Supplementary Information

Plasticity in binding confers selectivity in ligand induced protein degradation

Radosław P. Nowak[1,2], Stephen L. DeAngelo[1], Dennis Buckley[3, §], Zhixiang He[1,2], Katherine A. Donovan[1,2], Jian An[1,2], Nozhat Safaee[1,2], Mark P. Jedrychowski[4,5], Charles M. Ponthier[1], Mette Ishoey[3], Tinghu Zhang[1,2], Joseph D. Mancias[4], Nathanael S. Gray[1,2], James E. Bradner[3, §], Eric S. Fischer[1,2,*]

[1] Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

[2] Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts, USA.

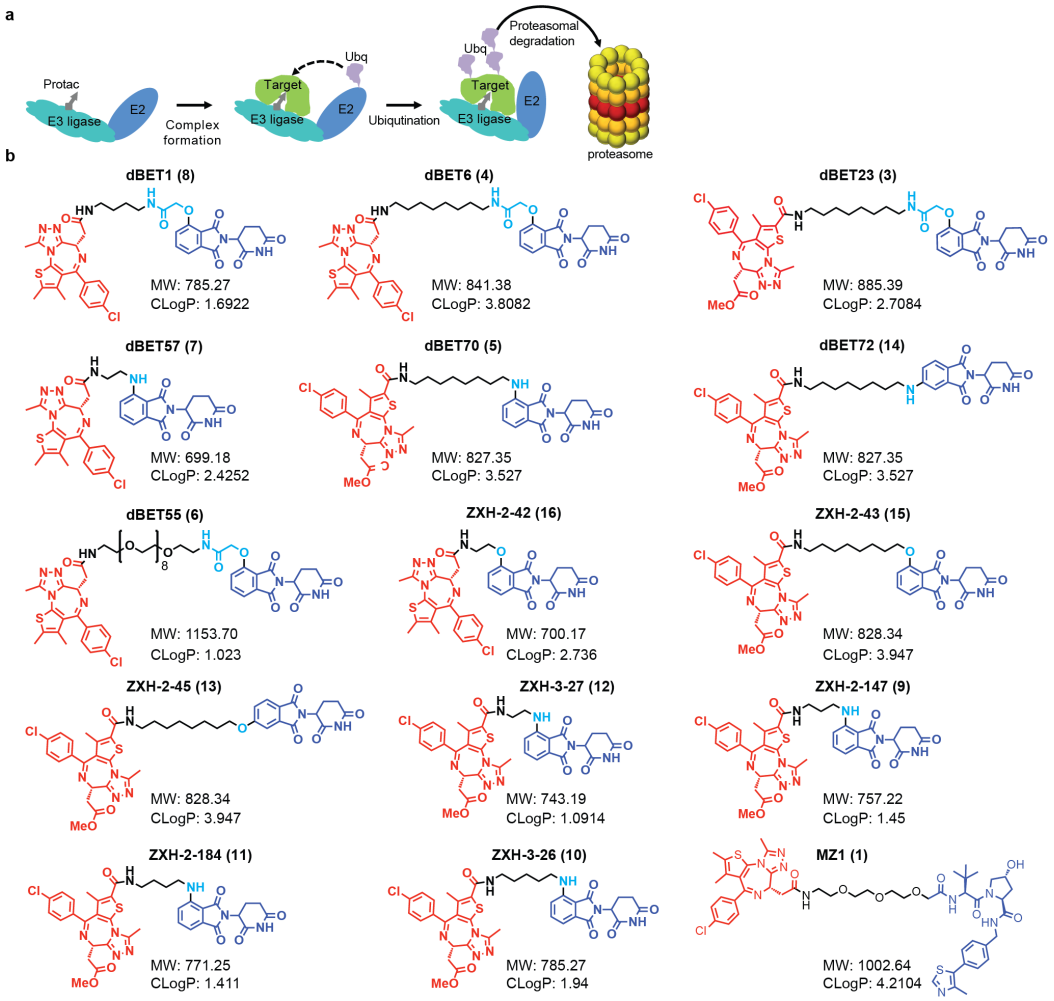
[3] Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

[4] Division of Genomic Stability and DNA Repair, Department of Radiation Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA

[5] Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, USA

[§] Present address: Novartis Institutes for Biomedical Research, Cambridge, Massachusetts, USA.

*Correspondence and request for materials to eric_fischer@dfci.harvard.edu (E.S.F.)

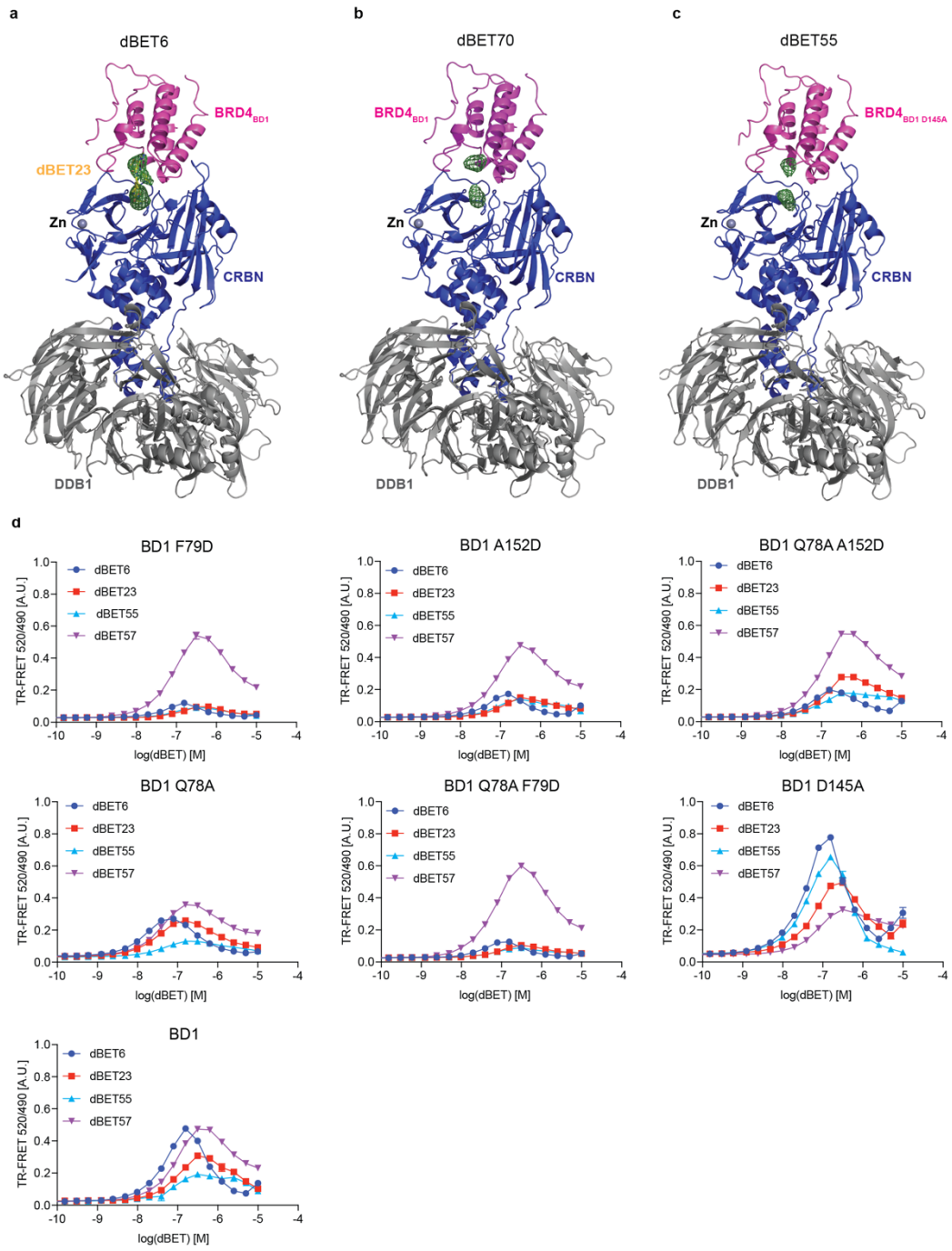


Supplementary Figure 1 | Structure of the DDB1ΔB-CRBN-dBET23-BRD4$_{BD1}$ complex.

(a) Schematic representation of heterobifunctional degrader (PROTAC) mediated degradation.

(b) Chemical structures, molecular weight and CLogP (calculated with ChemDraw Professional 16.0) for molecules used in this study. BET inhibitor JQ1-(S) colored in red, thalidomide moiety

colored in blue and the linker in black and cyan. **(c)** Multiple sequence alignment of BD1 and BD2 from different BET bromodomain paralogs, residue different between BRD2/3/4 are labelled, and **(d)** multiple sequence alignment of BD1 to BD2 from human BRD4. **(e)** Domain architecture of BRD4. A and B – DNA binding motifs, ET- extraterminal domain, SEED – Ser/Glu/Asp-rich region, CTM – C-terminal domain. **(f)** DDB1ΔB, CRBN, and BRD4 domain coloring and construct boundaries referencing figure 1b. Hatched lines indicate sequences omitted from the crystallized protein construct.

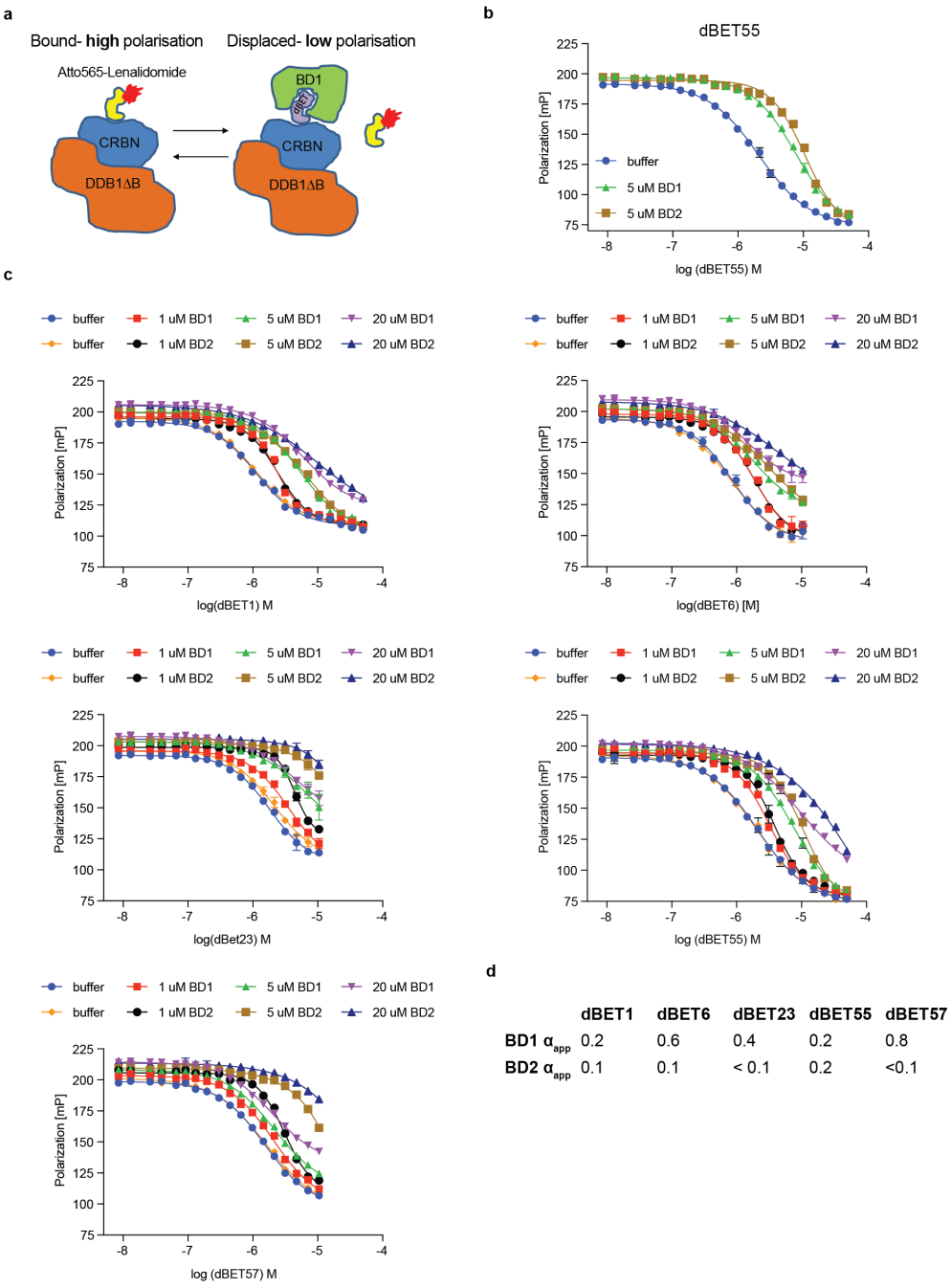


Supplementary Figure 2 | Structures of dBET6, dBET70 and dBET55 complexes.

(a) Cartoon representation of DDB1ΔB-CRBN-dBET6-BRD4$_{BD1}$. The F_O-F_C map is shown as green mesh for dBET6 contoured at 4.0σ. **(b)** Cartoon representation of DDB1ΔB-CRBN-dBET70-BRD4$_{BD1}$. The F_O-F_C map is shown as green mesh for dBET70 contoured at 4.0σ. **(c)** Cartoon representation of DDB1ΔB-CRBN-dBET55-BRD4$_{BD1/D145A.}$ The F_O-F_C map is shown as

green mesh contoured at 4.0σ. For **a, b** and **c** DDB1 is shown in grey, CRBN in blue, and $BRD4_{BD1}$ (wild type and mutant) in magenta. **(d)** TR-FRET data underlying bar charts shown in Fig. 2a, 4a – d and Supplementary Fig. 5d – i. TR-FRET data in this figure are three independent replicates presented as means ± s.d. (n=3).

It should be noted that the structures presented here may only represent a snapshot of possible binding conformations. The weak nature of the protein-protein interactions, the non-evolved contacts and the limited constraints of poly-carbon/PEG linkers will often allow for alternative conformations. While the mutational signatures for dBET23 and dBET6 suggest that the observed structures represent a major binding mode in solution, the mutational signature for dBET70 suggests that crystal packing stabilizes the observed conformation for this ligand. While the conformation observed in the crystal structure is possible with the dBET70 ligand, it may not represent the dominant binding mode in solution.



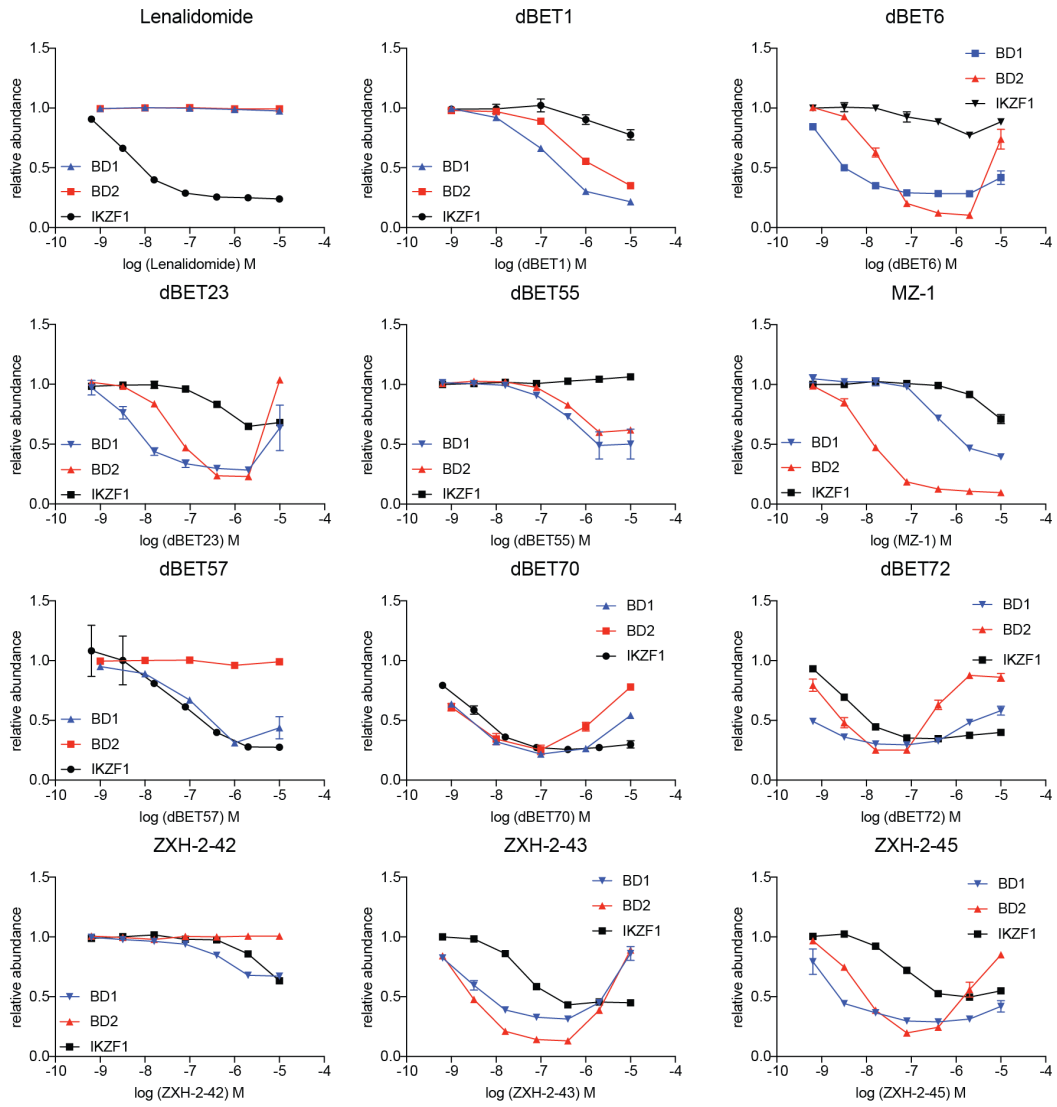
	dBET1	dBET6	dBET23	dBET55	dBET57
BD1 α_{app}	0.2	0.6	0.4	0.2	0.8
BD2 α_{app}	0.1	0.1	< 0.1	0.2	<0.1

Supplementary Figure 3 | Negative cooperativity governs CRBN-dBET-BRD4 interactions.

