



by
Boehringer Ingelheim

BET PROTAC

MZ1

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Summary

MZ1 is a BET protein family-specific PROTAC that originated from Alessio Ciulli's lab at the [University of Dundee](#), School of Life Sciences¹.

Chemical Structure

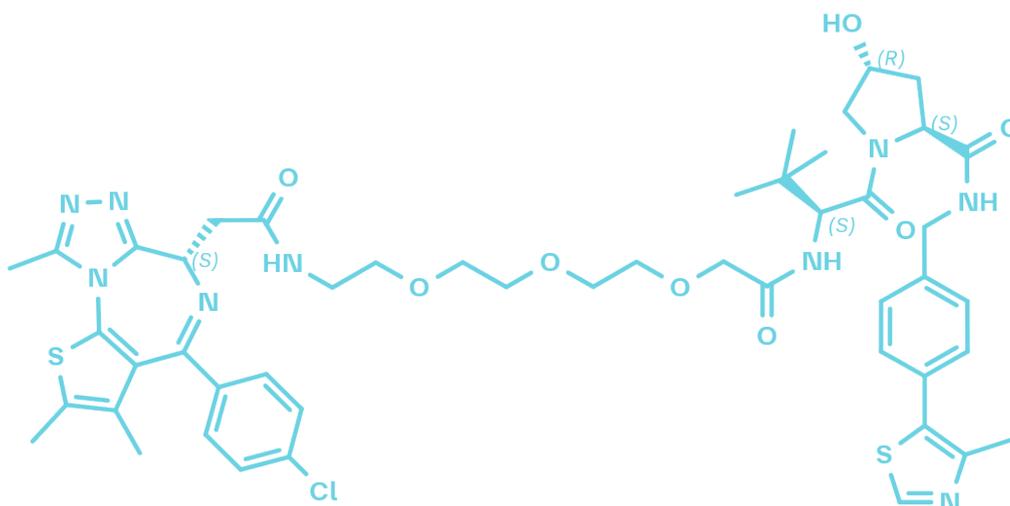


Figure 1: 2D structure of MZ 1

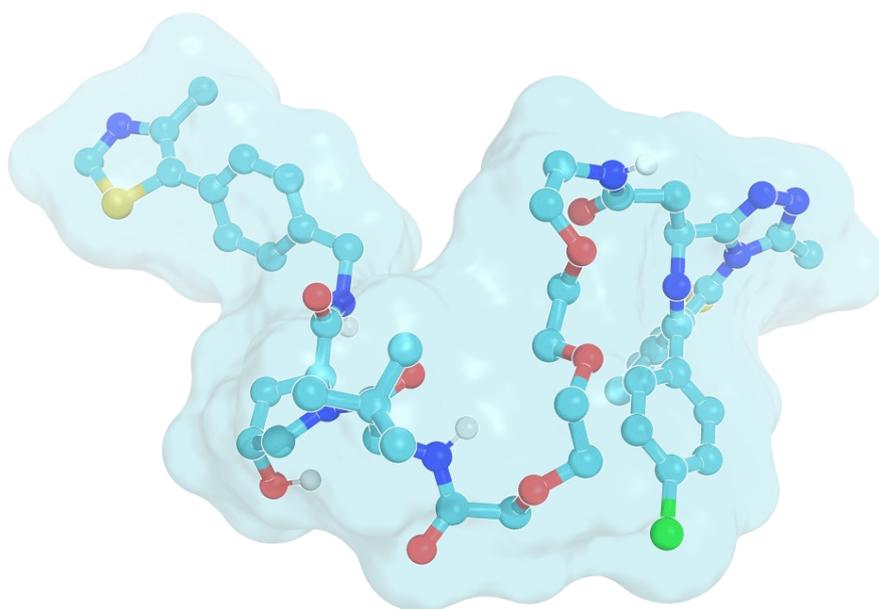


Figure 2: MZ 1, 3D conformation, as observed in complex with BRD4 bromodomain and VHL (PDB code: 5T35)

Highlights

MZ1 is a first in class PROTAC (proteolysis-targeting chimeras) that tethers JQ1 to a VHL E3 ubiquitin ligase ligand, aimed at triggering the intracellular destruction of BET proteins. It induces reversible, long-lasting, and preferential removal of BRD4 over BRD2 and BRD3 in cells. MZ1 and the negative control cis MZ1 have been discovered by Michael Zengerle, Kwok-Ho Chan, and Alessio Ciulli¹ at the Ciulli Lab at the [University of Dundee](#), School of Life Sciences. We are grateful to Alessio Ciulli for making this molecule available for scientific research via opnMe.

