expression are obtained from previous study.[3] Subsequent expression and purification was based on the methods in previous study with slight modifications. Single colonies from freshly transformed plasmid DNA in competent *E. coli* BL21(DE3) cells were grown overnight at 37 °C in 10 mL of LB medium with 50 µg/mL kanamycin. Starter culture was then diluted 1:100 in fresh Luria Broth (LB) medium with

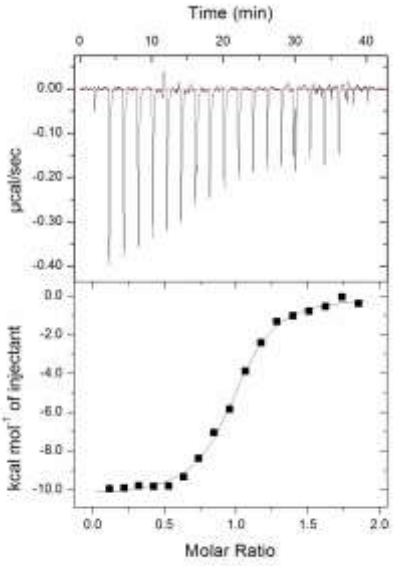
50 μg/mL of kanamycin. Cell growth was allowed at 37 °C and 200 rpm to an optical density of about 2.5 (OD600), at which point temperature was decreased to 18°C. Once the cultures equilibrated at 18 °C, protein expression was induced overnight at 18 °C with 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG). The bacteria was harvested the next day by centrifugation (8000 rpm for 10 minutes at 6 °C, JLA 8.1000 rotor on a Beckman Coulter Avanti J-20 XP centrifuge) and frozen at -20 °C as pellets for storage. Pellets of cells expressing His_6-tagged proteins were resuspended in lysis buffer (50 mM HEPES pH 7.5 at 25 °C, 150 mM NaCl, 40 mM Imidazole and 2 mM β-mercaptoethanol). One tablet of Complete Protease Inhibitor Cocktail (Roche) was added to the resuspension and cells were lysed using a French Press at 4 °C. Following a 20 min incubation period at room temperature with 10 μg/mL DNaseI and 10 mM MgCl2, the cell debris was removed by centrifugation, 20,000 x g at 4 °C. The lysate was purified via immobilized metal ion affinity chromatography on a His Trap HP 5mL Ni sepharose column (GE Healthcare Life Sciences) on an ÄKTApure system (GE Healthcare). His_6-tagged protein was eluted using a linear gradient to 250 mM imidazole in the same buffer. After Ni purification, the pooled elution fractions were concentrated to a volume of 4 mL and further purified by size exclusion chromatography on a Superdex 75 16/60 Hiload gel filtration column (GE Healthcare) on an ÄKTApure system using the following buffer: 20 mM HEPES pH 7.5, 150 mM NaCl. Samples were monitored by SDS-polyacrylamide gel electrophoresis to verify purity. Pure protein was then flash frozen with liquid nitrogen and stored at -80 °C. The mass and purity of the proteins were subsequently verified by mass spectrometry.

Isothermal Titration Calorimetry (ITC):

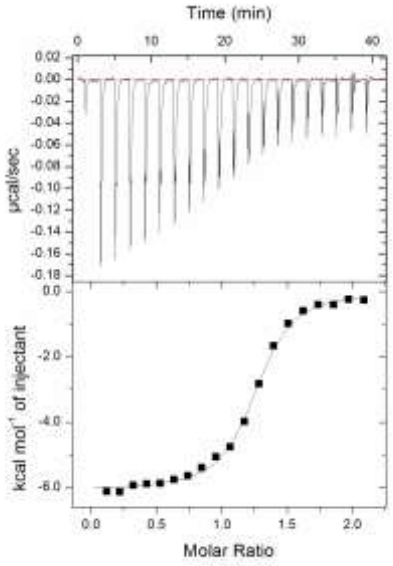
ITC experiments were carried out at an ITC 200 instrument from MicroCal™ with a concentration in the measuring cell of 15 μM and a syringe concentration of 150 μM. Experiments were conducted as PROTAC compound into protein titrations, except in the case of MZ3 where protein was titrated into PROTAC. BET protein experiments were conducted in a buffer containing 20 mM HEPES with 100 mM NaCl at pH 7.5 and a temperature of 30 °C. VBC protein experiments were carried out in a buffer containing 20 mM Bis-Tris, 150 mM NaCl and 2 mM Dithiothreitol (DTT) at pH 7 and a temperature of 25 °C.