(a) Schematic of fluorescence polarization based CRBN binding assay. Atto565-Lenalidomide fluorophore is displaced by degrader bound $BRD4_{BD1/2}$. **(b)** Fluorescence polarization competitive binding assay for dBET55 binding to DDB1ΔB-CRBN. Increasing concentrations of dBET55 titrated to preformed DDB1ΔB-CRBN-$lenalidomide_{Atto565}$ complex in presence or

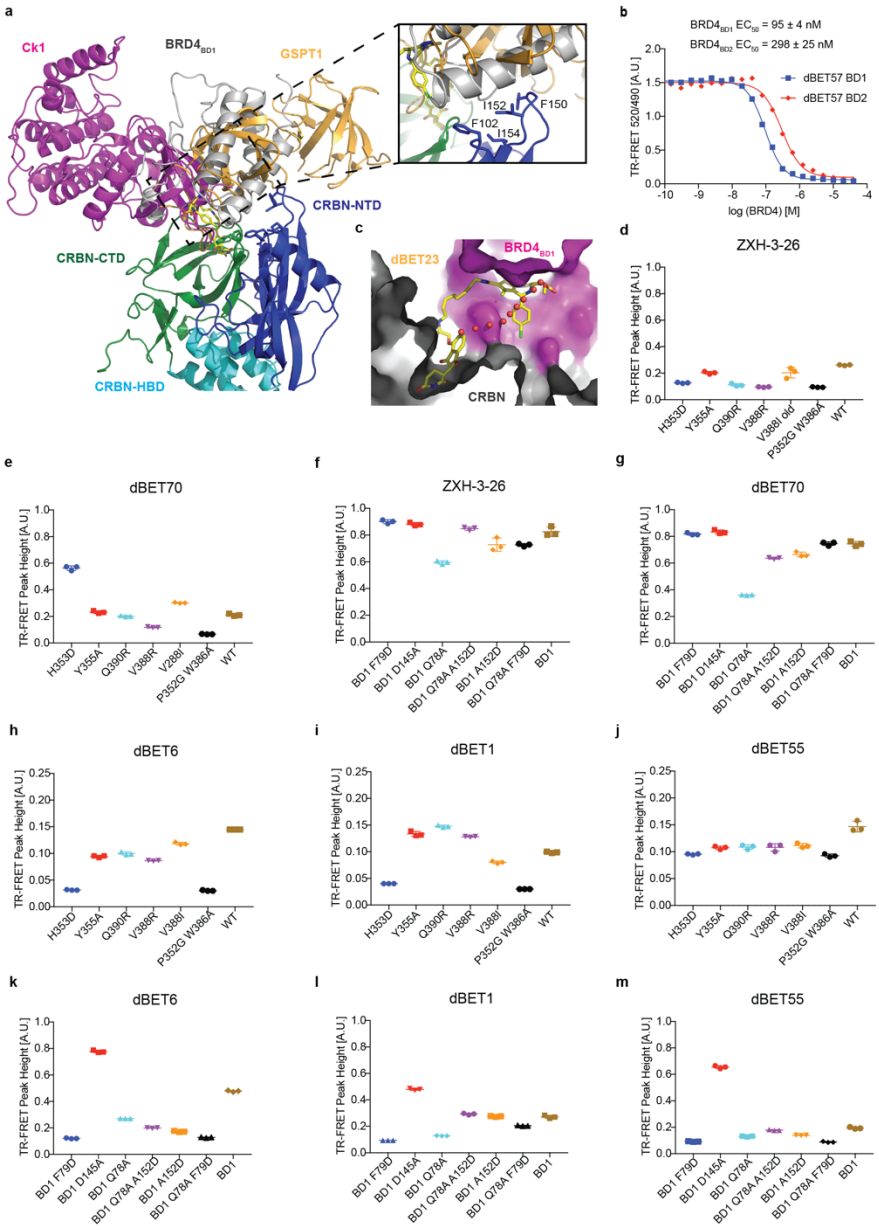
absence of $BRD4_{BD1}$ or $BRD4_{BD2}$. Data is plotted as means ± s.d. from two independent replicates (n=2) for BD1 and BD2 and four independent replicates (n=4) for buffer control. **(c)** Fluorescence polarization competitive binding assay for dBET1, dBET6, dBET23, dBET55, and dBET57 binding to DDB1ΔB-CRBN. Increasing concentrations of dBETs titrated to preformed DDB1ΔB-CRBN-lenalidomide$_{Atto565}$ complex in presence or absence of $BRD4_{BD1}$ or $BRD4_{BD2}$ at concentrations of 1 μM, 5 μM, and 20 μM. The data at 5 μM $BRD4_{BD1/2}$ was replotted for Fig. 2c – f and panel b of this figure. Data is presented as means ± s.d. of two independent replicates (n=2). **(d)** summary of apparent cooperativity factors α_{app}.

a



Supplementary Figure 4 | Cellular degradation of BRD4$_{BD1/2}$.

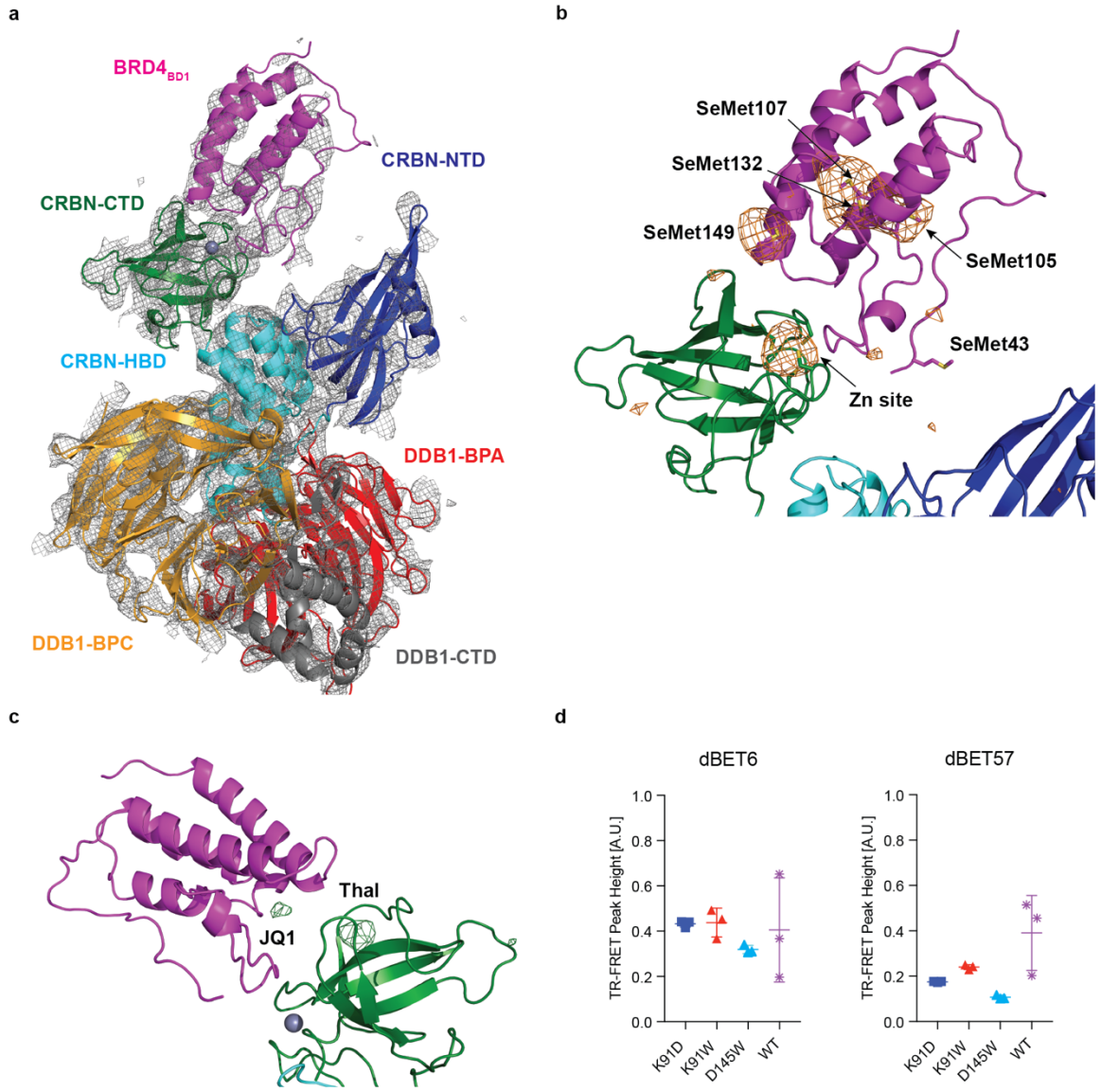
(a) Quantitative assessment of cellular degradation of a BRD4$_{BD1}$-EGFP/ BRD4$_{BD2}$-EGFP and IKZF1Δ-EGFP using flow cytometry analysis. Cells stably expressing BRD4$_{BD1}$-EGFP/ BRD4$_{BD2}$-EGFP or IKZF1Δ-EGFP with a mCherry reporter were treated with increasing concentrations of dBET1, dBET6, dBET23, dBET55, dBET57, dBET70, dBET72, MZ1, ZXH-2-42, ZXH-2-43, ZXH-2-45 and lenalidomide with the EGFP and mCherry signals quantified using flow cytometry analysis. Data is presented as means ± s.d. from three (six for lenalidomide) independent replicates.



Supplementary Figure 5 I Plasticity of CRBN-substrate interactions.

(a) CRBN utilizes different surfaces to interact with a variety with neo-substrates as illustrated by the superposition of DDB1ΔB-CRBN-dBET23-BRD4$_{BD1}$, DDB1ΔB-CRBN-lenalidomide-Ck1 α (pdb: 5fqd), and DDB1-CRBN-CC885-GSPT1 (pdb: 5hxb). Top right, close-up of the common hydrophobic interface between GSPT1-CRBN-NTD and BRD4$_{BD1}$-CRBN-NTD. **(b)** The structures of DDB1-CRBN-dBET23-BRD4$_{BD1}$ and DDB1-CRBN-lenalidomide-CK1a suggest

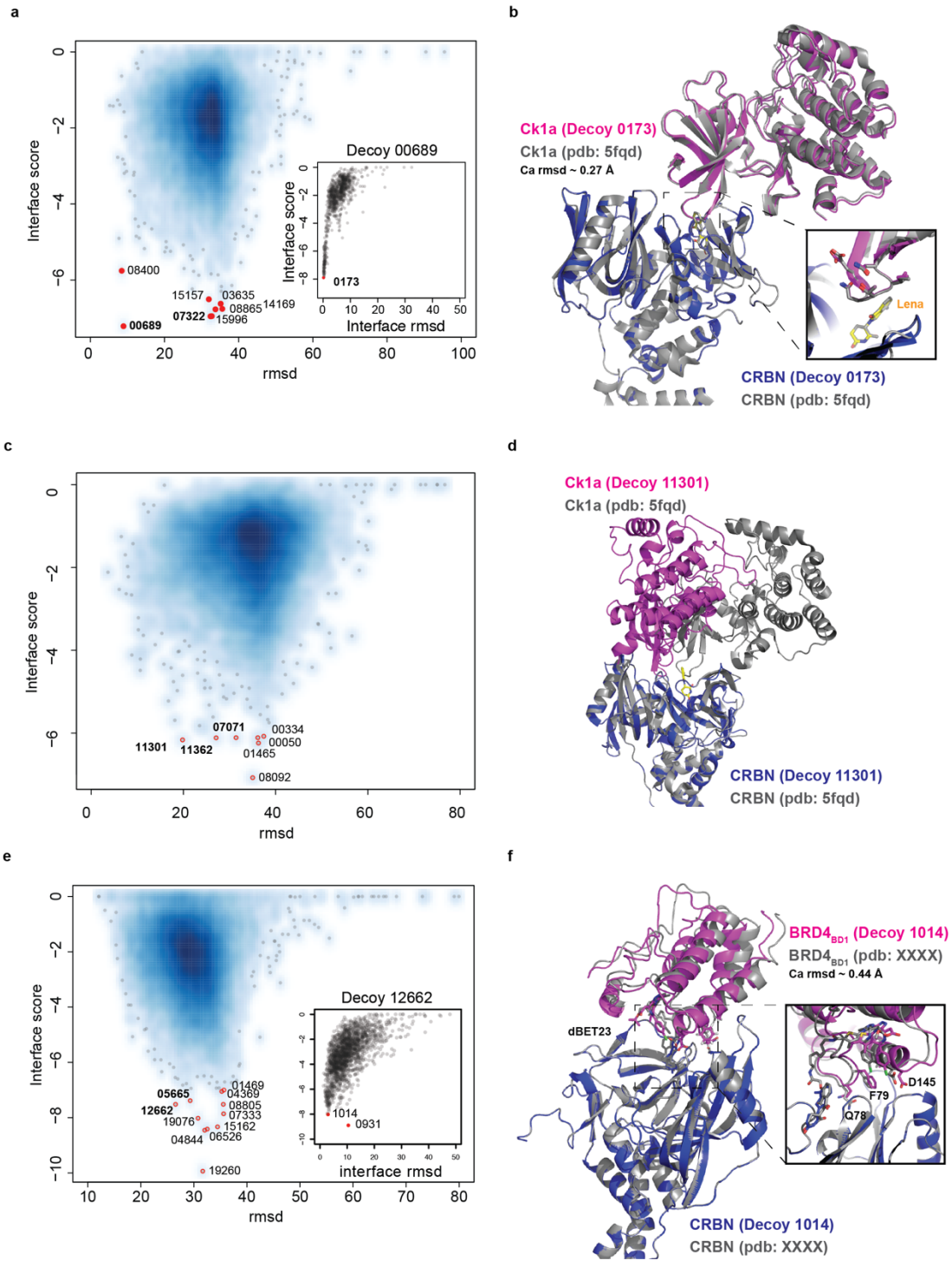
mutually exclusive binding of BRD4 with neo-substrates such as Ck1a or IKZF1/3, which is confirmed by titrating $BRD4_{BD1}$ or $BRD4_{BD2}$ into a preformed complex of DDB1-CRBN-dBET57-IKZF1Δ. Data is presented as mean and standard deviation of 10 technical replicates of a single experiment (n=1). **(c)** Surface representation of CRBN and $BRD4_{BD1}$ of DDB1-CRBN-dBET23-$BRD4_{BD1}$ crystal structure, showing dBET23 as stick representation. The hypothetical linker path from the acid position on JQ1 is shown with red spheres indicating the distance of a carbon-carbon bond and illustrating that the 2-carbon linker of dBET57 would be insufficient to bridge the gap. **(d)** TR-FRET. ZXH-3-26 degrader titrated to $BRD4_{BD1\text{-}SPYCATCHER\text{-}BODIPY}$ and Terbium-antiHis antibody, and wild type or various mutants of His6-DDB1-His6-CRBN complex. The peak height of the dose response curve for three independent replicates was quantified and is depicted as dot-plot. TR-FRET data in this figure are independent replicates presented as means ± s.d. (n=3). **(e, h - j)** as in **d** but for dBET70, dBET6, dBET1 and dBET55, respectively. **(f)** TR-FRET. ZXH-3-26 degrader titrated to $DDB1\Delta B\text{-}CRBN_{SPYCATCHER\text{-}BODIPY}$, Terbium-Streptavidin and wild type or mutants of $BRD4_{BD1\text{-}biotin}$. The peak height of the dose response curve for three independent replicates was quantified and is depicted as dot-plot. TR-FRET data in this figure are presented as means ± s.d. (n=3). **(g, k-m)** as in **f** but for dBET70, dBET6, dBET1 and dBET55, respectively.