Target information

Acetylation of lysine residues is a post-translational modification with broad relevance to cellular signalling and disease biology. Inhibitors that modulate the 'reading process' mediated by acetyl lysines are an area of extensive research. The principal readers of ϵ -N-acetyl lysine (Kac) marks are bromodomains (BRDs), which are a diverse family of evolutionary conserved protein-interaction modules. The conserved BRD fold contains a deep, largely hydrophobic acetyl lysine binding site, which represents an attractive pocket for the development of small, pharmaceutically active molecules. Proteins that contain BRDs have been implicated in the development of a large variety of diseases.

Traditionally small molecules have been used to inhibit the action of a target protein by occupying and blocking a functional region of the protein. An alternative innovative approach is the development of proteolysis targeting chimeras (PROTACs), i.e. heterobifunctional compounds consisting of one moiety that binds a Cullin RING E3 ubiquitin ligase linked to another that binds a desired protein of interest (POI), bringing the ligase and the POI into close spatial proximity. This hijacks the intrinsic catalytic activity of the E3 ligase from the natural and directs it toward the POI as a neo-substrate, triggering its poly-ubiquitination and subsequent proteasome-dependent degradation. As a result, a PROTAC acts as a degrader of the target as opposed to just an inhibitor, enabling the effective post-translational elimination of a target gene product in living organisms. This approach presents many advantages compared to conventional target inhibition. One of the most attractive features of the approach is that a PROTAC molecule acts sub-stoichiometrically, i.e. it only needs to bind a molecule of target once to induce its degradation, and then is released and set free to bind another molecule of target and carry on, as in a catalytic cycle. For this reason, the concentrations required for PROTACs to be active in cells tend to be much lower compared to those needed to be reached and maintained with inhibitors, which can lead to fewer off-target effects and a more selective chemical intervention on the desired target.

Structural elucidation of the key ternary ligase-PROTAC-target species and its impact on target degradation selectivity remain elusive. The crystal structure of BRD4 degrader MZ1 in complex with human VHL and the BRD4 bromodomain (BRD4^{BD2}) offers unique insights into

the first ternary complex structure of a low molecular weight degrader. The ligand folds into itself to allow formation of specific intermolecular interactions in the ternary complex. Isothermal titration calorimetry studies, supported by surface mutagenesis and proximity assays, are consistent with pronounced cooperative formation of ternary complexes with BRD4^{BD2}. Our results elucidate how PROTAC-induced de novo contacts dictate preferential recruitment of a target protein into a stable and cooperative complex with an E3 ligase for selective degradation^{1,2}.

Researchers can request up to two batches of 5 mg of MZ1 and one batch of cis MZ1 for free. Larger quantities of the compounds are available from [Tocris](#).