entry	compound	protein	Kd [nM]	ΔH [kcal/mol]	ΔS [cal/mol·K]	ΔG [kcal/mol]
1	MZ1	BRD2 BD1	307 ± 27.9	-10.0 ± 0.1	-4.29 ± 0.38	-9.05 ± 0.06
2		BRD2 BD2	228 ± 17.7	-6.08 ± 0.04	10.3 ± 0.2	-9.22 ± 0.05
3		BRD3 BD1	119 ± 4.81	-10.0 ± 0.06	-4.40 ± 0.20	-9.62 ± 0.02
4		BRD3 BD2	115 ± 10.9	-8.32 ± 0.05	-4.29 ± 0.26	-9.63 ± 0.06
5		BRD4 BD1	382 ± 13.5	-8.59 ± 0.03	1.04 ± 0.13	-8.91 ± 0.02
6		BRD4 BD2	120 ± 7.16	-6.86 ± 0.03	9.04 ± 0.16	-9.61 ± 0.04
7		VBC	149 ± 13.1	-6.87 ± 0.05	8.18 ± 0.24	-9.47 ± 0.05
8	MZ3		311 ± 51.2	-4.90 ± 0.11	13.4 ± 0.49	-9.04 ± 0.10
9	*cis*MZ1			no binding		