Supplementary Figure 6 | Experimental validation of DDB1-CRBN-dBET57-BRD4$_{BD1}$ structure.

(a) Cartoon representation of DDB1-CRBN-dBET57-BRD4$_{BD1}$ complex with the $2F_O$-F_C map contoured at 1.5 σ. Domains are colored as DDB1-BPA (red), DDB1-BPC (orange), DDB1-CTD (grey), CRBN-NTD (blue), CRBN-HBD (cyan), CRBN-CTD (green), and BRD4$_{BD1}$ (magenta). We note that CRBN was found in a not previously observed conformation, in which the thalidomide binding CRBN-CTD domain translates and rotates away from the CRBN-HBD and

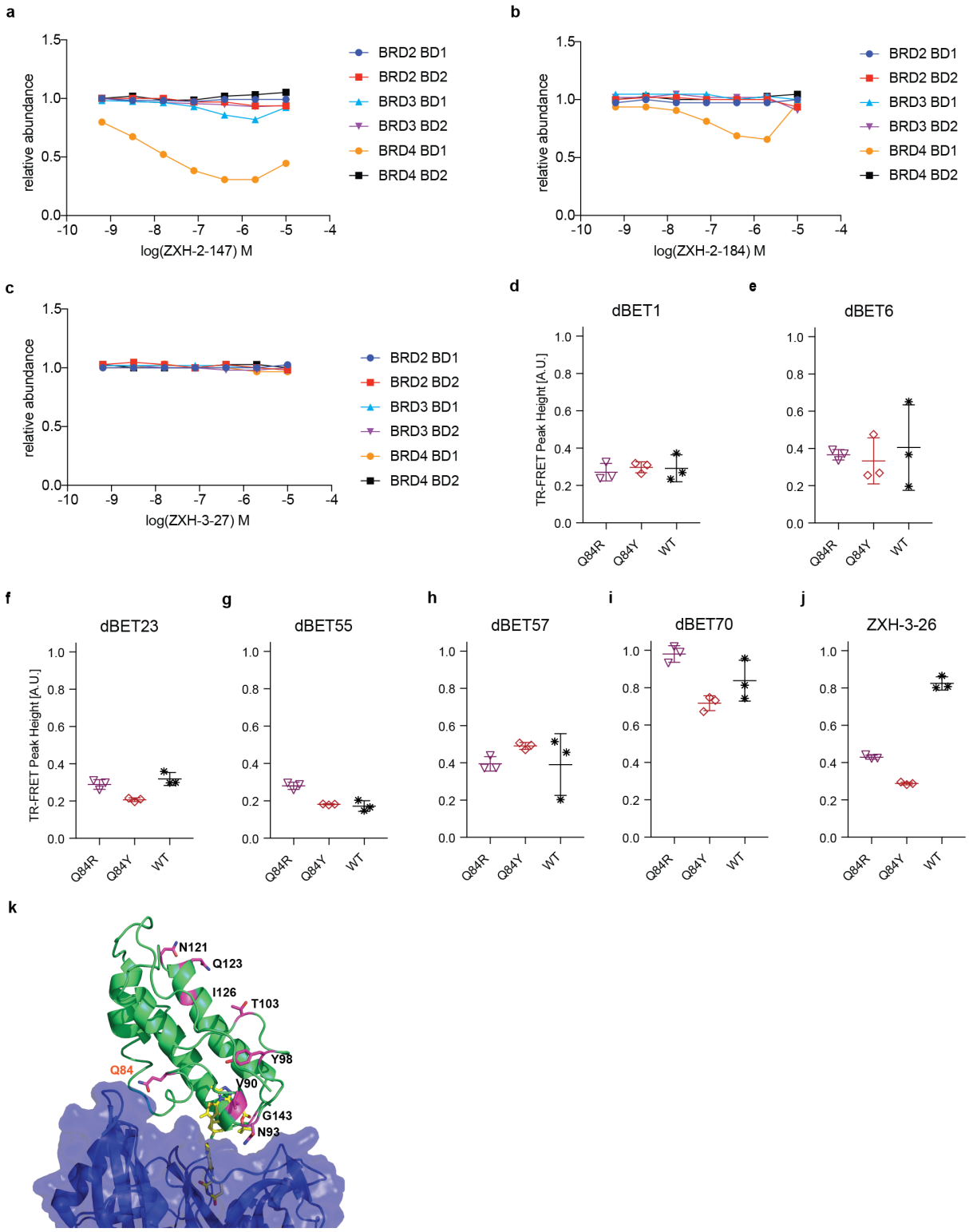
CRBN-NTD domains. This results in an open conformation that exposes large areas of CRBN that are typically buried. The high salt crystallization condition could be a driver of this structural rearrangement, and together with crystal contacts induce this conformation. We can, however, not exclude that this conformational dynamic is an intrinsic feature of CRBN to accommodate a variety of substrates and future studies are necessary to address this. Based on the compatibility of the observed $BRD4_{BD1}$ binding conformation with the open and closed CRBN conformations, we believe that for the interpretation of our data the conformational change is negligible. **(b)** Cartoon representation of DDB1-CRBN-dBET57-Se$MetBRD4_{BD1}$ complex. Anomalous difference map contoured at 3 σ shown in orange for data collected at the Se peak showing the position of the Se atoms and Zn. **(c)** F_O-F_C map of native DDB1-CRBN-dBET57-$BRD4_{BD1}$ contoured at 3.0 σ and shown in green, carved around the JQ1 and thalidomide sites. Positive difference density is observed for the Thalidomide (Thal) and JQ1 binding sites. **(d)** TR-FRET. dBET6 or dBET57 degrader titrated to DDB1ΔB-$CRBN_{SPYCATCHER\text{-}BODIPY}$, Terbium-Streptavidin and wild type or mutants of $BRD4_{BD1\text{-}biotin}$. The peak height of the dose response curve for three independent replicates was quantified and is depicted as dot-plot. TR-FRET data in this figure are independent replicates presented as means ± s.d. (n=3).



Supplementary Figure 7 | Rosetta docking of CRBN-lenalidomide-Ck1 complex

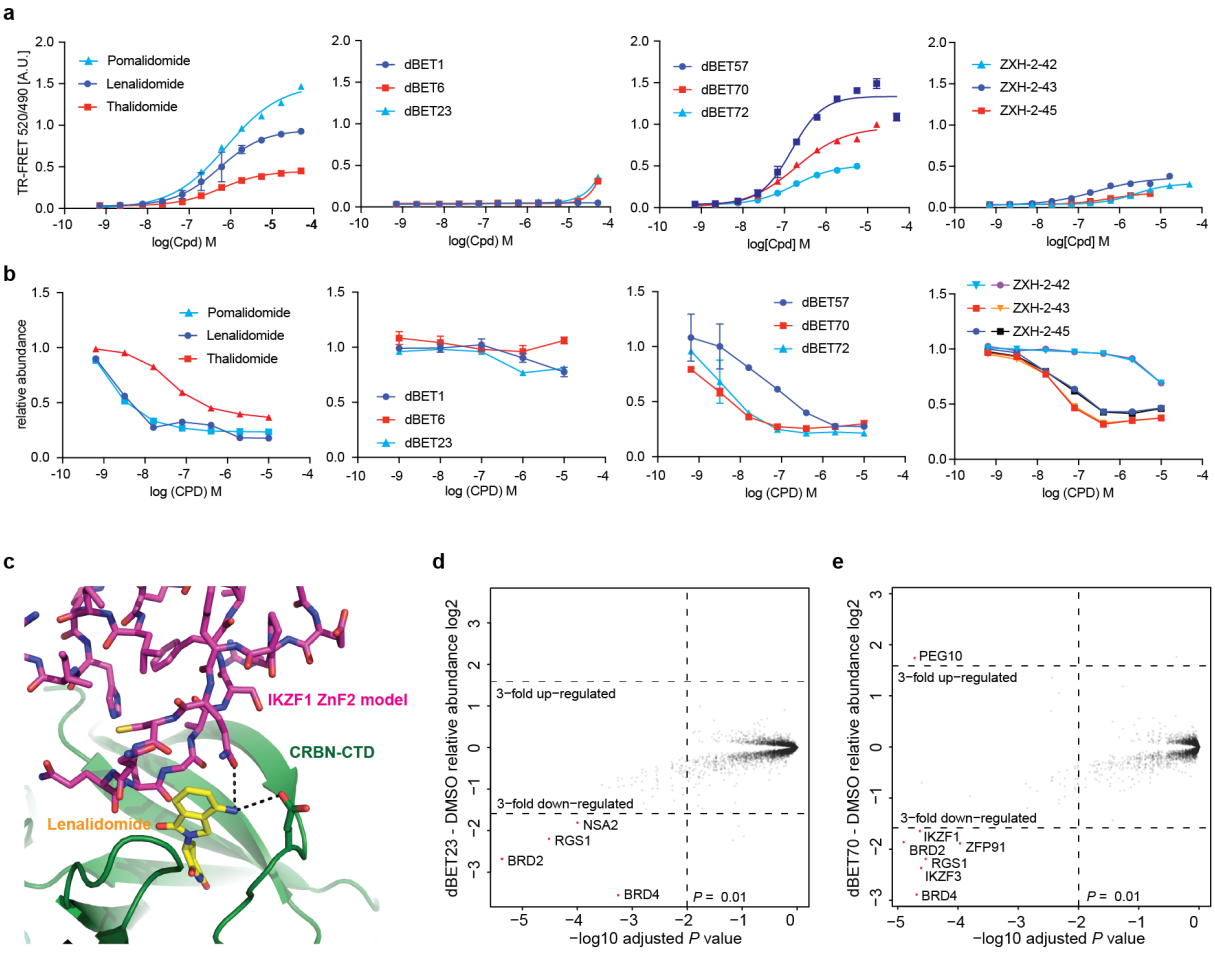
(a) Symmetric docking energy landscape for the binding of Ck1α to a CRBN-lenalidomide complex. Symmetric docking energy landscape for local perturbation docking experiments on a

lowest energy decoy 00689 is shown as insert. **(b)** Superposition of the DDB1ΔB-CRBN-lenalidomide-Ck1α structure (pdb: 5fqd) and the top solution, decoy 0173, from **a**. **(c)** Symmetric energy docking landscape for the binding of Ck1α to a CRBN-lenalidomide complex. The conformer parameter file for lenalidomide was restricted to a conformer not favorable of Ck1α binding. **(d)** Superposition of the DDB1ΔB-CRBN-lenalidomide-Ck1α structure (pdb: 5fqd) and the top solution from **c**. **(e)** Symmetric docking energy landscape for the binding of $BRD4_{BD1}$ to a CRBN-lenalidomide complex. The two low energy decoys that exhibit a conformation compatible with dBET binding are indicated by bold numbers. The symmetric docking energy landscape for local perturbation docking experiments on decoy 12662 compatible with dBET mediated binding is shown as insert. **(f)** Superposition of the DDB1ΔB-CRBN-dBET23-$BRD4_{BD1}$ structure (pdb: 6bn7) and the top solution from local perturbation of decoy 12662.



Supplementary Figure 8 | Selective degradation of BRD4.

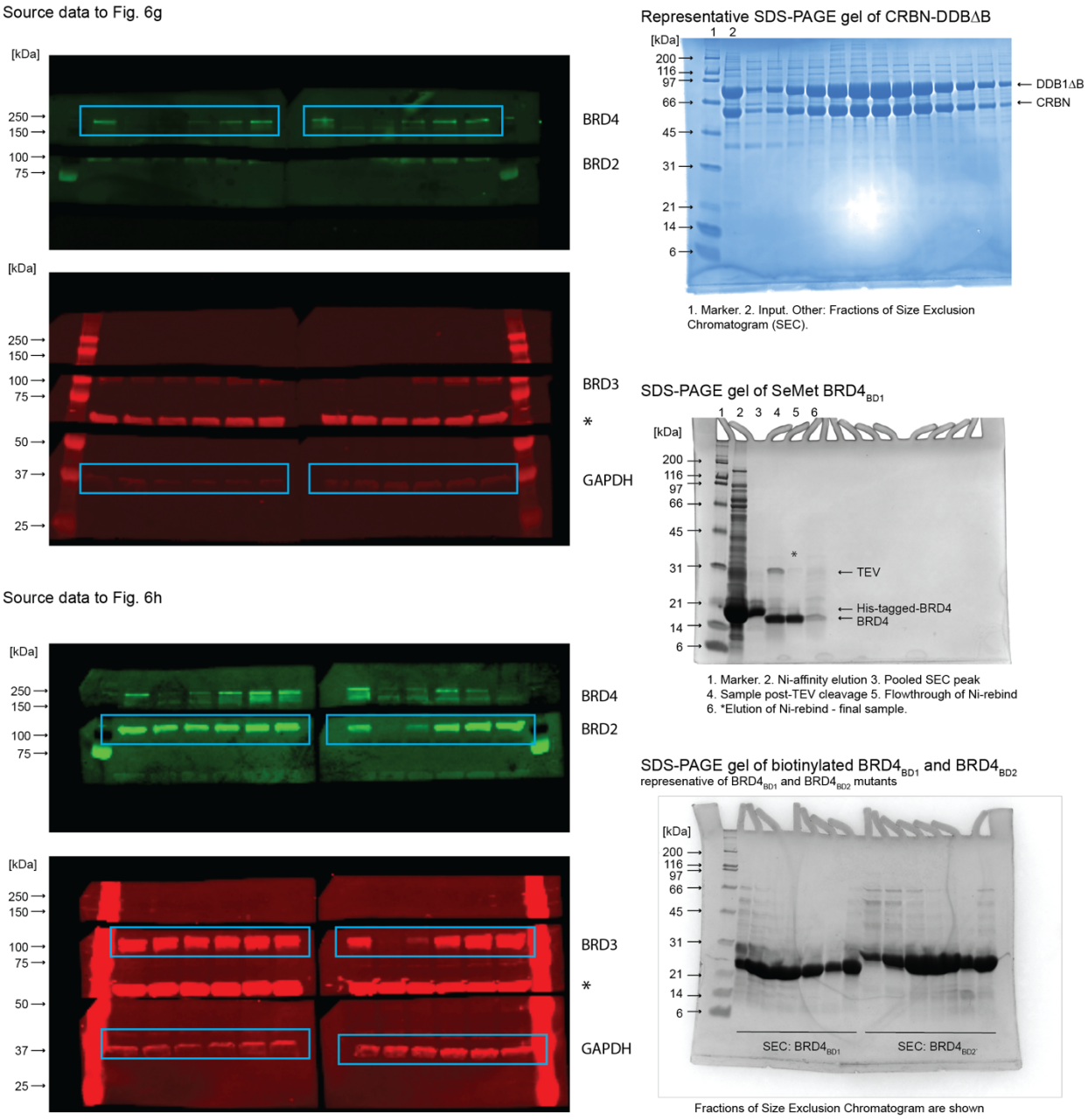
(a) Quantitative assessment of cellular degradation using EGFP/mCherry reporter assay. Cells stably expressing $BRD4_{BD1}$-EGFP (or constructs harbouring $BRD2_{BD1}$, $BRD2_{BD2}$, $BRD3_{BD1}$, $BRD3_{BD2}$, $BRD4_{BD2}$) and mCherry were treated with increasing concentrations of ZXH-2-147 and the EGFP and mCherry signals followed using flow cytometry analysis. **(b)** as in **a** but for ZXH-2-184. **(c)** as in **a** but for ZXH-3-27. Data in **a**-**c** are singlicate experiments (n=1). **(d)** TR-FRET. dBET1 degrader titrated to DDB1ΔB-$CRBN_{SPYCATCHER-BODIPY}$, Terbium-Streptavidin and wild type or mutants of $BRD4_{BD1-biotin}$. The peak height of the dose response curve for three independent replicates was quantified and is depicted as dot-plot. TR-FRET data in this figure are presented as means ± s.d. (n=3). **(e-j)** as in **d** but for dBET6, dBET23, dBET55, dBET57, dBET70 and ZXH-3-26 respectively. **(k)** Cartoon representation of docking pose from cluster 19 (see Figure 5) serving as a rationale for design of ZXH-3-26. $BRD4_{BD1}$ shown in green and CRBN in blue. Highlighted residues of BRD4 different between BRD2/3. Residue Q84 (R in BRD2, Y in BRD3) highlighted in orange.