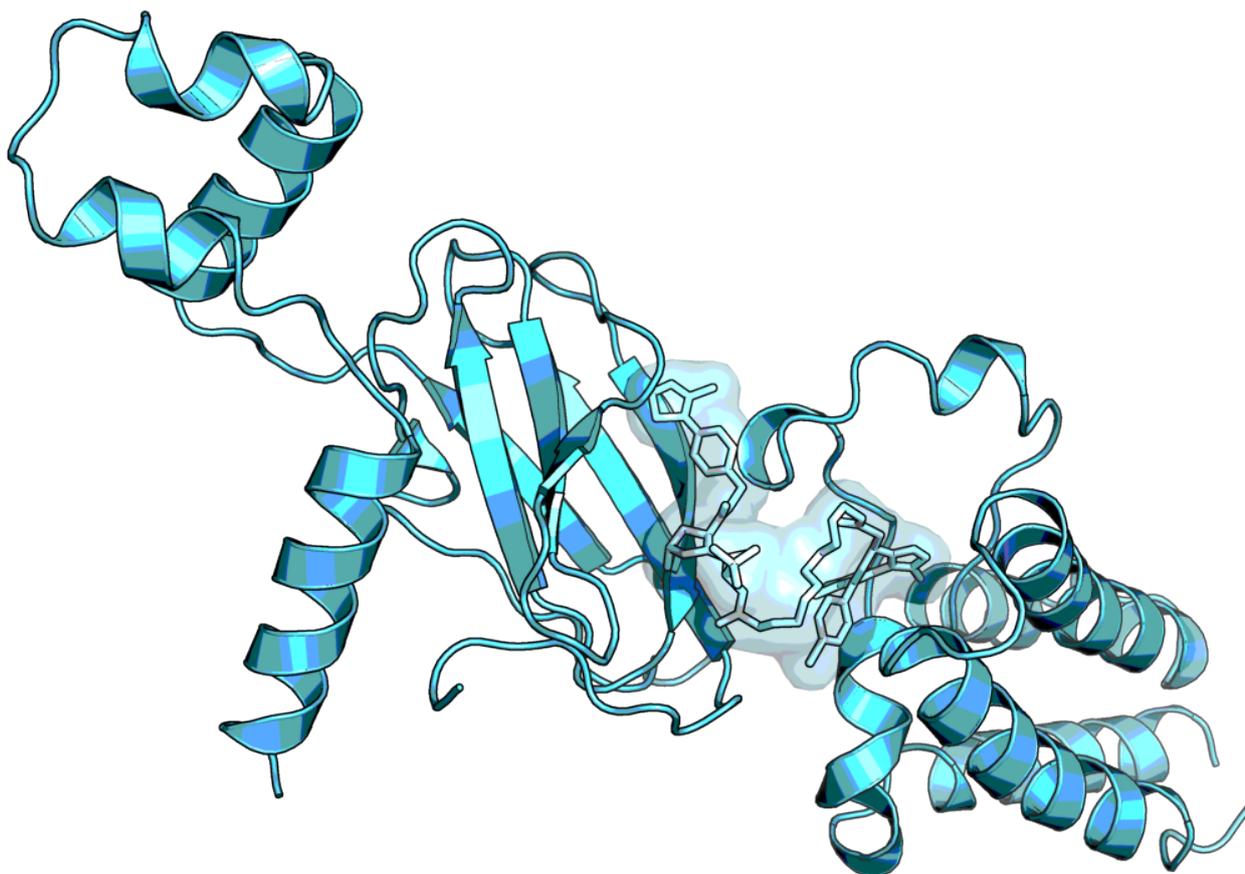


Figure 3: MZ 1, 3D conformation, as observed in complex with BRD4 bromodomain and VHL (PDB code: 5T35)

In vitro activity

The binary affinity for MZ1 binding to BRD4^{BD2} and to VCB (VHL-ElonginC-ElonginB) is 15 and 66 nM respectively (ITC). The affinity of the negative control “cis MZ1” to VCB is > 15 μ M (ITC). In presence of excess of BRD4^{BD2} MZ1 the ternary KD for the BRD4^{BD2}::MZ1::VCB is 3.7

nM. MZ1 degrades the BRD4 protein with a DC₅₀ of 2-20 nM depending on the cell line, and with selectivity on DC₅₀ of ~10-fold relative to BRD2 and BRD3.

Table: Binding affinities for binary and ternary complexes between MZ1, VCB and BET bromodomains as measured by isothermal titration calorimetry (for assay conditions see reference 2).

	PROBE NAME	MZ 1		
Protein in syringe	Species in cell	K _d (nM)	α	Δp<K _d
BRD2 ^{BD1}	MZ1	62 ± 6	-	-
BRD2 ^{BD2}	MZ1	60 ± 3	-	-
BRD3 ^{BD1}	MZ1	21 ± 5	-	-
BRD3 ^{BD2}	MZ1	13 ± 3	-	-
BRD4 ^{BD1}	MZ1	39 ± 9	-	-
BRD4 ^{BD2}	MZ1	15 ± 1	-	-
BRD2 ^{BD1} KEA	MZ1	69 ± 9	-	-
BRD4 ^{BD1} QVK	MZ1	22 ± 8	-	-
VCB ^a	MZ1 ^a	66 ± 6	-	-
VCB	MZ1:BRD2 ^{BD1}	24 ± 8	2.9	0.4 ± 0.2
VCB	MZ1:BRD2 ^{BD2}	28 ± 3	2.3	0.36 ± 0.06
VCB	MZ1:BRD3 ^{BD1}	19 ± 4	3.5	0.5 ± 0.1
VCB	MZ1:BRD3 ^{BD2}	7 ± 2	10.7	1.0 ± 0.2
VCB	MZ1:BRD4 ^{BD1}	28 ± 6	2.3	0.4 ± 0.1
VCB	MZ1:BRD4 ^{BD2}	3.7 ± 0.7	17.6	1.2 ± 0.09
VCB	MZ1:BRD2 ^{BD1} KEA	12 ± 7	7.9	0.8 ± 0.3
VCB	MZ1:BRD4 ^{BD2} QVK	14.9 ± 0.1	4.2	0.6 ± 0.04