MZ1 vs BRD2 BD1

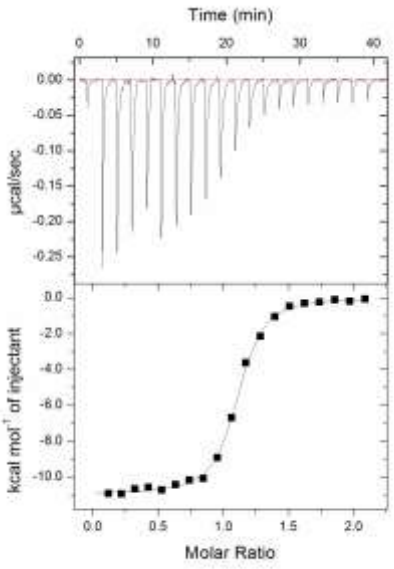


MZ1 vs BRD2 BD2

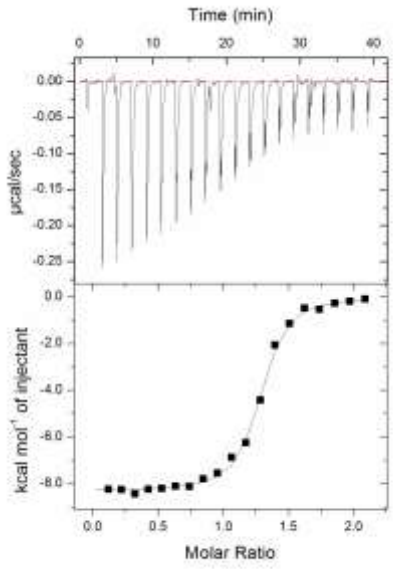


MZ1 vs BRD3 BD1

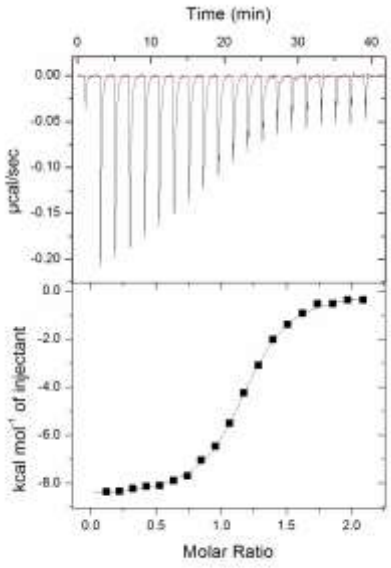
MZ1 vs BRD3 BD2



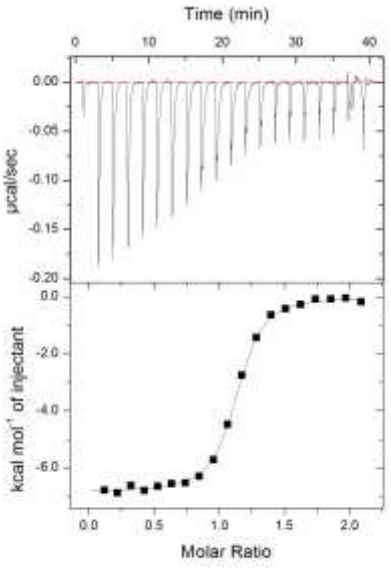
MZ1 vs BRD4 BD1



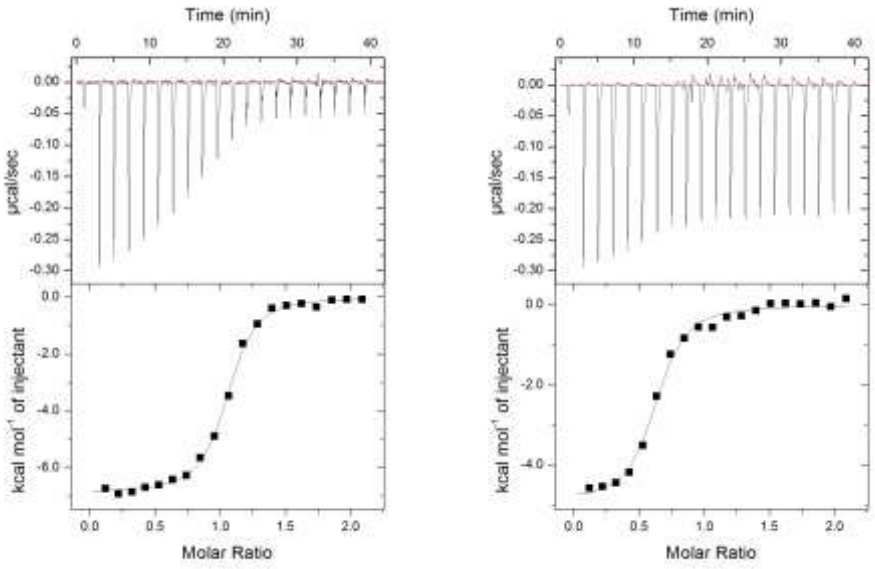
MZ1 vs BRD4 BD2



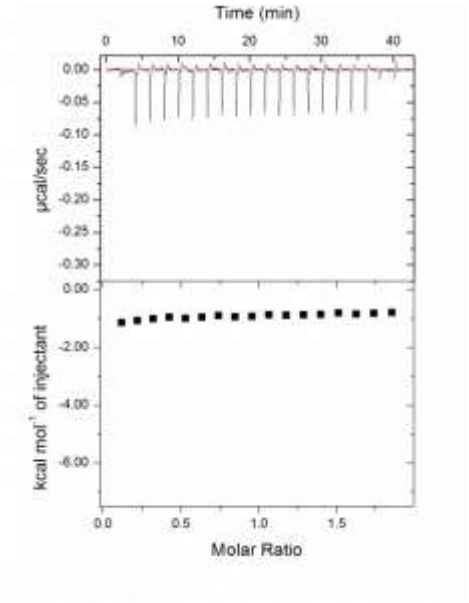
MZ1 vs VBC



MZ3 vs VBC



*cis*MZ1 vs VBC



Biology

Reagents

The proteasome inhibitor MG132 and radio immunoprecipitation assay buffer (RIPA-buffer) were purchased from Sigma Aldrich. DMEM media, phosphate buffered saline (PBS) and heat deactivated fetal bovine serum (FBS) were purchased from Gibco, Life Technologies. Complete Mini EDTA free Protease inhibitor cocktail was purchased from Roche. Lipofectamine® RNAiMAX Transfection Reagent from Life Technologies. siRNA from Life Technologies, cat. # 4390843,

4392420 (s12070), 4390824(s15545 & s23901). FuGene 6 Transfection Reagent from Promega (E2691).