Supplementary Figure 9 | Co-degradation of IMiD neo-substrates such as IKZF1/3.

(a) TR-FRET. Titration of the indicated molecules to DDB1ΔB-CRBN$_{SPYCATCHER-BODIPY}$, Terbium-streptavidin and IKZF1Δ$_{biotin}$. Data in this figure are presented as means ± s.d. from three independent replicates (n=3). **(b)** Quantitative assessment of cellular degradation of a IKZF1-EGFP reporter using flow cytometry analysis. Cells stably expressing IKZF1Δ-EGFP and mCherry were treated with increasing concentrations of the indicated molecules and the EGFP and mCherry signals followed using flow cytometry analysis. Data in this figure are presented as means ± s.d. from four cell culture replicates (n=4). **(c)** Model of a CRBN-IKZF1$_{ZnF2}$ complex (adapted from Petzold et al., 2016) bound to lenalidomide. Potential hydrogen bonds are indicated as dashed lines. **(d)** Scatter plot depicting the fold changes in relative abundance comparing dBET23 to DMSO control treatment (MM.1s) determined using quantitative

proteomics. Negative false discovery rate adjusted *P* Values are shown on the x-axis and log2 fold changes on the y-axis. Data shown are of three cell culture replicates measured in a single 10-plex TMT experiment. *P* Values were derived from a moderated *t*-statistic using the limma package[1] and corrected for multiple hypothesis testing (see **methods** for details). **(e)** as in **d** but for dBET70 to DMSO control. This data shows that certain degraders are capable of directly inducing binding of IKZF1/3 (and other IMiD targets) to CRBN, while others exhibit reduced or absent co-degradation of zinc-finger targets. In particular, we found that a secondary amine in the C4 position of the phthalimide (dBET57/70/72) leads to effective degradation of IKZF1/3 (and other IMiD targets), while replacing with an oxygen-ether linker (ZXH-2-42/43/45) reduces such activity, and using an oxy-acetamide linker (dBET1/6/23) fully prevents degradation of zinc finger targets. Thus, altering linker composition can be used as a strategy to tune co-degradation of IKZF1/3 and other IMiD targets.



Supplementary Figure 10 | Uncropped Immunoblots.

Boxed areas correspond to image regions represented in the indicated main text and Supplementary figures. Western blots have been flipped vertically to represent increasing concentrations of Compound. SDS-PAGE gel images for representative preparations of DDBΔB-CRBN, SeMet-BRD4$_{BD1}$, biotinylated BRD4$_{BD1}$ and biotinylated BRD4$_{BD2}$ are shown.

Supplementary Table 1a | Data collection and refinement statistics.

	DDB1ΔB-CRBN-dBET6-BRD4$_{BD1}$	DDB1ΔB-CRBN-dBET23-BRD4$_{BD1}$	DDB1ΔB-CRBN-dBET55-BRD4$_{BD1}$ D145A
Data collection			
Space group	P 6_5 2 2	P 6_5 2 2	P 6_5 2 2
Cell dimensions			
a, *b*, *c* (Å)	115.40, 115.40, 588.14	115.57, 115.57, 596.32	115.20, 115.20, 597.14
α, β, γ (°)	90, 90, 120	90, 90, 120	90, 90, 120
Resolution (Å)	49.79 - 3.33 (3.49 - 3.33)	49.87 - 3.49 (3.68 - 3.49)	149.28 - 3.99 (4.31 - 3.99)
R_{merge}	0.179 (5.471)	0.128 (3.561)	0.280 (2.227)
R_{pim}	0.032 (0.978)	0.041 (1.173)	0.072 (0.582)
$CC_{1/2}$	1.000 (0.469)	0.999 (0.328)	0.991 (0.452)
$I / \sigma I$	16.4 (0.9)	11.4 (0.7)	7.69 (1.1)
Completeness (%)	100.0 (100.0)	99.5 (97.3)	100.0 (100.0)
Redundancy	32.2 (33.2)	11.4 (10.5)	17.0 (16.1)
Refinement			
Resolution (Å)	49.79 - 3.33 (3.45 - 3.33)	49.35 - 3.50 (3.63 - 3.50)	99.77 - 3.99 (4.13 - 3.99)
No. reflections	35251 (3287)	30453 (2671)	21193 (2038)
R_{work}	0.1994 (0.3605)	0.2123 (0.3551)	0.2886 (0.3757)
R_{free}	0.2344 (0.4380)	0.2555 (0.3848)	0.3334 (0.3912)
No. atoms	10373	10331	10291
Protein	10313	10268	10290
Ligand/ion	60	63	1
Water	0	0	0
B-factors	175.87	204.01	189.70
Protein	176.08	204.26	189.70
Ligand/ion	140.19	162.21	133.84
Water	-	-	-
R.m.s. deviations			
Bond lengths (Å)	0.002	0.007	0.002
Bond angles (°)	0.51	0.48	0.46

*Each dataset was collected from one crystal. *Values in parentheses are for highest-resolution shell.

Supplementary Table 1b | Data collection and refinement statistics.

	DDB1ΔB-CRBN-dBET57-SeMetBRD4$_{BD1}$	DDB1ΔB-CRBN-dBET70-BRD4$_{BD1}$
Data collection		
Space group	I 4 2 2	P 6_5 2 2
Cell dimensions		
a, *b*, *c* (Å)	313.36, 313.36, 167.37	117.60, 117.60, 597.16
α, β, γ (°)	90, 90, 90	90 90 120
Resolution (Å)	147.60 - 6.34 (7.08 - 6.34)	149.29 - 4.38 (4.90 - 4.38)
R_{merge}	0.165 (2.952)	0.349 (3.276)
R_{pim}	0.036 (0.593)	0.059 (0.548)
$CC_{1/2}$	1.000 (0.627)	1.000 (0.768)
I / σI	15.3 (1.3)	8.5 (1.4)
Completeness (%)	98.1 (93.4)	99.7 (99.8)
Redundancy	25.4 (26.2)	36.9 (36.7)
Refinement		
Resolution (Å)	147.60-6.34 (6.57-6.34)	100.40 - 4.38 (4.54 - 4.38)
No. reflections	8964 (743)	16770 (1588)
R_{work}	0.3368 (0.4151)	0.2754 (0.3827)
R_{free}	0.3805 (0.5110)	0.3013 (0.4689)
No. atoms	10042	10314
Protein	10041	10313
Ligand/ion	1	1
Water	0	0
B-factors	484.99	278.70
Protein	485.00	278.71
Ligand/ion	465.40	197.08
Water	-	-
R.m.s. deviations		
Bond lengths (Å)	0.011	0.002
Bond angles (°)	1.48	0.469

*Each dataset was collected from one crystal. *Values in parentheses are for highest-resolution shell.

Supplementary Dataset 1 | Proteomics data of dBET23, dBET70 and ZXH-3-26 cellular effects.

Attached excel spreadsheet containing the raw output of statistical analysis using limma[1].

References

1 Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**, e47 (2015).

Synthetic procedures

Plasticity in binding confers selectivity in ligand induced protein degradation

Radosław P. Nowak[1,2], Stephen L. DeAngelo[1], Dennis Buckley[3, §], Zhixiang He[1,2], Katherine A. Donovan[1,2], Jian An[1,2], Nozhat Safaee[1,2], Mark Jedrychowski[4,5], Charles M. Ponthier[1], Mette Ishoey[3], Tinghu Zhang[1,2], Joseph D. Mancias[4], Nathanael S. Gray[1,2], James E. Bradner[3, §], Eric S. Fischer[1,2,*]

[1] Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02215, USA.

[2] Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA.

[3] Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA.

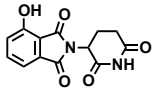
[4] Division of Genomic Stability and DNA Repair, Department of Radiation Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA

[5] Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA

[§] Present address: Novartis Institutes for Biomedical Research, Cambridge, MA, USA.

*Correspondence and request for materials to eric_fischer@dfci.harvard.edu (E.S.F.)

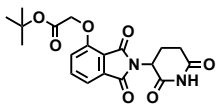
Synthetic chemistry methods for compounds in this study:



2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione (17)

3-Hydroxyphthalic anhydride (1.641 g, 10 mmol, 1 eq) and 3-aminopiperidine-2,6-dione hydrochloride (1.646 g, 10 mmol, 1 eq) were dissolved in pyridine (40 mL, 0.25 M) and heated to 110 °C. After 14 hours, the mixture was cooled to room temperature and concentrated under reduced pressure. Purification by column chromatography (ISCO, 24 g silica column, 0-10% MeOH/DCM) gave the desired product as a tan solid (2.424 g, 8.84 mmol, 88%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.08 (s, 2H), 7.65 (dd, *J* = 8.4, 7.2 Hz, 1H), 7.36 – 7.28 (m, 1H), 7.25 (dd, *J* = 8.4, 0.6 Hz, 1H), 5.07 (dd, *J* = 12.8, 5.4 Hz, 1H), 2.88 (ddd, *J* = 17.3, 14.0, 5.4 Hz, 1H), 2.63 – 2.50 (m, 2H), 2.08 – 1.95 (m, 1H).

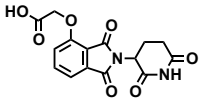


***tert*-butyl 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetate (18)**

2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindoline-1,3-dione (1.568 g, 5.71 mmol, 1 eq) was dissolved in DMF (57 mL, 0.1 M) at room temperature. Potassium carbonate (1.19 g, 8.58 mmol, 1.5 eq) and tert-butyl bromoacetate (0.843 mL, 5.71 mmol, 1 eq) were then added. After 2 hours, the mixture was diluted with EtOAc and washed once with water then twice with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 24 g silica column, 0-100%EtOAc/hexanes, 21 minute gradient) gave the desired product as a cream colored solid (2.06 g, 5.30 mmol, 93%).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.94 (s, 1H), 7.67 (dd, *J* = 8.4, 7.3 Hz, 1H), 7.52 (d, *J* = 6.8 Hz, 1H), 7.11 (d, *J* = 8.3 Hz, 1H), 4.97 (dd, *J* = 12.3, 5.3 Hz, 1H), 4.79 (s, 2H), 2.95 – 2.89 (m, 1H), 2.85 – 2.71 (m, 2H), 2.14 (dtd, *J* = 10.2, 5.0, 2.7 Hz, 1H), 1.48 (s, 9H).

LCMS 389.33 (M+H).

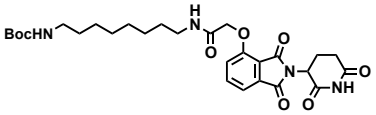


2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetic acid (19)

tert-butyl 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetate (2.06 g, 5.30 mmol, 1 eq) was dissolved in TFA (53 mL, 0.1M) at room temperature. After 4 hours, the solution was diluted with DCM and concentrated under reduced pressure. The resultant cream colored solid (1.484 g, 4.47 mmol, 84%) was deemed sufficiently pure and carried onto the next step without further purification.

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.11 (s, 1H), 7.79 (dd, *J* = 8.4, 7.4 Hz, 1H), 7.48 (d, *J* = 7.2 Hz, 1H), 7.39 (d, *J* = 8.6 Hz, 1H), 5.10 (dd, *J* = 12.8, 5.4 Hz, 1H), 4.99 (s, 2H), 2.93 – 2.89 (m, 1H), 2.63 – 2.51 (m, 2H), 2.04 (ddd, *J* = 10.5, 5.4, 3.1 Hz, 1H).

LCMS 333.25 (M+H).

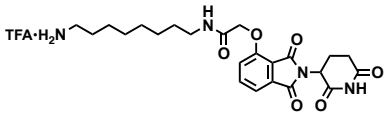


***tert*-butyl (8-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamido)octyl)carbamate (20)**

Boc-1,8-diaminooctane (2.10 g, 8.59 mmol, 1.1 eq) was dissolved in DMF (86 mL). In a separate flask, 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetic acid (2.60 g, 7.81 mmol, 1 eq) was dissolved in DMF (78 mL). The solution of Boc-1,8-diaminooctane in DMF was then added, followed by DIPEA (4.08 mL, 23.4 mmol. 3 eq) and HATU (2.97 g, 7.81 mmol, 1 eq). The mixture was stirred for 19 hours at room temperature, then diluted with EtOAc (600 mL). The organic layer was washed sequentially with 200 mL of half saturated sodium chloride, 200 mL 10% citric acid (aq.), 200 mL of half saturated sodium chloride, 200 mL of saturated sodium bicarbonate (aq.), 200 mL water and twice with 200 mL brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 40 g column, 0-5% MeOH/DCM, 35 minute gradient) gave the desired product as a white solid (3.53 g, 6.32 mmol, 81%).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.49 (s, 1H), 7.74 (dd, *J* = 8.3, 7.4 Hz, 1H), 7.55 (d, *J* = 7.2 Hz, 1H), 7.39 (t, *J* = 5.3 Hz, 1H), 7.19 (d, *J* = 8.4 Hz, 1H), 4.97 (dd, *J* = 12.4, 5.3 Hz, 1H), 4.63 (d, *J* = 2.2 Hz, 2H), 4.59 (d, *J* = 10.0 Hz, 1H), 3.36 (q, *J* = 6.9 Hz, 2H), 3.12 – 3.03 (m, 2H), 2.95 – 2.72 (m, 3H), 2.16 (ddt, *J* = 10.3, 5.2, 2.7 Hz, 1H), 1.59 (p, *J* = 7.1 Hz, 2H), 1.37 (d, *J* = 67.6 Hz, 19H).

LCMS 559.47 (M+H).

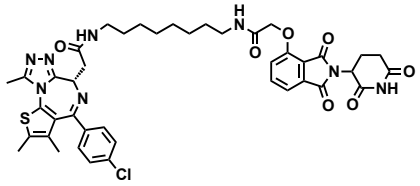


_N_-(8-aminooctyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamide trifluoroacetate (21)

tert-butyl (8-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamido)octyl)carbamate (3.53 g, 6.32 mmol, 1 eq) was dissolved in TFA (63 mL, 0.1M) and heated to 50 °C. After 1 hour, the mixture was cooled to room temperature, diluted with MeOH and concentrated under reduced pressure. The crude material was triturated with diethyl ether and dried under vacuum to give a white solid (2.93 g, 5.12 mmol, 81%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 7.82 (dd, *J* = 8.4, 7.4 Hz, 1H), 7.55 (d, *J* = 7.2 Hz, 1H), 7.44 (d, *J* = 8.4 Hz, 1H), 5.14 (dd, *J* = 12.5, 5.5 Hz, 1H), 4.76 (s, 2H), 3.33 (dd, *J* = 6.8, 1.8 Hz, 1H), 3.30 (s, 1H), 2.94 – 2.85 (m, 3H), 2.80 – 2.69 (m, 2H), 2.19 – 2.11 (m, 1H), 1.60 (dq, *J* = 24.8, 7.0 Hz, 4H), 1.37 (s, 8H).

LCMS 459.45 (M+H).



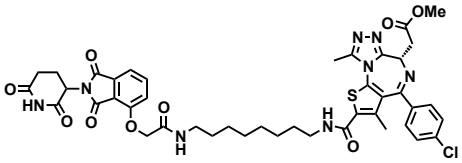
dBET6 (4)

(*S*)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetic acid (**JQ-acid**) (0.894 g, 2.23 mmol, 1 eq) and *N*-(8-aminooctyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamide trifluoroacetate (1.277 g) were dissolved in DMF (22.3 mL, 0.1M) at room temperature. DIPEA (1.17 mL, 6.69 mmol, 3 eq) was added, followed by HATU (0.848 g, 2.23 mmol, 1 eq). The mixture was stirred for 23 hours, and then diluted with EtOAc. The organic layer was washed with saturated sodium bicarbonate, water and three times with brine. The organic layer was then dried under sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 40 g column, 4-10% MeOH/DCM, 35 minute gradient) gave dBET6 as a cream colored solid (1.573 g, 1.87 mmol, 84%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 7.80 (dd, *J* = 8.3, 7.5 Hz, 1H), 7.53 (d, *J* = 7.3 Hz, 1H), 7.46 – 7.37 (m, 5H), 5.11 (ddd, *J* = 12.6, 8.2, 5.5 Hz, 1H), 4.75 (s, 2H), 4.63 (dd, *J* = 9.0, 5.2 Hz, 1H), 3.41 (ddd, *J* = 14.9, 9.0, 2.2 Hz, 1H), 3.30 – 3.14 (m, 5H), 2.86 (ddt, *J* = 19.8, 14.6, 5.2 Hz, 1H), 2.78 – 2.66 (m, 5H), 2.44 (s, 3H), 2.13 (ddq, *J* = 15.3, 7.7, 4.8, 3.8 Hz, 1H), 1.69 (s, 3H), 1.61 – 1.51 (m, 4H), 1.35 (s, 8H).