All ITC titrations were performed at 25 °C. Values reported are the mean ± S.E.M. from two independent measurements, except for VCB titration into MZ 1 (line ^a) for which values reported are the mean ± S.E.M. from eight independent measurements.

The binding thermodynamics and kinetics of ternary complex formation and dissociation of MZ1 with VHL and the various BET bromodomains was also later investigated by the Ciulli Lab,

in collaboration with Boehringer Ingelheim, using surface plasmon resonance (SPR). The data confirmed MZ1 forms the most stable and cooperative complexes between VHL and the second bromodomains (BD2s) of BET proteins. Specifically, the most stable, high-affinity and long-lived complex was formed with BRD4^{BD2}, with a single residue (Gly386) contributing to this “glueing” in an isoform-specific manner, as demonstrated by data obtained with reciprocal swap Gly-Glu mutants in BRD3^{BD2} and BRD4^{BD2.4}.

Table: Binding of MZ1 (binary complex formation) or MZ1:BET bromodomains (ternary complex formation) to surface-immobilized VCB as measured by surface plasmon resonance (for assay conditions/details see reference 4).

SPR BINDING TO VHL				
PROTAC	+bromodomain target	K _d (nM)	α	t _{1/2} (s)
MZ1	(binary)	29	-	-
MZ1	BRD2 ^{BD1} (ternary)	23	1.3	<0.7
MZ1	BRD2 ^{BD2} (ternary)	0.9	32.0	67.4
MZ1	BRD3 ^{BD1} (ternary)	12	2.4	<0.7
MZ1	BRD3 ^{BD2} (ternary)	8	3.6	6.0
MZ1	BRD3 ^{BD2} E344G (ternary)	2	14.0	58.7
MZ1	BRD4 ^{BD1} (ternary)	30	0.9	<0.7
MZ1	BRD4 ^{BD2} (ternary)	1	22.0	130.0
MZ1	BRD4 ^{BD2} G386E (ternary)	8	3.5	4

In vitro DMPK and CMC parameters

MZ1 is soluble at physiological pH, metabolically stable (hepatocytes) and a PGP substrate (high Caco-2 efflux ratio). Therefore, MZ1 is not suitable for oral administration but suitable for parenteral administration (*i.v.*, *i.p.* or *s.c.*). The compound shows moderate inhibition of CYP3A4 and species differences in Plasma Protein Binding.

PROBE NAME / NEGATIVE CONTROL	MZ1	CIS MZ 1
MW [Da, free base] ^a	1,002.6	1,002.6
logD @ pH 11	3.6	3.7

Solubility @ pH 7 [$\mu\text{g}/\text{mL}$]	19	12
Caco-2 permeability AB @ pH 7.4 [$*10^{-6}$ cm/s]	<0.34	0.3
Caco-2 efflux ratio	481	127
Microsomal stability (human/mouse/rat) [% Q_H]	88 / 79 / 73	>95 / >95 / 71
Hepatocyte stability (human/mouse/rat) + 50% Serum [% Q_H]	12 / 19 / 58	n.d.
Plasma Protein Binding (human/mouse/rat/) + 10%FCS [%]	97.5 / 99.7 / 97.6	n.d.
CYP 3A4 (IC_{50}) [μM]	5.5	2.4
CYP 2C8 (IC_{50}) [μM]	24	16
CYP 2C9 (IC_{50}) [μM]	>50	>50
CYP 2C19 (IC_{50}) [μM]	>50	>50
CYP 2D6 (IC_{50}) [μM]	37	26

^a For the salt form you will get, please refer to the label on the vial and for the molecular weight of the salt, please refer to the FAQs

In vivo DMPK parameters

MZ1 is suitable for a parenteral administration (*i.v.*, *i.p.* or *s.c.*). The compound shows high clearance in rats and low clearance in mice. High AUC levels can be obtained, when the compound is administered subcutaneously using a 25% HP- β -CD formulation. Because of the high Caco2 efflux ratio, the oral exposure is very low.