Tissue culture

HeLa and U2OS cells were cultured in DMEM supplemented with 10 % FBS, 1 % *L*-glutamine and 100U/ml of penicillin/streptomycin. Cells were maintained for no more than 30 passages at 37 °C and 5 % CO_2.

Cell treatment

Small interfering RNA

For siRNA inhibition studies, cells were plated in six-well plates and were grown to 50-60% confluence. Cells were transfected with siRNA targeting *BRD2*, *BRD3* or *BRD4* or negative control siRNA at a final concentration of 12 nM in the presence of lipofectamine reagent. After transfection, cells were cultured for another 24 hours for treatment with compound or harvested after 48 hours for gene expression study.

Single time point treatment

For treatment experiments cells were transferred in 6-well plates with 500 000 cells per well in 2 ml media. 12 h after settling 200 µl of media was removed and then replaced by a 10 fold concentrated compound solution in media. The final DMSO concentration was 0.01 % v/v.

Time course experiments

For time dependent treatment cells were transferred in 6-well plates with 300 000 cells per well in 2 ml media. For treatment 200 µl of media was removed and then replaced by a 10 fold concentrated compound solution in media. Treatment was conducted at given time points prior to harvest.

Protein recovery experiment

Cells were transferred in 6-well plates with 300 000 cells per well in 2 ml media. For treatment 200 µl of media was removed and then replaced by a 10 fold concentrated compound solution in media. 4 h after treatment the media was aspirated and replaced by fresh media without treatment. Cells were harvested at given time points.

Western blotting

For protein extracts the dishes were placed on ice. The media was aspirated and the tissue layer washed twice with ice cold PBS. 120 µl of RIPA-buffer containing Protease inhibitor was added and the cells detached from the surface with a cell scraper. After removal of the insoluble fraction by centrifugation the protein concentration of the supernatant was determined by a Pierce™ BCA Protein Assay Kit. Protein extracts were fractionated by SDS-PAGE on 3-8% Tris-Acetate NuPage® Novex® (Life Technologies) polyacrylamide gels and transferred to a nitrocellulose membrane using i-Blot® 2 from Life Technologies. The membrane was then blocked with 3.5 % Bovine Serum albumin (BSA) in Tris-buffered saline (TBS) with 0.1 % Tween-20. For detecting proteins the following primary antibodies in the given concentrations were used: anti-BRD2 (Abcam, ab139690, EPR7642) 1:2000, anti-BRD3 (Abcam, ab50818, 2088C3a) 1:500, anti-BRD4 (Abcam, ab128874, EPR5150(2)) 1:1000, anti-Hif-1α (BD Biosciences, 610959, clone 54) 1:1000, anti-VHL (Cell Signaling Technology, 2738S) 1:750, anti-β-Actin (Cell Signaling Technology, 4970S, 13E5) 1:2000, anti-hHR23b (Abcam, ab86781) 1:2000, anti-DDB1 (Abcam, ab109027, EPR6089) 1:50000. For visualisation a Li-Cor Biosciences Odyssey system with the following secondary fluorescent Antibodies from Li-Cor Biosciences was used: IRDye800CW Goat Anti-Mouse (926-32210), IRDye800CW Donkey Anti-Rabbit (926-32213), both in concentrations of 1:10 000. Membranes were incubated with the corresponding antibodies either at 4 °C for 12 h or at 25 °C for 4 h. Between incubation with the different antibodies membranes were stripped with 0.25 M solution of Glycine·HCl at pH 2.