^{13}C NMR (126 MHz, MeOD) δ 174.49, 172.65, 171.30, 169.80, 168.28, 167.74, 166.18, 157.03, 156.24, 152.18, 138.19, 138.08, 137.97, 134.92, 133.52, 133.23, 132.02, 131.99, 131.33, 129.76, 121.65, 119.30, 117.94, 69.36, 55.27, 50.57, 40.49, 40.13, 38.84, 32.19, 30.49, 30.34, 30.31, 30.22, 27.92, 27.82, 23.64, 14.42, 12.92, 11.60.

LCMS 841.48 (M+H).



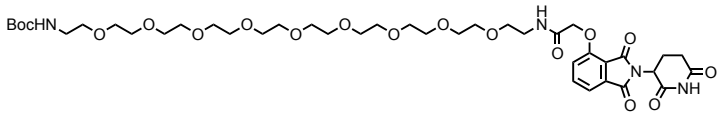
dBET23 (3)

A 0.1 M solution of *N*-(8-aminooctyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamide trifluoroacetate in DMF (220 microliters, 0.0220 mmol, 1 eq) was added to (*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-carboxylic acid (9.87 mg, 0.0220 mmol, 1 eq) at room temperature. DIPEA (11.5 microliters, 0.0660 mmol, 3 eq) and HATU (8.4 mg, 0.0220 mmol, 1 eq) were added. The mixture was then stirred for 21 hours, then diluted with EtOAc and washed with saturated sodium bicarbonate, water and brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 4 g silica column, 0-10% MeOH/DCM, 25 minute gradient) gave the desired product as a white solid (8.84 mg, 0.00998 mmol, 45%).

^{1}H NMR (400 MHz, Methanol-d_4) δ 7.81 (dd, *J* = 8.4, 7.4 Hz, 1H), 7.53 (d, *J* = 7.3 Hz, 1H), 7.50 – 7.39 (m, 5H), 5.12 (dd, *J* = 12.6, 5.4 Hz, 1H), 4.75 (s, 2H), 4.68 (t, *J* = 7.2 Hz, 1H), 3.76 (s, 3H), 3.54 (d, *J* = 7.2 Hz, 2H), 3.39 – 3.32 (m, 3H), 3.29 (s, 1H), 2.90 – 2.83 (m, 1H), 2.79 – 2.68 (m, 5H), 2.14 (dd, *J* = 8.9, 3.7 Hz, 1H), 1.99 (s, 3H), 1.65 – 1.53 (m, 4H), 1.36 (d, *J* = 6.5 Hz, 8H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 175.4, 173.7, 172.5, 169.4, 169.3, 168.2, 165.6, 163.6, 157.7, 157.3, 153.0, 139.6, 139.2, 139.1, 138.1, 137.8, 135.7, 133.0, 132.9, 132.6, 131.2, 123.0, 119.5, 118.7, 74.9, 70.3, 62.9, 56.1, 54.3, 51.5, 41.0, 38.8, 33.6, 31.6 (d, *J* = 4.0 Hz), 31.3 (d, *J* = 3.3 Hz), 29.0, 28.9, 24.6, 18.7, 14.0.

LCMS 885.47 (M+H).

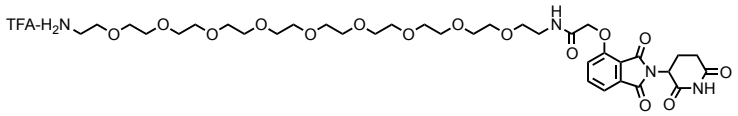


***tert*-butyl (1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-2-oxo-6,9,12,15,18,21,24,27,30-nonaoxa-3-azadotriacontan-32-yl)carbamate (22)**

tert-butyl (29-amino-3,6,9,12,15,18,21,24,27-nonaoxanonacosyl) carbamate (422.53 mg, 0.759 mmol, 1eq) as a solution in 15.18 ml DMF (0.1 M) was added to 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy) acetic acid (252.26 mg, 0.759, 1eq). DIPEA (376.45 µl, 2.277 mmol, 3eq) was added, followed by HATU (288.6 mg, 0.759 mmol, 1eq). The mixture was stirred for 17 hours at room temperature. The mixture was then diluted with EtOAc and washed with saturated sodium bicarbonate, water then brine. The organic layer was dried over sodium sulfate, filtered and condensed to give a white solid (255.8 mg, 39 % yield). The crude material was purified by column chromatography (ISCO, 12 g silica column, 0 to 10% MeOH/DCM 25 minute gradient) to give a white solid (105.3 mg, 16 % yield).

^{1}H NMR (500 MHz, Methanol-d_4) δ 7.80 (dd, *J* = 8.4, 7.3 Hz, 1H), 7.50 (dd, *J* = 7.3 Hz, 1H), 7.43 (dd, *J* = 8.5 Hz, 1H), 5.12 (dd, *J* = 12.8, 5.5 Hz, 1H), 3.61 (m, *J* = 8.2, 5.6, 2.6 Hz, 36H), 3.50 (dd, *J* = 5.6, 1.9 Hz, 4H), 3.22 (q, *J* = 5.5 Hz, 2H), 2.90 (ddd, *J* = 17.5, 13.9, 5.3 Hz, 1H), 2.80 – 2.70 (m, 2H), 2.17 (m, *J* = 13.1, 5.8, 2.8 Hz, 1H), 1.43 (s, 9H).

LCMS 871.35 (M+H)

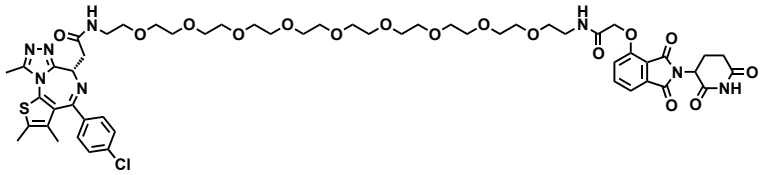


***N*-(29-amino-3,6,9,12,15,18,21,24,27-nonaoxanonacosyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamide trifluoroacetate salt (23)**

tert-butyl (1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-2-oxo-6,9,12,15,18,21,24,27,30-nonaoxa-3-azadotriacontan-32-yl)carbamate (105.3 mg, 0.121mmol, 1eq) was added to 1.21 ml TFA (0.1M) and was stirred for 2 hours at 50^{o} C. The mixture was diluted with methanol and condensed to give a white solid (104.28, 97 % yield) with no further purification.

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.11 (s, 1H), 8.00 (s, *J* = 5.8 Hz, 1H), 7.82 (dd, *J* = 7.9 Hz, 1H), 7.75 – 7.71 (s, 2H), 7.50 (dd, *J* = 7.3 Hz, 1H), 7.40 (dd, *J* = 8.6 Hz, 1H), 5.11 (dd, *J* = 12.8, 5.4 Hz, 1H), 4.79 (s, 2H), 3.91 – 3.41 (m, 36H), 3.32 (t, *J* = 5.7 Hz, 2H), 2.98 (m, *J* = 5.5 Hz, 2H), 2.90 (ddd, *J* = 18.1, 14.0, 5.3 Hz, 1H), 2.63 – 2.54 (m, 2H), 2.05 (dd, *J* = 12.3, 6.1 Hz, 1H).

LCMS 771.80 (M+H)



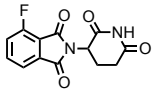
dBET55 (6)

A 0.1 M solution of *N*-(29-amino-3,6,9,12,15,18,21,24,27-nonaoxanonacosyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)acetamide trifluoroacetate in DMF (200 microliters, 0.020 mmol, 1 eq) was added to JQ-acid (8.0 mg, 0.020 mmol, 1 eq) at room temperature. DIPEA (10.5 microliters, 0.060 mmol, 3 eq) and HATU (7.6 mg, 0.020 mmol, 1 eq) were added. After 18 hours the mixture was diluted with EtOAc and washed with saturated sodium bicarbonate, water and brine. The combined organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 4 g silica column, 0-10% MeOH/DCM, 25 minute gradient) gave the desired product (10.55 mg, 0.00914 mmol, 46%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 7.82 (dd, *J* = 8.4, 7.4 Hz, 1H), 7.55 (d, *J* = 7.0 Hz, 1H), 7.49 – 7.41 (m, 5H), 5.13 (dd, *J* = 12.6, 5.5 Hz, 1H), 4.80 (s, 2H), 4.65 (dd, *J* = 9.1, 5.1 Hz, 1H), 3.68 – 3.58 (m, 36H), 3.53 – 3.44 (m, 5H), 2.94 – 2.86 (m, 1H), 2.81 – 2.70 (m, 5H), 2.46 (s, 3H), 2.19 – 2.13 (m, 1H), 1.74 – 1.69 (m, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 175.4, 172.5, 172.3, 169.5, 169.4, 168.1, 165.6, 157.8, 157.6, 152.4, 139.6, 139.4, 137.9, 135.7, 134.9, 132.8, 132.5, 132.2, 131.1, 123.0, 119.4, 118.7, 72.4 (d, *J* = 4.0 Hz), 72.3, 71.9, 71.5, 70.1, 65.7, 62.9, 56.5, 51.5, 41.3, 41.1, 40.1, 33.6, 24.6, 16.7, 15.3, 13.9.

LCMS 1153.59 (M+H).

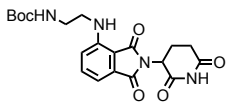


2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (24)

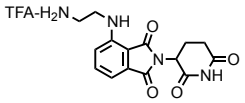
3-fluorophthalic anhydride (1.66g, 10 mmol, 1 eq) and 3-aminopiperidine-2,6-dione hydrochloride salt (1.81 g, 11 mmol, 1.1 eq) were dissolved in AcOH (25 mL) followed by potassium acetate (3.04 g, 31 mmol, 3.1 eq). The mixture was fitted with an air condenser and heated to 90 °C. After 16 hours, the mixture was diluted with 100 mL water and cooled over ice. The slurry was then centrifuged (4000 rpm, 20 minutes, 4 °C) and decanted. The remaining solid was then resuspended in water, centrifuged and decanted again. The solid was then dissolved in MeOH and filtered through a silica plug (that had been pre-wetted with MeOH), washed with 50% MeOH/DCM and concentrated under reduced pressure to yield the desired product as a grey solid (2.12 g, 7.68 mmol, 77%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.13 (s, 1H), 7.98 – 7.91 (m, 1H), 7.79 (d, *J* = 7.3 Hz, 1H), 7.74 (t, *J* = 8.8 Hz, 1H), 5.16 (dd, *J* = 12.9, 5.4 Hz, 1H), 2.89 (ddd, *J* = 17.2, 14.0, 5.5 Hz, 1H), 2.61 (ddd, *J* = 17.1, 4.4, 2.4 Hz, 1H), 2.57 – 2.50 (m, 1H), 2.06 (dtd, *J* = 13.0, 5.4, 2.3 Hz, 1H).

LCMS 277.21 (M+H).



***tert*-butyl (2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethyl)carbamate (25)**
A stirred solution of 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (174 mg, 0.630 mmol, 1 equiv) in DMF (6.3 mL, 0.1 M) was added DIPEA (220 µL, 1.26 mmol, 2 equiv) and 1-Boc-ethylendiamine (110 µL, 0.693 mmol, 1.1 equiv). The reaction mixture was heated to 90 °C overnight, whereupon it was cooled to room temperature and taken up in EtOAc (30 mL) and water (30 mL). The organic layer was washed with brine (3x20 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (0→10% MeOH in CH_2Cl_2) to give the title compound as a yellow solid (205 mg, 79%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (bs, 1H), 7.50 (dd, *J* = 8.5, 7.1 Hz, 1H), 7.12 (d, *J* = 7.1 Hz, 1H), 6.98 (d, *J* = 8.5 Hz, 1H), 6.39 (t, *J* = 6.1 Hz, 1H), 4.96 – 4.87 (m, 1H), 4.83 (bs, 1H), 3.50 – 3.41 (m, 2H), 3.41 – 3.35 (m, 2H), 2.92 – 2.66 (m, 3H), 2.16 – 2.09 (m, 1H), 1.45 (s, 9H).
LCMS 417.58 (M+H).

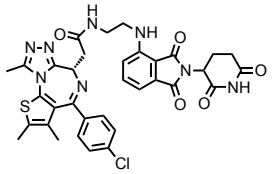


4-((2-aminoethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (26)

A stirred solution of *tert*-butyl (2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethyl)carbamate (205 mg, 0.492 mmol, 1 equiv) in dichloromethane (2.25 mL) was added trifluoroacetic acid (0.250 mL). The reaction mixture was stirred at room temperature for 4 h, whereupon the volatiles were removed *in vacuo*. The title compound was obtained as a yellow solid (226 mg, >95%), that was used without further purification.

^{1}H NMR (500 MHz, MeOD) δ 7.64 (d, *J* = 1.4 Hz, 1H), 7.27 – 7.05 (m, 2H), 5.10 (dd, *J* = 12.5, 5.5 Hz, 1H), 3.70 (t, *J* = 6.0 Hz, 2H), 3.50 – 3.42 (m, 2H), 3.22 (t, *J* = 6.0 Hz, 1H), 2.93 – 2.85 (m, 1H), 2.80 – 2.69 (m, 2H), 2.17 – 2.10 (m, 1H).

LCMS 317.53 (M+H).



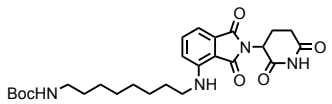
dBET57 (7)

JQ-acid (8.0 mg, 0.0200 mmol, 1 eq) and 2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)ethan-1-aminium 2,2,2-trifluoroacetate (8.6 mg, 0.0200 mmol, 1 equiv) were dissolved in DMF (0.200 mL, 0.1 M) at room temperature. DIPEA (17.4 µL, 0.100 mmol, 5 equiv) and HATU (7.59 mg, 0.0200 mmol, 1 equiv) were then added and the mixture was stirred at room temperature overnight. The reaction mixture was taken up in EtOAc (15 mL), and washed with satd. $NaHCO_3$ (aq) (15 mL), water (15 mL) and brine (3x15 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (0→10% MeOH in CH_2Cl_2, R_f = 0.3 (10% MeOH in CH_2Cl_2)) to give the title compound as a bright yellow solid (11.2 mg, 80%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (bs, 0.6H), 8.39 (bs, 0.4H), 7.51 – 7.43 (m, 1H), 7.38 (d, *J* = 7.8 Hz, 2H), 7.29 (dd, *J* = 8.8, 1.7 Hz, 2H), 7.07 (dd, *J* = 7.1, 4.9 Hz, 1H), 6.97 (dd, *J* = 8.6, 4.9 Hz, 1H), 6.48 (t, *J* = 5.9 Hz, 1H), 6.40 (t, *J* = 5.8 Hz, 0.6H), 4.91 – 4.82 (m, 0.4H), 4.65 – 4.60 (m, 1H), 3.62 – 3.38 (m, 6H), 2.87 – 2.64 (m, 3H), 2.63 (s, 3H), 2.40 (s, 6H), 2.12 – 2.04 (m, 1H), 1.67 (s, 3H), rotamers;

^{13}C NMR (126 MHz, DMSO-d_6) δ 172.8, 170.2, 170.0, 168.6, 167.3, 163.1, 155.1, 149.8, 146.3, 136.7, 136.2, 135.2, 132.22 (d, *J* = 5.9 Hz), 130.7, 130.1, 129.8, 129.5, 128.4, 117.2, 110.6, 109.3, 53.7, 48.5, 37.6, 31.0, 29.0, 22.1, 14.0, 12.7, 11.3.

LCMS 700.34 (M+H).



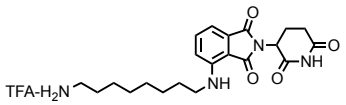
***tert*-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)octyl)carbamate (27)**

2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (552.4 mg, 2.0 mmol, 1 eq) and *tert*-butyl (8-aminooctyl)carbamate (537.6 mg, 2.2 mmol, 1.1 eq) were dissolved in NMP (10 mL). DIPEA (697 microliters, 4.0 mmol, 2 eq) was added and the mixture was heated to 90 °C. After 21 hours the mixture was cooled to room temperature and diluted with EtOAc. The organic layer was washed with 10% citric acid (aq), brine, saturated sodium bicarbonate (aq), water, and three times with brine. The organic layer was then dried over sodium sulfate, filtered and concentrated under reduced pressure. The material was purified by column chromatography (ISCO, 12 g column, 0-5% MeOH/DCM, 25 minute gradient) to give the desired product as a yellow solid (0.62 g, 1.24 mmol, 62%).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.51 (s, 1H), 7.49 – 7.44 (m, 1H), 7.06 (d, *J* = 7.1 Hz, 1H), 6.86 (d, *J* = 8.6 Hz, 1H), 6.22 (t, *J* = 5.4 Hz, 1H), 4.91 (dd, *J* = 12.2, 5.3 Hz, 1H), 4.56 (s, 1H), 3.24 (q, *J* = 6.7 Hz, 2H), 3.07 (t, *J* = 12.7 Hz, 2H), 2.89 – 2.67 (m, 3H), 2.11 (dq, *J* = 10.3, 3.6, 2.7 Hz, 1H), 1.64 (p, *J* = 7.0 Hz, 2H), 1.36 (d, *J* = 61.0 Hz, 19H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.47, 169.60, 168.68, 167.73, 156.06, 147.06, 136.15, 132.57, 116.71, 111.39, 109.91, 79.11, 48.95, 42.68, 40.66, 31.49, 30.09, 29.25, 29.20, 28.51, 26.89, 26.75, 22.89.