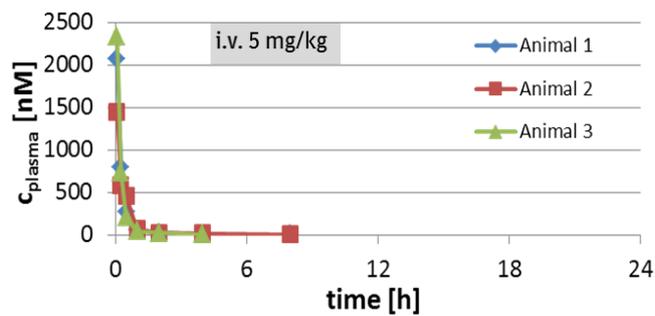
MZ1	RAT ^A	MOUSE ^B
Clearance [% Q_H]	156	20.7
Mean residence time after <i>i.v.</i> dose [h]	0.61	0.34
$t_{1/2,term}$ [h]	1.52	1.04
V_{ss} [L/kg]	4.05	0.38
AUC_{0-inf} [nM.h]	1,300	3,760

c_{max} [nM]	163	2,070
t_{max} [h]	2	0.5
$t_{1/2,term}$ [h]	5.13	2.95
c_{max} [nM]	-	<2

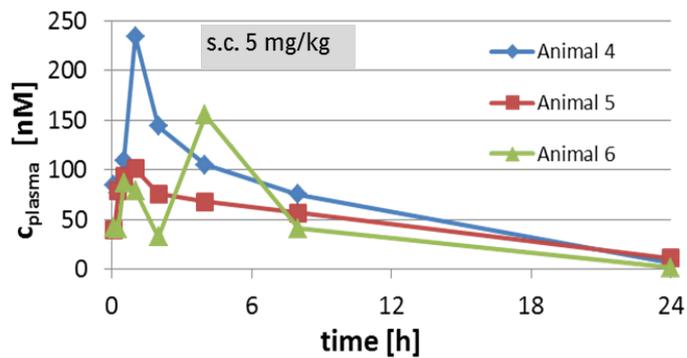
^a rat *i.v.* dose: 5 mg/kg; rat *s.c.* dose: 5 mg/kg

^b mouse *i.v.* dose: 5 mg/kg, mouse *s.c.* dose: 5 mg/kg

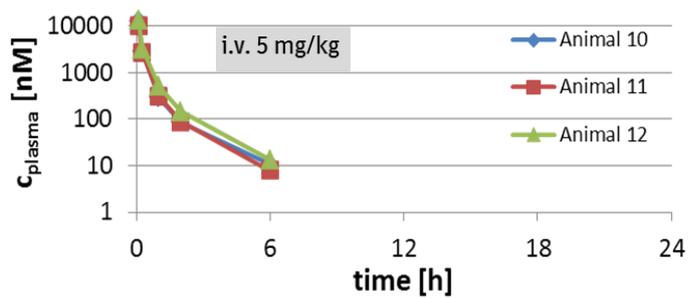
A



B



C



D

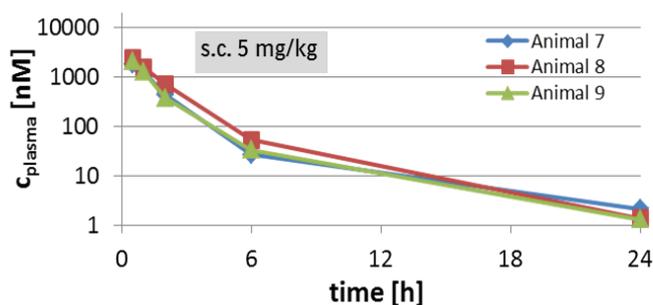


Figure 4: PK curves: A Rat *i.v.* dose: 5 mg/kg, B Rat *s.c.* dose: 5 mg/kg, C mouse *i.v.* dose: 5 mg/kg, D mouse *s.c.* dose: 5 mg/kg

In vitro pharmacology

MZ1 shows antiproliferative and Myc-suppression activity in AML MV4;11 and HL60 cells. BET Reduction by MZ1 in HeLa Cells, Antiproliferative Activity, and BRD4/cMyc Reduction in AML Cells^{a 3}.

