# Electronic Supplementary Information

# Structural rationalization of GSPT1 and IKZF1 degradation by thalidomide molecular glue derivatives

Radosław P. Nowak, [a,b] Jianwei Che,[a,b] Silas Ferrao,[a] Nikki R. Kong,[a,b] Hu Liu, [a,b] Breanna L. Zerfas[a,b] and Lyn H. Jones[a,b]*

[a.] *Center for Protein Degradation, Dana-Farber Cancer Institute, Boston MA, USA*
[b.] *Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston MA, USA*
**Corresponding author: lyn_jones@dfci.harvard.edu*

Contents

Compound toolkit

Thalidomide, 4-OH-Thal, 5-OH-Thal, Pomalidomide, 5-$NH_2$-Thal, EM12, 4-OH-EM12, 5-OH-EM12, 6-OH-EM12, Lenalidomide, 5-$NH_2$-EM12, 6-$NH_2$-EM12 and 7-$NH_2$-EM12 are commercially available (Sigma Aldrich). 7-OH-EM12 was prepared using a previously reported procedure.[1] All compounds should be treated as teratogens.

Cellular CRBN NanoBRET engagement assay[2]

HEK293T cells were transduced with lentivirus and put under puromycin selection (5 μg/mL) for two weeks to produce a cell line stably expressing CRBN with N-terminally fused NanoLuc luciferase (NanoLuc-CRBN). After antibiotic selection, cells were cultured in DMEM (Gibco, Life Technologies) supplemented with 10% FBS and 1 μg/mL puromycin to maintain stable NanoLuc-CRBN expression. To run the assay, cells were cultured to confluency in 10 $cm^2$ tissue culture treated plates (Corning, 430165), washed with PBS and trypsinized at room temperature to detach from the cell culture plate. After 3-4 min, the trypsin was quenched with 5x volume DMEM media (Gibco, Life Technologies) with 10% FBS and cells were collected by centrifugation (1000 rpm, 5 min). The supernatant was removed by vacuum aspiration and the pellet was then resuspended in Opti-MEM without phenol red. The density of this cell suspension was determined by diluting the cells 1:1 with trypan blue and counting using a Countess II (Thermo Fischer Scientific). The required volume of the cell suspension was prepared at 2 x $10^5$ viable cells/mL in Opti-MEM I (Gibco, Life Technologies). To this suspension was added the CRBN engagement tracer (stock at 10 μM in 31.25% PEG-400, 12.5 mM HEPES, pH 7.5, filtered using a 0.22 μm nitrocellulose membrane; final concentration in cell suspension for assay at 250 nM). Cells were then plated in a white/opaque cell culture treated 384-well plate (Corning, 3570) at volume of 50 μL/well. After plating, the assay plate was centrifuged (500 x g, 5 min) and covered in aluminum foil. Compounds for testing were added to the plate using a D300e Digital Dispenser (HP) in duplicate 12-pt titrations from a 10 mM stock in DMSO, with DMSO normalized to 1% total volume. The plate was then placed in an incubator at 37 °C, 5% $CO_2$ for two hours. After incubation, the plate was removed and set on the bench to cool to room temperature (~10-15 min). The NanoLuc substrate (500X solution) and extracellular inhibitor (1500X solution) were diluted in Opti-MEM I (Gibco, Life Technologies) to prepare a 3X solution. This was then added to each well (25 μL/well). The plate was read on a Pherastar FSX microplate reader with simultaneous dual emission capabilities

to read 384-well plates at 450 and 520 nm. The NanoBRET ratio was calculated by dividing the signal at 520 nm by the signal at 450 nm for each sample. Duplicate points were averaged and plotted against [compound, M] to generate an $EC_{50}$ curve. The Nluc substrate and extracellular inhibitor were purchased as a kit from Promega Corporation and used as is from the box - Promega NanoBRET Nano-Glo Substrate/Inhibitor; Promega Catalog number N2161 for 10,000 assay kit.

Cellular GSPT1-GFP/mCherry degradation assay using Operetta

GSPT1 GFP/mCherry (Flp293T based) cells were maintained in DMEM high glucose media (11965118, Thermo Fisher) supplemented with 10% FBS (FSP500, ExCellBio), 1% penicillin/streptomycin (SV30010, Hyclone) and 5 µg/mL hygromycin B (10687010, Invitrogen) in T-75 tissue culture flasks. To prepare an assay plate media was aspirated carefully from T-75 flask with confluent cells, following by a wash with 10 mL dPBS. After aspiration of dPBS 1 mL of 0.05% Trypsin (25300062, Invitrogen) was added to the cells on the plate and swirled around to evenly disperse on the surface and incubated in the hood for 2 min at room temperature. 10 mL of fresh culture media was then added to the plate with the trypsin and striped cells by pipetting up and down around the plate. Afterwards all the cells were transferred to a 50 mL Falcon tube, spun down for 5 mins at 1000 rpm. After spinning down, media was aspirated, and the cell pellet resuspend in 5 mL assay media (FluoroBrite DMEM (A1896701, Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin). At this point cells were counted and made up to 40 mL of 0.16 x 10^6/mL cells in assay media (8000 cells/well). Cell mix was plated in the 384 well plate (781090, Corning) by adding 50 µL/well using multi-drop dispenser (Multi-dropCombi, Thermo Fisher). After plating, the assay plate was spun down in centrifuge for 5 mins at 500xg and incubated at 37ºC, 5% $CO_2$ overnight, allowing the cells to adhere. Compounds were dispensed the next day using Labcyte acoustic dispenser (POD810), 100 µM top, 3 times dilution, and 11-point dose response in duplicate with the final DMSO concentration of 1%. Positive control (CC-885) had a top concentration of 0.1 µM. After dispensing compounds, the assay plate was returned to incubator for 5h. After 5h incubation, 15 µl of 16% formaldehyde (28908, Pierce) was dispense into each well directly using multi-drop (Multi-dropCombi, Thermo Fisher) using standard tube dispensing cassette (24072670, Thermo Fisher) with the final concentration of formaldehyde of 4%. The assay plate was then spun down at 500 rpm, for 1 min, then incubated in the dark for 15 mins. Following the incubation media was decanted by inverting plate and flicking out media over