LCMS 501.39 (M+H).

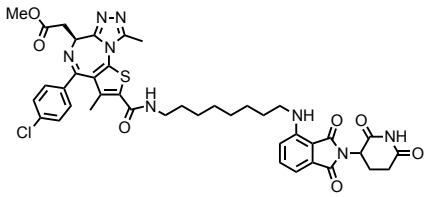


4-((8-aminooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (28)

tert-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)octyl)carbamate (0.55 g, 1.099 mmol, 1 eq) was dissolved in TFA (11 mL) and heated to 50 °C. After 40 minutes, the mixture was concentrated under reduced pressure, triturated with Et_2O, and dried under high vacuum to yield a yellow residue (523 mg, 1.016 mmol, 93%) that was used without further purification.

^{1}H NMR (500 MHz, Methanol-d_4) δ 7.59 – 7.51 (m, 1H), 7.04 (dd, *J* = 7.9, 1.7 Hz, 2H), 5.06 (dd, *J* = 12.4, 5.5 Hz, 1H), 3.34 (d, *J* = 7.0 Hz, 2H), 2.95 – 2.81 (m, 3H), 2.79 – 2.66 (m, 2H), 2.15 – 2.08 (m, 1H), 1.67 (tt, *J* = 12.2, 7.2 Hz, 4H), 1.43 (d, *J* = 22.2 Hz, 8H).

LCMS 401.39 (M+H).



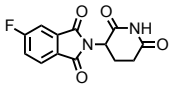
dBET70 (5)

(*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-carboxylic acid (201 mg, 0.452 mmol, 1 eq) and 4-((8-aminooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (232.5 mg, 0.452 mmol, 1 eq) were dissolved in DMF (4.5 mL). DIPEA (236 microliters, 1.355 mmol, 3 eq) and HATU (171.9 mg, 0.452 mmol, 1 eq) were added and the mixture was stirred for 18 hours at room temperature. The mixture was then diluted with EtOAc, and washed three times with 1M HCl (aq), then once with brine, saturated sodium bicarbonate, water and brine. The organic layer was then dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 24 g silica column, 0-6% MeOH/DCM, 35 minute gradient) to give the desired product as a yellow solid (224.92 mg, 0.2719 mmol, 60%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 7.54 – 7.50 (m, 1H), 7.45 (d, *J* = 8.5 Hz, 2H), 7.42 – 7.38 (m, 2H), 7.00 (dd, *J* = 7.8, 2.9 Hz, 2H), 5.00 (ddd, *J* = 12.8, 5.4, 3.1 Hz, 1H), 4.66 (t, *J* = 7.1 Hz, 1H), 3.75 (s, 3H), 3.53 (d, *J* = 7.3 Hz, 2H), 3.37 (dq, *J* = 15.7, 8.3, 7.7 Hz, 2H), 3.29 (d, *J* = 6.9 Hz, 2H), 2.85 (ddd, *J* = 18.3, 13.9, 5.1 Hz, 1H), 2.77 – 2.64 (m, 5H), 2.11 – 2.05 (m, 1H), 1.97 (s, 3H), 1.64 (dq, *J* = 20.8, 6.8 Hz, 4H), 1.41 (d, *J* = 21.1 Hz, 8H).

^{13}C NMR (126 MHz, MeOD) δ 174.60, 173.08, 171.59, 170.79, 169.25, 165.54, 163.65, 156.83, 152.44, 148.25, 138.20, 137.94, 137.86, 137.81, 137.22, 133.86, 132.42, 131.84, 131.26, 129.89, 117.96, 111.73, 110.94, 54.89, 52.47, 50.16, 43.37, 41.25, 37.13, 32.21, 30.29, 30.22, 30.17, 27.87, 27.78, 23.79, 16.57, 11.68.

LCMS 827.60 (M+H)

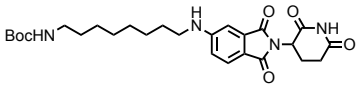


2-(2,6-dioxopiperidin-3-yl)-5-fluoroisoindoline-1,3-dione (29)

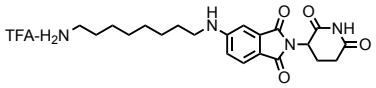
4-fluorophthalic anhydride (3.32 g, 20 mmol, 1 eq) and 3-aminopiperidine-2,6-dione hydrochloride salt (3.620 g, 22 mmol, 1.1 eq) were dissolved in AcOH (50 mL) followed by potassium acetate (6.08 g, 62 mmol, 3.1 eq). The mixture was fitted with an air condenser and heated to 90 °C. After 16 hours, the mixture was diluted with 200 mL water and cooled over ice. The slurry was then centrifuged (4000 rpm, 20 minutes, 4 °C) and decanted. The remaining solid was then resuspended in water, centrifuged and decanted again. The solid was then dissolved in MeOH and filtered through a silica plug (that had been pre-wetted with MeOH), washed with 50% MeOH/DCM and concentrated under reduced pressure to yield the desired product as a grey solid (2.1883 g, 7.92 mmol, 40%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.13 (s, 1H), 8.01 (dd, *J* = 8.3, 4.5 Hz, 1H), 7.85 (dd, *J* = 7.4, 2.2 Hz, 1H), 7.72 (ddd, *J* = 9.4, 8.4, 2.3 Hz, 1H), 5.16 (dd, *J* = 12.9, 5.4 Hz, 1H), 2.89 (ddd, *J* = 17.2, 13.9, 5.5 Hz, 1H), 2.65 – 2.51 (m, 2H), 2.07 (dtd, *J* = 12.9, 5.3, 2.2 Hz, 1H).

LCMS 277.22 (M+H).



***tert*-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)octyl)carbamate (30)**
2-(2,6-dioxopiperidin-3-yl)-5-fluoroisoindoline-1,3-dione (294 mg, 1.06 mmol, 1 eq) and *tert*-butyl (8-aminooctyl)carbamate (286 mg, 1.17 mmol, 1.1 eq) were dissolved in NMP (5.3 mL). DIPEA (369 microliters, 2.12 mmol, 2 eq) was added and the mixture was heated to 90 °C. After 19 hours the mixture was cooled to room temperature and diluted with EtOAc. The organic layer was washed with water and three times with brine. The organic layer was then dried over sodium sulfate, filtered and concentrated under reduced pressure. The material was purified by column chromatography (ISCO, 12 g column, 0-10% MeOH/DCM, 30 minute gradient) to give the desired product as a brown solid (0.3345 g, 0.6682 mmol, 63%).
^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.12 (s, 1H), 7.62 (d, *J* = 8.3 Hz, 1H), 7.02 (s, 1H), 6.81 (d, *J* = 7.2 Hz, 1H), 4.93 (dd, *J* = 12.3, 5.3 Hz, 1H), 4.51 (s, 1H), 3.21 (t, *J* = 7.2 Hz, 2H), 3.09 (d, *J* = 6.4 Hz, 2H), 2.90 (dd, *J* = 18.3, 15.3 Hz, 1H), 2.82 – 2.68 (m, 2H), 2.16 – 2.08 (m, 1H), 1.66 (p, *J* = 7.2 Hz, 2H), 1.37 (d, *J* = 62.3 Hz, 20H).
LCMS 501.41 (M+H).

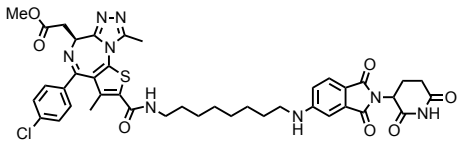


5-((8-aminooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (31)

tert-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)amino)octyl)carbamate (334.5 mg, 0.668 mmol, 1 eq) was dissolved in TFA (6.7 mL) and heated to 50 °C. After 50 minutes, the mixture was cooled to room temperature, diluted with DCM and concentrated under reduced pressure, triturated with Et_2O, and dried under high vacuum to yield a dark yellow foam (253 mg, 0.492 mmol, 74%) that was used without further purification.

^{1}H NMR (500 MHz, Methanol-d_4) δ 7.56 (d, *J* = 8.4 Hz, 1H), 6.97 (d, *J* = 2.1 Hz, 1H), 6.83 (dd, *J* = 8.4, 2.2 Hz, 1H), 5.04 (dd, *J* = 12.6, 5.5 Hz, 1H), 3.22 (t, *J* = 7.1 Hz, 2H), 2.94 – 2.88 (m, 2H), 2.85 – 2.68 (m, 3H), 2.09 (ddd, *J* = 10.4, 5.4, 3.0 Hz, 1H), 1.70 – 1.61 (m, 4H), 1.43 (d, *J* = 19.0 Hz, 8H).

LCMS 401.36 (M+H).



dBET72 (14)

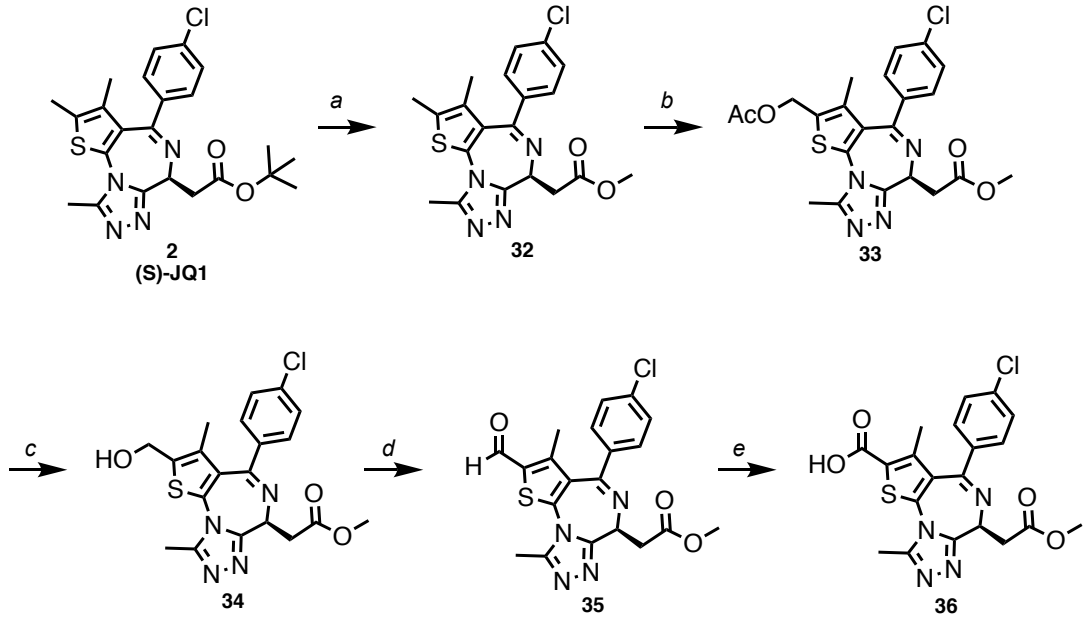
5-((8-aminooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione trifluoroacetate salt (10.3 mg, 0.020 mmol, 1 eq) in DMF (200 microliters) was added to (*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-carboxylic acid (8.9 mg, 0.020 mmol, 1 eq) at room temperature. DIPEA (10.5 microliters, 0.060 mmol, 3 eq) was added, followed by HATU (7.6 mg, 0.020 mmol, 1 eq). After 27 hours, the mixture was diluted with EtOAc then washed with saturated sodium bicarbonate, water and brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. . The mixture was purified by column chromatography (ISCO, 4 g column, 0-10% MeOH/DCM, 25 minute gradient) to give the desired product as a yellow solid (4.98 mg, 0.00602 mmol, 30%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 7.54 (d, *J* = 8.4 Hz, 1H), 7.49 – 7.40 (m, 4H), 6.96 (d, *J* = 2.1 Hz, 1H), 6.82 (dd, *J* = 8.4, 2.1 Hz, 1H), 5.02 (dd, *J* = 12.7, 5.5 Hz, 1H), 4.67 (t, *J* = 7.1 Hz, 1H), 3.76 (s, 3H), 3.54 (d, *J* = 7.2 Hz, 2H), 3.41 – 3.33 (m, 2H), 3.20 (t, *J* = 7.0 Hz, 2H), 2.85 (ddd, *J* = 19.2, 14.0, 5.3 Hz, 1H), 2.77 – 2.65 (m, 5H), 2.11 – 2.04 (m, 1H), 1.99 (s, 3H), 1.64 (dt, *J* = 19.3, 7.1 Hz, 4H), 1.43 (d, *J* = 21.8 Hz, 8H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 175.5, 173.7, 172.8, 170.4, 169.8, 165.6, 163.6, 157.3, 157.1, 153.0, 139.2, 139.1, 138.1, 137.8, 136.8, 133.1, 132.9, 132.6, 131.2, 127.7, 118.4, 75.1, 72.4, 65.7, 56.1, 54.3, 51.3, 45.1, 38.8, 33.6, 30.9, 29.1, 29.0, 24.9, 18.8, 14.0.

LCMS 827.46 (M+H).

(*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-carboxylic acid (**36**) was synthesized at ChemPartner from (S)-JQ1 using the following procedures:



a) MeOH, H_2SO_4; b) $Mn(OAc)_3 \cdot 2H_2O$, Ac_2O, H_2SO_4(s), AcOH; c) K_2CO_3, MeOH; d) Dess-Martin, CH_2Cl_2; e) $NaClO_2$, H_2O_2, NaH_2PO_4, CH_3CN

Compound **32**

(*S*)-JQ1 (4.57 g, 10 mmol) was dissolved in MeOH (0.25 M). conc.H_2SO_4 (50 drops) was added to the solution. The mixture was refluxed overnight. The mixture was concentrated *in vacuo*, poured into water, extracted with AcOEt, and washed with brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (AcOEt/MeOH) to give title compound 3.93 g (95 %).

Compound **33**

To a mixture of acetic acid (52 mL) and acetic anhydride (30 mL) was added dropwise concentrated sulfuric acid (8 mL). Compound **33** (6.04 g, 14.6 mmol) was added, and manganese acetate (III)*dihydrate (8 g, 29.4 mmol) was further added. The mixture was stirred at room temperature for 3 days. The reaction mixture was poured into ice water, and extracted twice with ethyl acetate (300 mL). The organic layer was washed twice with saturated brine (300 mL). The residue was dried over Na_2SO_4, and the solvent was evaporated to give an oil (5 g), which was used without further purification.

Compound **34**

Compound **33** (6.0 g, 12.7 mmol) and K_2CO_3 (1.2 eq) were suspended in MeOH (0.1 M). The mixture was stirred at room temperature for 2 hours. The mixture was neutralized with 1N HCl, then concentrated *in vacuo*. The residue was poured into water, and extracted with DCM. The organic layer was dried over Na_2SO_4, filtrated, and concentrated *in vacuo*. The residue was purified with flash column chromatography (AcOEt/MeOH) to give title compound 2 g (32 % over 2 steps).

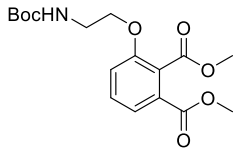
Compound **35**

Compound **34** (867 mg, 2.01 mmol) was dissolved in DCM (20 mL). Dess-Martin periodinane (1.2 eq) was added to the solution at 0 ^{o}C. The mixture was stirred at room temperature for 2 hours. The mixture was diluted with DCM, washed with saturated $NaHCO_3$ solution, dried over Na_2SO_4, and concentrated *in vacuo* to give an oil (850 mg). The crude product was used directly without further purification.

Compound **36**

Compound **35** (850 mg, 1.98 mmoL) was suspended in CH_3CN (8 mL). Sodium phosphate monobasic (0.97 eq) in H_2O (3 mL) solution was added to the suspension. Hydrogen peroxide (5 eq) was added to the solution dropwise. Sodium chlorite (1.4 eq) in H_2O (2 mL) solution was added to the suspension. The mixture was stirred for 3 hours. The mixture was diluted with EtOAc, quenched with $Na_2S_2O_3$ aq, then, acidified with 1N HCl (pH<4). The mixture was extracted with EtOAc, washed with brine. The organic layer was dried over Na_2SO4, filtered, and concentrated *in vacuo*. The residue was purified by prep-HPLC to give compound **5** (667 mg, 75 %).