RNA extraction and real-time PCR

Expression levels of genes of interest were analysed by RT-PCR. After treatment described above, cells were harvested and RNA was extracted with Qiagen RNeasy Mini Kit (cat. #: 74104). Reverse transcription were performed using 250-500 ng of extracted RNA with Bio-Rad iScript cDNA synthesis Kit (cat. #: 170-8891). The cDNA samples were diluted by 25-fold. Gene-specific primers designed with aid of UCSC Genome Browser.[4]

Table S1. Primers for RT-PCR

Name	Sequence (5′→ 3′)
AREG-fw	AAGGAGAAGCTGAGGAACGAA
AREG-rv	TGGCTATGACTTGGCAGTGA
FAS-fw	AGAACTTGGAAGGCCTGCAT
FAS-rv	GTCTGGTTCATCCCCATTGA
FGFR1-fw	CTGACCACAGAATTGGAGGC
FGFR1-rv	GCAGGTGTAGTTGCCCTTGT
MYC-fw	CCGCTTCTCTGAAAGGCTCT
MYC-rv	AAGCTAACGTTGAGGGGCAT
P21-fw	TGGAGACTCTCAGGGTCGAA
P21-rv	GGATTAGGGCTTCCTCTTGG
TYRO3-fw	AACTACGAAGATCGGGGGAC
TYRO3-rv	CCAGGCCTTTTAGGTTGTGA
GAPDH-fw	AACGGGAAGCTTGTCATCAATGGAAA
GAPDH-rv	GCATCAGCAGAGGGGGCAGAG

All PCR reactions were performed using the Bio-Rad CFX96 Touch Real-Time PCR system and the amplifications were done using the Quanta PerfeCTa® SYBR® Green FastMix for iQ (cat. # 95071). The thermal cycling conditions were composed of 95°C for 10 min, 45 cycles at 95°C for 10s and 60°C for 30s followed by a ramping temperature step to 95°C for melt-curve analysis. The experiments were carried out in triplicate for each data point. The data was analysed using CFX Manager software from Bio-Rad and the relative quantification in gene expression was determined by normalising to the control gene *GAPDH*.

Fluorescence microscopy:

U2OS cells transiently expressing GFP-tagged BRD4 were prepared. Plasmid pcDNA5/FRT/TO-GFP containing full-length wild-type BRD4 is obtained as described in previous study.[3] U2OS cells were plated onto a glass bottom microwell dish (MatTek, P35G-1.5-14-C) in 2.5 mL medium and were grown to 50-60% confluence. Then the cells were transfected with 4 μg of plasmid DNA in the presence of FuGene 6 Transfection Reagent (Promega, E2691). Twelve hours after

transfection, medium was removed in exchange of fresh medium supplemented with 0.5 µg/mL tetracycline to induce GFP-BRD4 expression. After 18 hours, medium was removed again in exchange of fresh medium without tetracycline. After 6 hours, compound MZ1 or *cis*-MZ1 were added to the plate. Immediately after the treatment, fluorescence given out from individual cells plate were observed on a DeltaVision Elite imaging system with excitation at 480 nm and emission at 525 nm. Images of individual cells were made at regular time interval to observe changes in fluorescence over time.

Legends to Supporting Information videos:

Supporting video a: BRD4 depletion by MZ1 monitored by live fluorescence imaging.

U2OS cells transfected with GFP-BRD4 were treated with 5 µM of MZ1 over a time course of 4 hours. GFP-BRD4 degradation was followed by live fluorescence imaging using a DeltaVision Elite Imaging system. The pictures taken every 2 minutes were combined to a 10 second time lapse video.

Supporting video b: Intracellular amount of GFP-BRD4 does not change in the presence of *cis*MZ1.

U2OS cells transfected with GFP-BRD4 were treated with 5 µM of *cis*MZ1 over a time course of 4 hours. Live fluorescence imaging using a DeltaVision Elite Imaging system showed no reduction in fluorescence. The pictures taken every 2 minutes were combined to a 10 second time lapse video.

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