MZ 1											
	pDC ₅₀ /Dmax (%) in HeLa cells				pIC ₅₀		Emax (%)		BRD4/cMyc depletion (%)		(α)
	BR D4 short	BR D4 long	BR D3	BR D2	MV4; 11	HL6 0	MV4; 11	HL6 0	MV4; 11	HL 60	
MZ 1	8.1 / 98	8.6 / 100	7.0 / 100	7.4 / 98	7.57 ±0.03	6.66 ±0.05	96.1 ±0.3	92.1 ±0.4	96 / 84	82 / 68	7.4

^a DC50: concentration in molar causing 50% reduction of protein level relative to vehicle control treatment in 24h. Dmax: maximum reduction of protein level relative to vehicle control treatment. pIC50 was measured after 48h treatment. Errors on pIC50 values reflect the quality of the curve fitting. Protein depletion % are for 50 nM treatments (4h) in MV4;11/HL60. For more details please refer to reference 3.

Negative control

Cis MZ1 is the (S) hydroxy diastereoisomer of MZ1. While exhibiting MZ1 comparable Bromodomain binding affinity it no longer is able to bind and recruit VCB and therefore is not degrading BET proteins in cells. The affinity of the cis MZ1 to VCB is $> 15 \mu\text{M}$ (ITC).

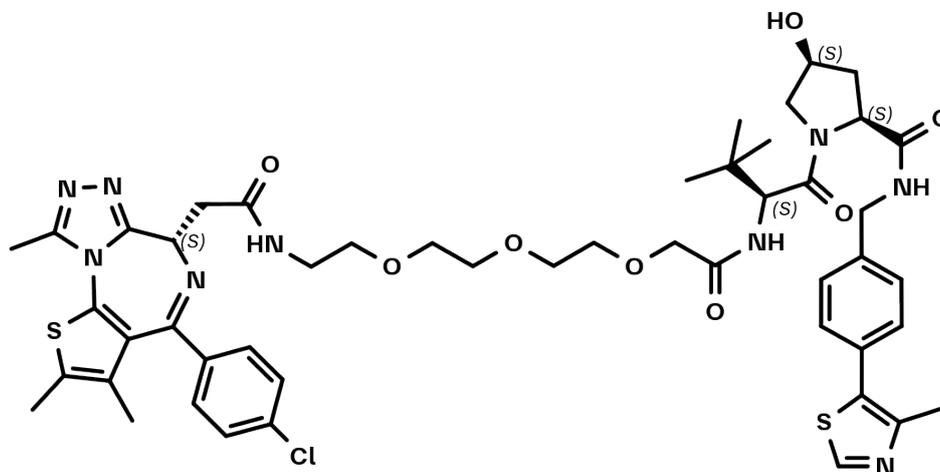


Figure 5: cis MZ1 which serves as a negative control

Selectivity

SELECTIVITY DATA AVAILABLE	MZ 1	CIS MZ 1
SafetyScreen44™ with kind support of eurofins	Yes	Yes
Invitrogen®	No	No
DiscoverX®	No	No
Dundee	No	No

To assess the cellular selectivity of MZ1 for BRD4/3/2 degradation and identify potential degradation off-targets, multiplexed isobaric tagging mass spectrometry proteomic experiments were performed by the Ciulli Lab to monitor protein levels in a quantitative and unbiased manner. HeLa cells were treated in duplicate with DMSO, 1 mM MZ1, or 1 mM cis MZ1 for 24 h. Among the 5,674 proteins quantified in this analysis, of those that met the criteria for a statistically significant change in abundance, markedly selective degradation of BET proteins was observed, and in particular preferential degradation of BRD4 over BRD3 and BRD2. As expected, BRD4 was not depleted by treatment with negative control cis MZ1. Protein levels of other bromodomain-containing proteins remained unaffected².