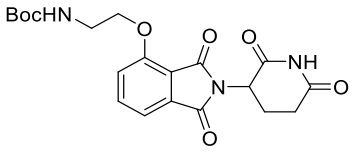
^{1}H NMR (400 MHz, Methanol-d_4) δ 7.44 (q, *J* = 8.8 Hz, 4H), 4.68 (t, *J* = 7.2 Hz, 1H), 3.76 (s, 3H), 3.54 (d, *J* = 7.2 Hz, 2H), 2.74 (s, 3H), 2.09 (s, 3H).



dimethyl 3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)phthalate (37)

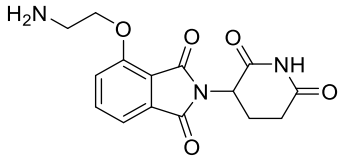
tert-butyl (2-bromoethyl)carbamate (280 mg, 1.25 mmol, 1 eq) and dimethyl 3-hydroxyphthalate (263 mg, 1.25 mmol, 1 eq) were dissolved in DMF (6.25 mL, 0.2 M) followed by potassium carbonate (345 mg, 2.5 mmol, 2 eq). The mixture was stirred at 50°C. After the reaction completed, the mixture was cooled down to room temperature, diluted with EtOAc and washed once with water then twice with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 12 g silica column, 0-40% EtOAc/hexane) gave the desired product as a white solid (268 mg, 61%).

LCMS 354 (M+H).



***tert*-butyl (2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)ethyl)carbamate (38)**
dimethyl 3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)phthalate (268 mg, 0.76 mmol, 1 eq) was dissolved in EtOH (3.8 mL, 0.2 M) followed by aqueous 3M NaOH (760 µL, 2.28 mmol, 3 eq), then the mixture was heated to 80°C for 4 hours. The mixture was then cooled down to room temperature, diluted with 14 ml DCM and 5.5 ml 0.5M HCl. The layers were separated and the organic layer was washed with 7 mL water. The aqueous layers were combined and extracted three times with 14 mL chloroform. The combined organic layers were dried over sodium sulfate, filtered and condensed to give the material that was carried forward without further purification. **LCMS** 326.

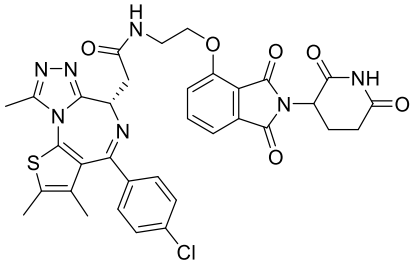
The resultant material and 3-aminopiperidine-2,6-dione hydrochloride (125 mg, 0.76 mmol, 1 eq) were dissolved in pyridine (3.8 mL, 0.2 M) and heated to 110°C overnight. Then the mixture was cooled to room temperature and concentrated under reduced pressure, purified by column chromatography (ISCO, 12 g silica column, 0-6% MeOH/DCM) to give the desired product (152 mg, 48% for two steps).
LCMS 417.



4-(2-aminoethoxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (39)

tert-butyl (2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)ethyl)carbamate (152 mg, 0.37 mmol) was dissolved in TFA (3.7 mL, 0.1 M) and heated to 50 °C for 3 hours. The mixture was cooled to room temperature, diluted with Methanol and concentrated under reduced pressure. The material was purified by column chromatography (ISCO, 4 g silica column, 0-20% 1.75N NH_3•MeOH/DCM) to give the free base product (101 mg, 86%).

LCMS 317 (M+H).



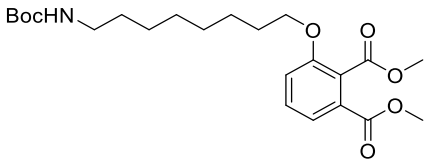
ZXH-2-42 (16)

(*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 (24 mg, 0.06 mmol, 1 eq) was dissolved in 1 mL DMF followed by 4-(2-aminoethoxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (19 mg, 0.06 mmol, 1 eq), and then DIEA (30 µL, 0.18 mmol, 3 eq), HATU (27 mg, 0.072 mmol, 1.2 eq) were added. The mixture was stirred at room temperature overnight and then purified by HPLC to give the product as TFA salt (3.8 mg, 8%).

1H NMR (500 MHz, DMSO-d_6) δ 11.10 (s, 1H), 8.51 (s, 1H), 7.83 (t, *J* = 7.8 Hz, 1H), 7.59 (d, *J* = 8.6 Hz, 1H), 7.51 – 7.48 (m, 1H), 7.45 – 7.39 (m, 4H), 5.09 (s, 1H), 4.57 – 4.49 (m, 1H), 4.30 (t, *J* = 5.8 Hz, 2H), 3.35 (s, 3H), 3.18 – 3.13 (m, 2H), 2.86 (s, 1H), 2.60 (s, 3H), 2.42 (s, 2H), 1.62 (s, 3H), 1.27 (s, 2H).

^{13}C NMR (201 MHz, DMSO-d_6) δ 173.3, 170.7, 170.4, 167.3, 165.7, 163.5, 155.5, 150.3, 137.6, 135.6, 132.7, 131.2, 130.6, 130.0, 128.9, 120.7, 120.0, 116.1, 115.5, 54.2, 42.3, 37.9, 31.4, 22.5, 18.5, 17.2, 14.5, 12.9, 11.8.

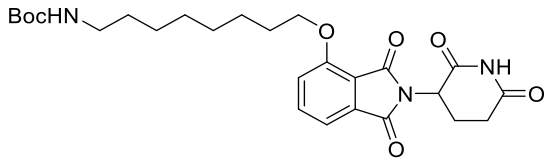
LCMS 700 (M+H).



dimethyl 3-((8-((*tert*-butoxycarbonyl)amino)octyl)oxy)phthalate (40)

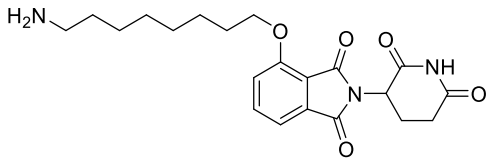
tert-butyl (8-bromooctyl)carbamate (308 mg, 1 mmol, 1 eq) and dimethyl 3-hydroxyphthalate (210 mg, 1 mmol, 1 eq) were dissolved in DMF (5 mL, 0.2 M) followed by potassium carbonate (276 mg, 2 mmol, 2 eq). The mixture was stirred at 50°C. After the reaction completed, the mixture was cooled down to room temperature, diluted with EtOAc and washed once with water then twice with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 12 g silica column, 0-25% EtOAc/hexane) gave the desired product as a white solid (315 mg, 72%).

LCMS 438 (M+H).



***tert*-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)octyl)carbamate (41)**
dimethyl 3-((8-((tert-butoxycarbonyl)amino)octyl)oxy)phthalate (315 mg, 0.72 mmol, 1 eq) was dissolved in EtOH (3.6 mL, 0.2 M) followed by aqueous 3M NaOH (720 µL, 2.16 mmol, 3 eq), then the mixture was heated to 80°C for 4 hours. The mixture was then cooled down to room temperature, diluted with 13 mL DCM and 5 mL 0.5M HCl. The layers were separated and the organic layer was washed with 6.5 mL water. The aqueous layers were combined and extracted three times with 13 mL chloroform. The combined organic layers were dried over sodium sulfate, filtered and condensed to give the material that was carried forward without further purification. **LCMS** 410.

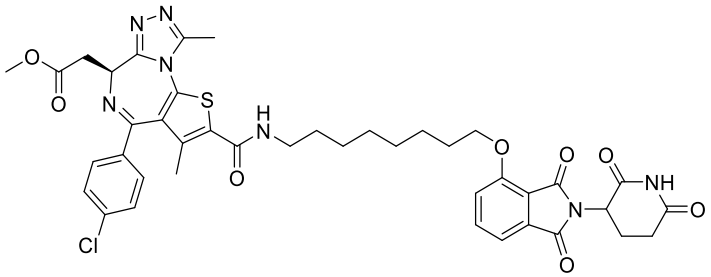
The resultant material and 3-aminopiperidine-2,6-dione hydrochloride (118 mg, 0.72 mmol, 1 eq) were dissolved in pyridine (3.6 mL, 0.2 M) and heated to 110°C overnight. Then the mixture was cooled to room temperature and concentrated under reduced pressure, purified by column chromatography (ISCO, 12 g silica column, 0-5% MeOH/DCM) to give the desired product (217 mg, 54% for two steps). **LCMS** 502.



4-((8-aminooctyl)oxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (42)

tert-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)octyl)carbamate (217 mg, 0.43 mmol) was dissolved in TFA (4.3 mL, 0.1 M) and heated to 50°C for 3 hours. The mixture was cooled to room temperature, diluted with MeOH and concentrated under reduced pressure. The material was purified by column chromatography (ISCO, 4 g silica column, 0-20% 1.75N NH_3•MeOH/DCM) to give the free base product (152 mg, 88%).

LCMS 402 (M+H).



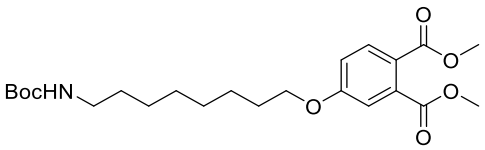
ZXH-2-43 (15)

(*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine-2-carboxylic acid (20 mg, 0.045 mmol, 1 eq) was dissolved in 1 mL DMF followed by 4-((8-aminooctyl)oxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (19 mg, 0.045 mmol, 1 eq), and then DIEA (23 µL, 0.14 mmol, 3 eq), HATU (21 mg, 0.05 mmol, 1.2 eq) were added. The mixture was stirred at room temperature overnight and then purified by HPLC, then by column chromatography (ISCO, 4 g silica column, 0-8% 1.75 N NH_3 in Methanol/DCM) to give the free base product (22.1 mg, 59%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.10 (s, 1H), 8.30 (t, *J* = 5.7 Hz, 1H), 7.80 (dd, *J* = 8.5, 7.2 Hz, 1H), 7.51 (d, *J* = 2.6 Hz, 1H), 7.50 (d, *J* = 2.7 Hz, 2H), 7.48 – 7.43 (m, 3H), 5.08 (dd, *J* = 12.8, 5.5 Hz, 1H), 4.58 (dd, *J* = 7.7, 6.6 Hz, 1H), 4.20 (t, *J* = 6.4 Hz, 2H), 3.68 (s, 3H), 3.47 (qd, *J* = 16.6, 7.2 Hz, 2H), 3.34 (s, 1H), 3.29 – 3.20 (m, 2H), 2.89 (ddd, *J* = 16.9, 13.9, 5.4 Hz, 1H), 2.65 (s, 3H), 2.06 – 1.99 (m, 1H), 1.91 (s, 3H), 1.76 (p, *J* = 6.6 Hz, 2H), 1.50 (dt, *J* = 33.3, 7.3 Hz, 4H), 1.33 (s, 6H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 175.4, 173.7, 172.6, 169.5, 168.0, 165.6, 163.6, 158.7, 157.3, 153.0, 139.7, 139.2, 139.1, 138.1, 137.8, 135.9, 133.0, 132.9, 132.6, 131.2, 122.4, 118.9, 117.8, 71.4, 56.1, 54.3, 51.4, 38.8, 33.6, 31.6, 31.3, 31.2, 31.0, 29.0, 27.9, 24.6, 18.7, 14.0.

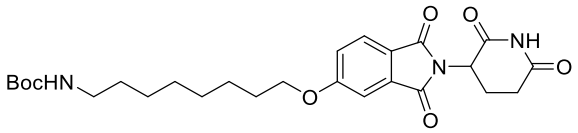
LCMS 828 (M+H).



dimethyl 4-((8-((*tert*-butoxycarbonyl)amino)octyl)oxy)phthalate (43)

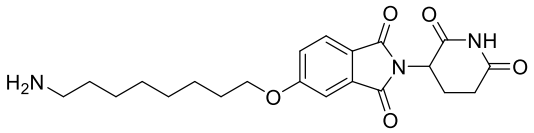
tert-butyl (8-bromoethyl)carbamate (182 mg, 0.87 mmol, 1 eq) and dimethyl 4-hydroxyphthalate (267 mg, 0.87 mmol, 1 eq) were dissolved in DMF (4.4 mL, 0.2 M) followed by potassium carbonate (239 mg, 1.73 mmol, 2 eq). The mixture was stirred at 50°C. After the reaction completed, the mixture was cooled down to room temperature, diluted with EtOAc and washed once with water then twice with brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (ISCO, 12 g silica column, 0-30% EtOAc/hexane) gave the desired product as a white solid (296 mg, 78%).

LCMS 438 (M+H).



***tert*-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)oxy)octyl)carbamate (44)**
dimethyl 4-((8-((tert-butoxycarbonyl)amino)octyl)oxy)phthalate (296 mg, 0.68 mmol, 1 eq) was dissolved in EtOH (3.4 mL, 0.2 M) followed by aqueous 3M NaOH (680 µL, 2.04 mmol, 3 eq), then the mixture was heated to 80°C for 4 hours. The mixture was then cooled down to room temperature, diluted with 12 mL DCM and 4.7 mL 0.5 M HCl. The layers were separated and the organic layer was washed with 6.2 mL water. The aqueous layers were combined and extracted three times with 12 mL chloroform. The combined organic layers were dried over sodium sulfate, filtered and condensed to give the material that was carried forward without further purification. **LCMS** 410.

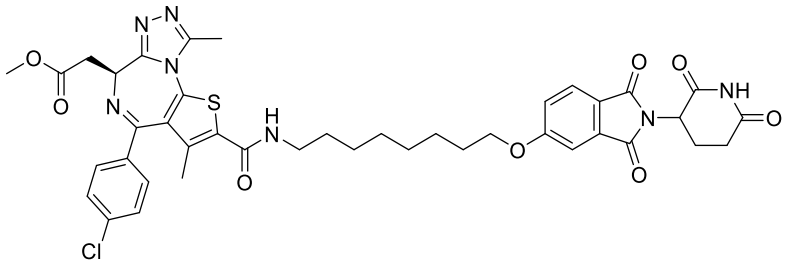
The resultant material and 3-aminopiperidine-2,6-dione hydrochloride (112 mg, 0.68 mmol, 1 eq) were dissolved in pyridine (3.4 mL, 0.2 M) and heated to 110°C overnight. Then the mixture was cooled to room temperature and concentrated under reduced pressure, purified by column chromatography (ISCO, 12 g silica column, 0-7% Methanol/DCM) to give the desired product (170 mg, 50% for two steps). **LCMS** 502.



5-((8-aminooctyl)oxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (45)

tert-butyl (8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)oxy)octyl)carbamate (170 mg, 0.34 mmol, 1 eq) was dissolved in TFA (3.4 mL, 0.1 M) and heated to 50 °C for 3 hours. The mixture was cooled to room temperature, diluted with MeOH and concentrated under reduced pressure. The material was purified by column chromatography (ISCO, 4 g silica column, 0-20% 1.75N NH3•MeOH/DCM) to give the free base product (111 mg, 82%).

LCMS 402 (M+H).



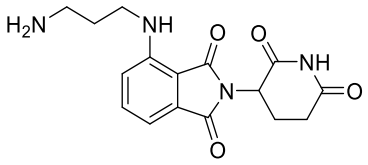
ZXH-2-45 (13)

(*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine-2-carboxylic acid (24 mg, 0.054 mmol, 1 eq) was dissolved in 1 mL DMF followed by 5-((8-aminooctyl)oxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (22 mg, 0.054 mmol, 1 eq), and then DIEA (27 µL, 0.16 mmol, 3 eq), HATU (25 mg, 0.065 mmol, 1.2 eq) were added. The mixture was stirred at room temperature overnight and then purified by HPLC to give the product as TFA salt (18.3 mg, 36%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.11 (s, 1H), 8.33 (t, *J* = 5.7 Hz, 1H), 7.82 (d, *J* = 8.3 Hz, 1H), 7.50 (d, *J* = 8.8 Hz, 2H), 7.46 (d, *J* = 8.6 Hz, 2H), 7.41 (d, *J* = 2.2 Hz, 1H), 7.34 (dd, *J* = 8.4, 2.3 Hz, 1H), 5.12 (dd, *J* = 12.8, 5.4 Hz, 1H), 4.58 (dd, *J* = 7.7, 6.6 Hz, 1H), 4.17 (t, *J* = 6.5 Hz, 2H), 3.68 (s, 3H), 3.47 (qd, *J* = 16.6, 7.3 Hz, 2H), 3.25 (dq, *J* = 17.2, 6.7 Hz, 2H), 3.17 (s, 1H), 2.90 (ddd, *J* = 16.8, 13.8, 5.4 Hz, 1H), 2.65 (s, 3H), 2.09 – 2.01 (m, 1H), 1.91 (s, 3H), 1.75 (p, *J* = 6.8 Hz, 2H), 1.53 (t, *J* = 6.9 Hz, 2H), 1.42 (q, *J* = 7.0 Hz, 2H), 1.33 (d, *J* = 3.8 Hz, 6H).