a container. Finally, 50 µl of dPBS (18007771, Corning) was added into each well and the plate was read plate using Operetta CLS (HH1600, Perkin Elmer). The imaging was performed with non-confocal optical mode using 10x Air, NA 0.3 objective with binning set to 2. The channels were defined as: Ch1 - Digital Phase Contrast, 10 ms acquisition, 10% intensity and 0 µm offset; Ch2 – EGFP, 200 ms acquisition, 100% intensity and 0 µm offset; Ch3 – mCherry, 200 ms acquisition, 100% intensity and 0 µm offset. The EGFP intensity threshold was set to 10,000. For data analysis the %GFP positive cells were calculated as:

%GFP positive cells = (# Cells with more than 10,000 EGFP) / (# Cells with mCherry) * 100%.

Data was reported as %DMSO treatment, where:

%DMSO treatment = (%GFP positive cells sample) / (Average %GFP positive cells MAX) * 100%

and analyzed using GraphPad Prism 9 and dose response curves were fitted using Variable Slope equation.

Generation of HiBit knock-in line in MOLT4 cells

MOLT4 T-ALL cells were electroporated with the CRISPR/Cas9 ribonucleoprotein (RNP) complex with single guide (sg) RNAs targeting *IKZF1* at the ATG start codon (5'-GAGGACCATGGATGCTGATGAGG-3'). The RNP complexes were co-electroporated along with a single-stranded oligodeoxynucletide (ssODN) donor template containing the coding sequence of the 11-amino acid HiBiT peptide (Promega) and homologous sequences to the endogenous *IKZF1* gene (5' starting at Hg38 Chr7:50,318,982; 3' ending at Hg38 Chr7: 50,319,144). Cells were cultured in RPMI media (Gibco, Life Technologies) with 10% FBS at 37ºC, 5% $CO_2$ for 48h, followed by initial HiBiT lytic assay (Promega) to identify successfully targeted cells. Pools with the highest HiBiT signals were then sorted as single cell into 96-well plates and cultured for 2 weeks, until clones were observed, collected, and assessed again using the HiBiT lytic assay. After DNA sequencing confirmation, one IKZF1-HiBiT clone was selected for subsequent compound screening.

Cellular IKZF1 HiBit degradation assay

To run the assay, compounds were first dispensed ranging from 10 µM in 3-fold dilution into 384-well white flat bottom TC-treated plates (Corning, 3570). Then 4000 cells per well in 50 µL of

media were plated. Cells were mixed, centrifuged at 500 rpm for 1 min, and incubated for 24h at 37ºC, 5% $CO_2$. Before reading, plates were equilibrated to room temperature, while HiBiT lytic reagent was prepared according to manufacturer's instructions (Promega): for 10 mL of reagent, dilute 200 µL HiBiT substrate and 100 µL of LgBiT protein in 10 mL of lytic buffer. 12.5 µL of prepared reagent was added to each well (1:4 dilution). Plates were shaken at room temperature for 10 min under aluminum foil cover, then read on an EnVision plate reader (Perkin Elmer). For data analysis, DMSO-treated samples were averaged, and %DMSO treated was calculated for each test sample. Data were analyzed using GraphPad Prism 9 and dose response curves were fitted using Variable Slope equation model.

Docking and molecular dynamics

Cocrystal structures of CRBN with GSPT1 (PDB 5HXB) and IKZF1 (PDB 6H0F) were used for generating docking grid files. Glide from the Schrodinger suite (version 2022-2) was used for all docking calculations. Three hydrogen bonds in the glutarimide binding site (i.e. Trp380 and His378) of CRBN were required as constraint. Default values were chosen for other parameters. The final binding mode for each molecule was chosen visually from the top 5 poses to make sure they were consistent with known IMiD binding modes. Molecular dynamics simulations for 5-OH-EM12 in GSPT1/CRBN/DDB1, 5-$NH_2$-EM12 in GSPT1/CRBN/DDB1, 5-$NH_2$-EM12 in IKZF1/CRBN/DDB1, and 4-OH-Thal in IKZF1/CRBN/DDB1 were carried out using Desmond in Schrodinger suite (version 2022-2). The simulations were run for 50 ns with the TIP3P water box with 12 Å buffer. DDB1 backbone atoms were constrained with a force constant of 4 kcal/mol/Å$^2$ during the simulations. Default relaxation protocol was used to equilibrate the system before the production run. Statistics of interactions were gathered with the simulation interaction module in Schrodinger suite.

## References

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