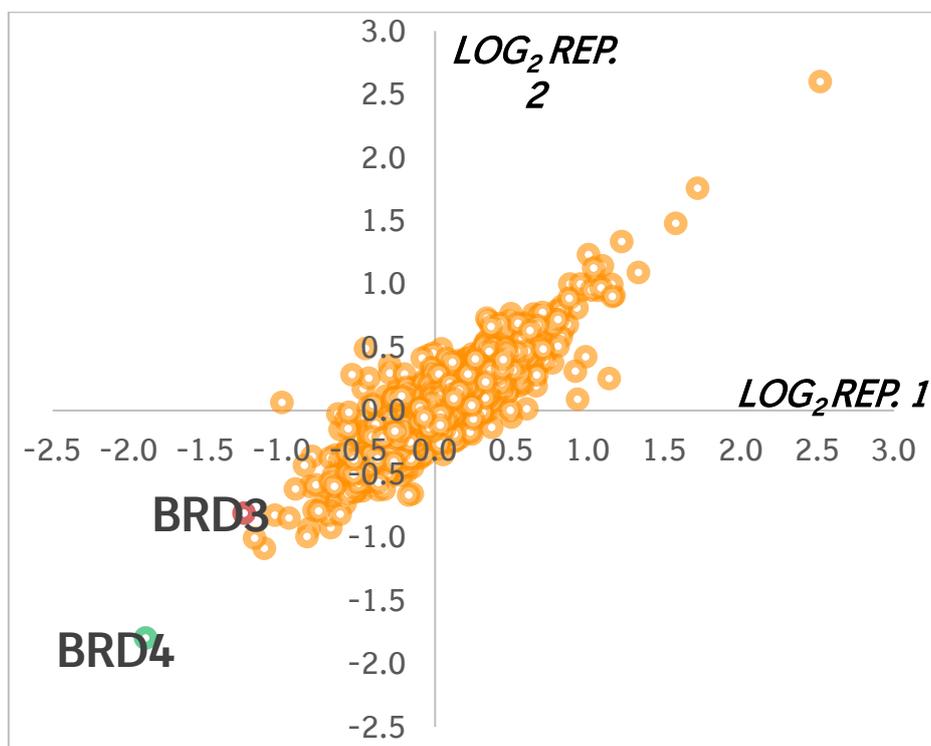


Figure 6: Impact of MZ1 (1 μ M, 24 h) on the cellular proteome using global protein quantification by TMT-labeling mass spectrometry. Data plotted as fold change (\log_2) of replicate 1 versus replicate 2, for a total of 5,674 proteins quantified (for assay conditions/details see reference 2).

The PROTAC MZ1 was also tested by the Eric Fischer Laboratory - Dana-Farber Cancer Institute as part of their Degradation Proteomics Initiative^{5,6}. It induces selective degradation of BRD2, BRD3 and BRD4 after 5 h of treatment at 1 μ M in the neuroblastoma cell line Kelly Cells.

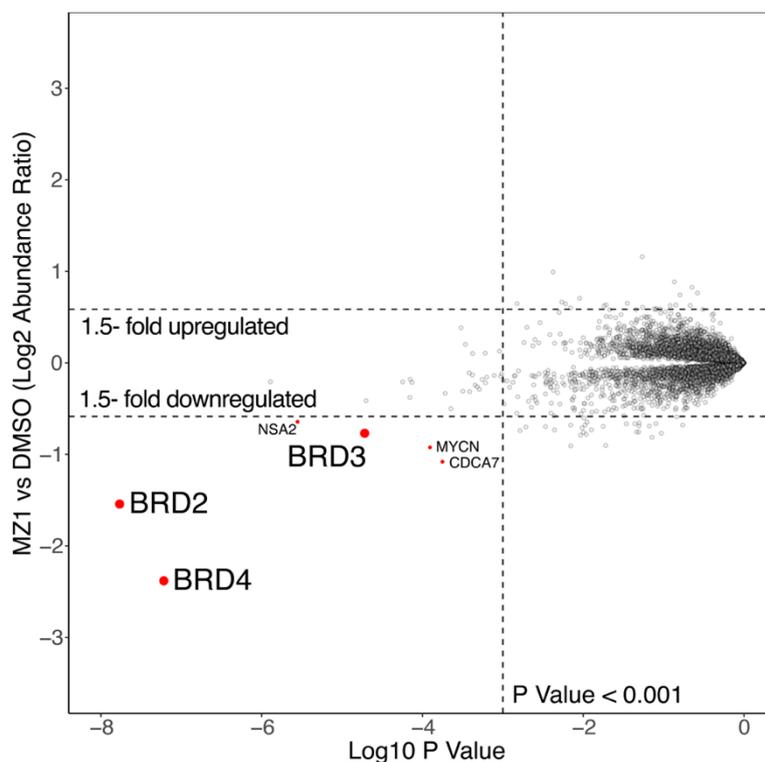


Figure 7: Global protein quantification was used to explore the unbiased proteome-wide selectivity of MZ1 induced degradation. Whole cell protein quantification was performed using label free quantification with the Fischer lab's diaPASEF workflow.