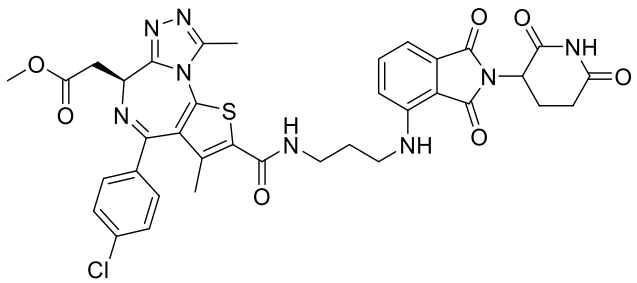
^{13}C NMR (126 MHz, DMSO-d_6) δ 172.8, 171.0, 169.9, 166.8 (d, *J* = 9.5 Hz), 164.1, 163.0, 160.9, 157.9, 157.7, 154.6, 150.3, 136.5 (d, *J* = 7.1 Hz), 135.4, 135.1, 133.9, 130.4, 130.2, 130.0, 128.5, 125.3, 122.8, 120.7, 118.5, 116.1, 108.8, 68.8, 53.4, 51.6, 48.9, 36.2, 30.9, 28.9, 28.6, 28.3, 26.3, 25.3, 22.1, 16.1, 11.3.

LCMS 828 (M+H).



4-((3-aminopropyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (46)
A stirred solution of 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (277 mg, 1 mmol, 1 eq) in DMF (5 mL) was added DIPEA (330 μL, 2 mmol, 2 equiv) and *tert*-butyl (3-aminopropyl)carbamate (191 mg, 1.1 mmol, 1.1 equiv). The reaction mixture was heated to 90 ℃ overnight. Cooled to room temperature, the mixture was diluted with EtOAc and washed once with water then twice with brine, dried over Na_2SO_4 and concentrated in *vacuo* and then carried forward without further purification. **LCMS** 431 (M+H).

The resultant material (1 mmol, 1 eq) was dissolved in DCM (6 mL) and then added TFA (2 mL), stirred at room temperature until the reaction completed. And then the mixture was concentrated under reduced pressure, purified by column chromatography (ISCO, 12 g silica column, 0-15% 1.75N NH_3•MeOH/DCM) to give the free base product (236 mg, 72% for 2 steps). **LCMS** 331 (M+H).



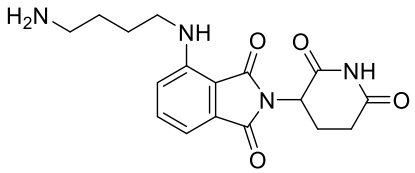
ZXH-2-147 (9)

(*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-carboxylic acid (25 mg, 0.056 mmol, 1 eq) was dissolved in DMF (1 mL), followed by 4-((3-aminopropyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (19 mg, 0.056 mmol, 1 eq), and then HATU (25 mg, 0.067 mmol, 1.2 eq), DIPEA (28 μL, 0.168 mmol, 3 eq) were added. The mixture was stirred at room temperature overnight and then purified by HPLC to give the product as TFA salt (3.5 mg, 8%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.09 (s, 1H), 8.43 – 8.36 (m, 1H), 7.61 – 7.44 (m, 5H), 7.17 – 7.11 (m, 1H), 7.06 (dd, *J* = 20.6, 7.8 Hz, 1H), 6.73 (d, *J* = 19.8 Hz, 1H), 5.06 (dd, *J* = 12.7, 5.5 Hz, 1H), 4.58 (ddd, *J* = 8.0, 6.6, 1.3 Hz, 1H), 4.53 – 4.48 (m, 1H), 3.68 (s, 3H), 3.50 – 3.44 (m, 3H), 3.34 (d, *J* = 11.6 Hz, 2H), 2.93 – 2.84 (m, 1H), 2.66 (s, 3H), 2.59 (d, *J* = 19.8 Hz, 1H), 2.42 – 2.36 (m, 1H), 2.31 – 2.25 (m, 2H), 2.03 (d, *J* = 7.0 Hz, 1H), 1.93 (s, 3H), 1.82 (p, *J* = 6.8 Hz, 2H).

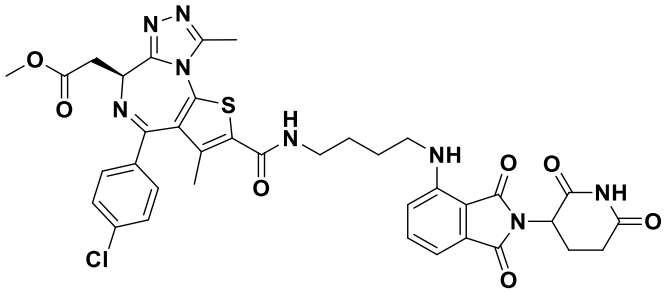
^{13}C NMR (126 MHz, DMSO-d_6) δ 175.5, 175.2, 173.7, 172.7, 172.0, 170.4, 170.0, 165.6, 163.9, 157.3, 153.0, 148.9, 148.7, 139.2 (d, *J* = 4.6 Hz), 138.1, 135.3, 132.9, 131.2, 119.8, 119.2, 113.1, 112.8, 112.6, 111.8, 56.1, 54.3, 53.9, 39.7, 38.8, 33.6, 33.2, 24.8, 18.8, 14.0.

LCMS 757 (M+H).



4-((4-aminobutyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (47)
A stirred solution of 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (277 mg, 1 mmol, 1 eq) in DMF (5 mL) was added DIPEA (330 μL, 2 mmol, 2 equiv) and *tert*-butyl (4-aminopropyl)carbamate (207 mg, 1.1 mmol, 1.1 equiv). The reaction mixture was heated to 90 ℃ overnight. Cooled to room temperature, the mixture was diluted with EtOAc and washed once with water then twice with brine, dried over Na_2SO_4 and concentrated in *vacuo* and then carried forward without further purification. **LCMS** 445 (M+H).

The resultant material (1 mmol, 1 eq) was dissolved in DCM (6 mL) and then added TFA (2 mL), stirred at room temperature until the reaction completed. And then the mixture was concentrated under reduced pressure, purified by column chromatography (ISCO, 12 g silica column, 0-20% 1.75N NH_3•MeOH/DCM) to give the free base product (224 mg, 65% for 2 steps). **LCMS** 345 (M+H).



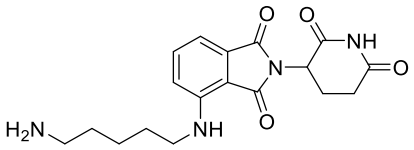
ZXH-2-184 (11)

(*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-carboxylic acid (37 mg, 0.08 mmol, 1 eq) was dissolved in DMF (1 mL), followed by 4-((4-aminobutyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (28 mg, 0.08 mmol, 1 eq), and then HATU (37 mg, 0.1 mmol, 1.2 eq), DIPEA (40 μL, 0.24 mmol, 3 eq) were added. The mixture was stirred at room temperature overnight and then purified by HPLC to give the product as TFA salt (4.8 mg, 7%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.09 (s, 1H), 8.37 (t, *J* = 5.7 Hz, 1H), 7.56 (dd, *J* = 8.6, 7.1 Hz, 1H), 7.51 (d, *J* = 8.7 Hz, 2H), 7.46 (d, *J* = 8.6 Hz, 2H), 7.12 (d, *J* = 8.6 Hz, 1H), 7.01 (d, *J* = 7.0 Hz, 1H), 6.59 (t, *J* = 6.1 Hz, 1H), 5.05 (dd, *J* = 12.8, 5.5 Hz, 1H), 4.58 (dd, *J* = 7.7, 6.6 Hz, 1H), 3.68 (s, 3H), 3.47 (qd, *J* = 16.5, 7.2 Hz, 2H), 3.31 (d, *J* = 5.6 Hz, 2H), 2.89 (ddd, *J* = 17.0, 13.8, 5.4 Hz, 1H), 2.64 (s, 3H), 2.59 (ddd, *J* = 17.0, 4.4, 2.4 Hz, 1H), 2.03 (ddd, *J* = 10.0, 6.2, 2.2 Hz, 1H), 1.90 (s, 3H), 1.61 (q, *J* = 2.8, 2.4 Hz, 4H), 1.24 (s, 2H).

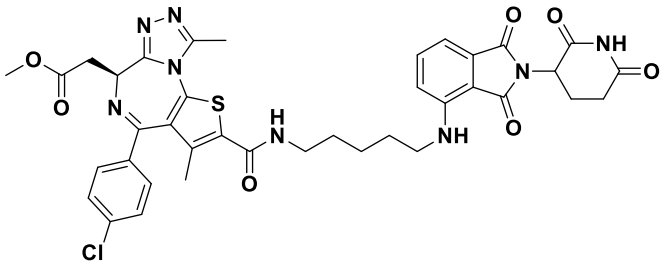
^{13}C NMR (126 MHz, DMSO-d_6) δ 175.5, 173.7, 172.7, 171.5, 169.9, 165.6, 163.7, 157.3, 153.0, 149.0, 139.2 (d, *J* = 2.5 Hz), 138.9, 138.1, 137.9, 134.8, 132.9, 132.6, 131.2, 119.9, 113.0, 111.7, 56.1, 54.3, 51.2, 44.1, 38.8, 33.6, 29.1, 28.8, 24.8, 18.7, 14.0.

LCMS 771 (M+H).



4-((5-aminopentyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (48)
A stirred solution of 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (219 mg, 0.8 mmol, 1 eq) in DMF (4 mL) was added DIPEA (264 μL, 1.6 mmol, 2 equiv) and *tert*-butyl (5-aminopropyl)carbamate (177 mg, 0.88 mmol, 1.1 equiv). The reaction mixture was heated to 90 ℃ overnight. Cooled to room temperature, the mixture was diluted with EtOAc and washed once with water then twice with brine, dried over Na_2SO_4 and concentrated in *vacuo* and then carried forward without further purification. **LCMS** 458 (M+H).

The resultant material (0.8 mmol, 1 eq) was dissolved in DCM (6 mL) and then added TFA (2 mL), stirred at room temperature until the reaction completed. And then the mixture was concentrated under reduced pressure, purified by column chromatography (ISCO, 12 g silica column, 0-20% 1.75N NH_3•MeOH/DCM) to give the free base product (194 mg, 68% for 2 steps). **LCMS** 359 (M+H).



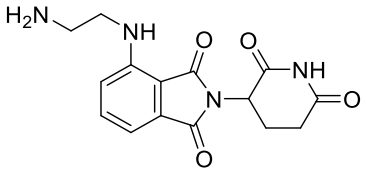
ZXH-3-26 (10)

(*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-carboxylic acid (62 mg, 0.14 mmol, 1 eq) was dissolved in DMF (1 mL), followed by 4-((5-aminopentyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (50 mg, 0.14 mmol, 1 eq), and then HATU (64 mg, 0.168 mmol, 1.2 eq), DIPEA (70 μL, 0.42 mmol, 3 eq) were added. The mixture was stirred at room temperature overnight and then purified by HPLC to give the product as TFA salt (18.4 mg, 15%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.09 (s, 1H), 8.32 (t, *J* = 5.7 Hz, 1H), 7.57 (dd, *J* = 8.6, 7.1 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 2H), 7.46 (d, *J* = 8.6 Hz, 2H), 7.10 (d, *J* = 8.6 Hz, 1H), 7.02 (d, *J* = 7.0 Hz, 1H), 6.55 (t, *J* = 6.0 Hz, 1H), 5.04 (ddd, *J* = 12.8, 5.5, 1.1 Hz, 1H), 4.58 (dd, *J* = 7.7, 6.6 Hz, 1H), 3.68 (s, 3H), 3.47 (qd, *J* = 16.6, 7.3 Hz, 2H), 3.31 – 3.23 (m, 4H), 2.89 (ddd, *J* = 16.9, 13.8, 5.5 Hz, 1H), 2.65 (s, 3H), 2.59 (ddd, *J* = 17.0, 4.4, 2.5 Hz, 1H), 2.05 – 1.99 (m, 1H), 1.90 (s, 3H), 1.60 (dp, *J* = 21.6, 7.2 Hz, 4H), 1.40 (h, *J* = 7.4, 6.5 Hz, 2H).

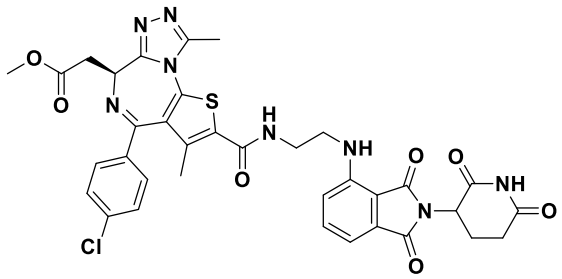
^{13}C NMR (126 MHz, DMSO-d_6) δ 172.8, 171.0, 170.1, 168.9, 167.3, 163.0, 161.0, 154.7, 150.3, 146.4, 136.5 (d, *J* = 5.6 Hz), 136.2, 135.4, 135.2, 132.2, 130.3, 130.2, 130.0, 128.5, 117.2, 110.4, 109.0, 53.4, 51.6, 48.6, 48.5, 41.7, 36.2, 31.0, 28.7, 28.4, 23.7, 22.1, 16.1, 11.3.

LCMS 785 (M+H).



4-((2-aminoethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (49)
A stirred solution of 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (180 mg, 0.65 mmol, 1 eq) in DMF (4 mL) was added DIPEA (214 μL, 1.3 mmol, 2 equiv) and *tert*-butyl (2-aminopropyl)carbamate (114 mg, 0.72 mmol, 1.1 equiv). The reaction mixture was heated to 90 ℃ overnight. Cooled to room temperature, the mixture was diluted with EtOAc and washed once with water then twice with brine, dried over Na_2SO_4 and concentrated in *vacuo* and then carried forward without further purification. **LCMS** 417 (M+H).

The resultant material (0.65 mmol, 1 eq) was dissolved in DCM (6 mL) and then added TFA (2 mL), stirred at room temperature until the reaction completed. And then the mixture was concentrated under reduced pressure, purified by column chromatography (ISCO, 12 g silica column, 0-15% 1.75N NH_3•MeOH/DCM) to give the free base product (100 mg, 49% for 2 steps). **LCMS** 317 (M+H).



ZXH-3-27 (12)

(*S*)-4-(4-chlorophenyl)-6-(2-methoxy-2-oxoethyl)-3,9-dimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-2-carboxylic acid (44 mg, 0.1 mmol, 1 eq) was dissolved in DMF (1 mL), followed by 4-((2-aminoethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (31 mg, 0.1 mmol, 1 eq), and then HATU (46 mg, 0.12 mmol, 1.2 eq), DIPEA (50 μL, 0.3 mmol, 3 eq) were added. The mixture was stirred at room temperature overnight and then purified by HPLC to give the product as TFA salt (6.6 mg, 8%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.09 (s, 1H), 8.49 (dt, *J* = 5.7, 2.9 Hz, 1H), 7.61 (ddd, *J* = 8.6, 7.0, 1.6 Hz, 1H), 7.54 – 7.48 (m, 2H), 7.45 (d, *J* = 8.4 Hz, 2H), 7.24 (d, *J* = 8.6 Hz, 1H), 7.04 (d, *J* = 7.1 Hz, 1H), 6.83 (t, *J* = 6.0 Hz, 1H), 5.05 (ddd, *J* = 12.8, 5.5, 1.4 Hz, 1H), 4.58 (ddd, *J* = 7.6, 6.6, 0.9 Hz, 1H), 3.68 (s, 3H), 3.57 – 3.42 (m, 6H), 2.89 (ddd, *J* = 17.8, 13.9, 5.4 Hz, 1H), 2.64 (d, *J* = 1.1 Hz, 3H), 2.59 (ddd, *J* = 15.0, 4.7, 2.4 Hz, 1H), 2.01 (dtd, *J* = 12.5, 5.2, 2.2 Hz, 1H), 1.90 (d, *J* = 2.6 Hz, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 172.8, 171.0, 170.0, 168.7, 167.3, 162.9, 161.6, 154.6, 150.3, 146.3, 136.7, 136.5, 136.2, 135.8, 135.4, 132.2, 130.2, 130.0, 129.7, 128.5, 117.2, 110.6, 109.3, 53.4, 51.6, 48.5, 41.1, 36.2, 31.0, 22.1, 16.1, 11.3.

LCMS 743 (M+H).