Of the 7,084 proteins quantified in this experiment after in-house filtering for data quality, Donovan *et al.* found a high degree proteome-wide selectivity for BRD2, BRD3 and BRD4, confirming preferential degradation of BRD4, over BRD2 and BRD3. Statistical analysis was performed comparing degrader treatment to DMSO vehicle control using a moderated t-test in Bioconductor's limma package to generate hit lists containing log₂ Fold Change and P-values for each protein.

Co-crystal structure of the MZ1 in complex with target protein and VCB

The X-ray of the PROTAC MZ1 in complex with the second bromodomain of BRD4 and pVHL:ElonginC:ElonginB is available in the PDB: 5T35².

Reference molecule(s)

dBET (CRBN based PROTAC) is a BET bromodomain degrader which is less specific than MZ1^{7,8}.

Supplementary data

2D structure files can be downloaded free of charge from [opnMe](#).

References

1. Zengerle M., Chan K.-H., Ciulli A. Selective Small Molecule Induced Degradation of the BET Bromodomain Protein BRD4 *ACS Chem Biol* **2015**, 10(8), 1770–1777. DOI: [10.1021/acschembio.5b00216](#), PubMed: [26035625](#).
2. Gadd M. S., Testa A., Lucas X., Chan K.-H., Chen W., Lamont D. J., Zengerle M., Ciulli A. Structural basis of PROTAC cooperative recognition for selective protein degradation *Nat Chem Biol* **2017**, 13(5), 514–521. DOI: [10.1038/nchembio.2329](#), PubMed: [28288108](#).
3. Chan K.-H., Zengerle M., Testa A., Ciulli A. Impact of Target Warhead and Linkage Vector on Inducing Protein Degradation: Comparison of Bromodomain and Extra-Terminal (BET) Degraders Derived from Triazolodiazepine (JQ1) and Tetrahydroquinoline (I-BET726) BET Inhibitor Scaffolds *J Med Chem* **2018**, 61(2), 504–513. DOI: [10.1021/acs.jmedchem.6b01912](#), PubMed: [28595007](#).
4. Roy M. J., Winkler S., Hughes S. J., Whitworth C., Galant M., Farnaby W., Rumpel K., Ciulli A. Spr-Measured Dissociation Kinetics of PROTAC Ternary Complexes Influence Target Degradation Rate *ACS Chem Biol* **2019**, 14(3), 361–368. DOI: [10.1021/acschembio.9b00092](#), PubMed: [30721025](#).
5. The data can be accessed via the following link: [Proteomics Dashboard \(fischerlab.org\)](#)
6. Donovan K. A., Ferguson F. M., Bushman J. W., Eleuteri N. A., Bhunia D., Ryu S., Tan L., Shi K., Yue H., Liu X., Dobrovolsky D., Jiang B., Wang J., Hao M., You I., Teng M., Liang Y., Hatcher J., Li Z., Manz T. D., Groendyke B., Hu W., Nam Y., Sengupta S., Cho H., Shin I., Agius M. P., Ghobrial I. M., Ma M. W., Che J., Buhrlage S. J., Sim T., Gray N. S., Fischer E. S. Mapping the Degradable Kinome Provides a Resource for Expedited Degradation Development *Cell* **2020**, 183(6), 1714-1731.e10. DOI: [10.1016/j.cell.2020.10.038](#), PubMed: [33275901](#).
7. DeMars K. M., Yang C., Castro-Rivera C. I., Candelario-Jalil E. Selective degradation of BET proteins with dBET1, a proteolysis-targeting chimera, potently reduces pro-inflammatory responses in lipopolysaccharide-activated microglia *Biochem Biophys Res Commun* **2018**, 497(1), 410–415. DOI: [10.1016/j.bbrc.2018.02.096](#), PubMed: [29448097](#).

8. Winter G. E., Buckley D. L., Paulk J., Roberts J. M., Souza A., Dhe-Paganon S., Bradner J. E. Drug DEVELOPMENT. Phthalimide conjugation as a strategy for in vivo target protein degradation *Science* **2015**, 348(6241), 1376–1381. [DOI: 10.1126/science.aab1433](https://doi.org/10.1126/science.aab1433), [PubMed: 25999370](https://pubmed.ncbi.nlm.nih.gov/25999370/).