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Corresponding Author:	Jan Kroenke Universitat Ulm Ulm, BW GERMANY
Corresponding Author's Institution:	Universitat Ulm
Corresponding Author E-Mail:	jan.kroenke@uni-ulm.de
Order of Authors:	Stefanie Lindner
	Christian Steinebach
	Hannes Kehm
	Martin Mangold
	Michael Gütschow
	Jan Kroenke
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TITLE:

Chemical Inactivation of the E3 Ubiquitin Ligase Cereblon by Pomalidomide-Based Homo-PROTACs

AUTHORS & AFFILIATIONS:

Stefanie Lindner[1*], Christian Steinebach[2*], Hannes Kehm[1], Martin Mangold[2], Michael Gütschow[2], Jan Krönke[1†]

[1]Department of Internal Medicine III, University Hospital Ulm, Ulm, Germany
[2]Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Bonn, Germany
[*]These authors contributed equally.

Corresponding Author:

Jan Krönke (jan.kroenke@uni-ulm.de)
Tel. +49 731 500 45718

Email Addresses of Co-Authors:

Stefanie Lindner (stefanie.lindner@uni-ulm.de)
Christian Steinebach (c.steinebach@uni-bonn.de)
Hannes Kehm (hannes.kehm@uni-ulm.de)
Martin Mangold (martinmangold@uni-bonn.de)
Michael Gütschow (guetschow@uni-bonn.de)

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CRBN, PROTAC, IMiD, pomalidomide, ubiquitin ligase, multiple myeloma, proteasome

SUMMARY:

This work describes the synthesis and characterization of a pomalidomide-based, bifunctional homo-PROTAC as a novel approach to induce ubiquitination and degradation of the E3 ubiquitin ligase cereblon (CRBN), the target of thalidomide analogs.

ABSTRACT:

The immunomodulatory drugs (IMiDs) thalidomide and its analogs, lenalidomide and pomalidomide, all FDA approved drugs for the treatment of multiple myeloma, induce ubiquitination and degradation of the lymphoid transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) via the cereblon (CRBN) E3 ubiquitin ligase for proteasomal degradation. IMiDs have recently been utilized for the generation of bifunctional proteolysis targeting chimeras (PROTACs) to target other proteins for ubiquitination and proteasomal degradation by the CRBN E3 ligase. We designed and synthesized pomalidomide-based homobifunctional PROTACs and analyzed their ability to induce self-directed ubiquitination and degradation of CRBN. Here, CRBN serves as both, the E3 ubiquitin ligase and the target at the same time. The homo-PROTAC compound **8** degrades CRBN with a high potency with only minimal remaining effects on IKZF1 and IKZF3. CRBN inactivation by compound **8** had no effect on cell viability and proliferation of different multiple myeloma cell lines. This homo-PROTAC abrogates the effects of IMiDs in

multiple myeloma cells. Therefore, our homodimeric pomalidomide-based compounds may help to identify CRBN's endogenous substrates and physiological functions and investigate the molecular mechanism of IMiDs.

INTRODUCTION:

The immunomodulatory drugs (IMiDs) thalidomide and its analogs, lenalidomide and pomalidomide, all approved for the treatment of multiple myeloma, bind to the E3 ubiquitin ligase cereblon (CRBN), a substrate adaptor for cullin4A-RING E3 ubiquitin ligase ($CRL4^{CRBN}$)[1-3]. Binding of IMiDs enhances the affinity of $CRL4^{CRBN}$ to the lymphoid transcription factors Ikaros (IKZF1) and Aiolos (IKZF3), leading to their ubiquitination and degradation (**Figure 1**)[4-8]. Since IKZF1 and IKZF3 are essential for multiple myeloma cells, their inactivation results in growth inhibition. SALL4 was recently found as an additional IMiD-induced neo-substrate of CRBN that is likely responsible for the teratogenicity and the so-called Contergan catastrophe in the 1950s caused by thalidomide[9,10]. In contrast, casein kinase 1α (CK1α) is a lenalidomide-specific substrate of CRBN that is implicated in the therapeutic effect in myelodysplastic syndrome with chromosome 5q deletions[11].

The ability of small-molecules to target a specific protein for degradation is an exciting implication for modern drug development. While the mechanism of thalidomide and its analogs was discovered after their first use in humans, so called **Pr**oteolysis **Ta**rgeting **C**himeras (PROTACs) have been designed to specifically target a protein of interest (POI) (**Figure 2**)[12-18]. PROTACs are heterobifunctional molecules that consist of a specific ligand for the POI connected via a linker to a ligand of an E3 ubiquitin ligase like CRBN or von-Hippel-Lindau (VHL)[18-22]. PROTACs induce the formation of a transient ternary complex, directing the POI to the E3 ubiquitin ligase, resulting in its ubiquitination and proteasomal degradation. The major advantages of PROTACs over conventional inhibitors is that binding to a POI is sufficient rather than its inhibition and therefore PROTACs can potentially target a far wider spectrum of proteins including those that were considered to be undruggable like transcription factors[15]. In addition, chimeric molecules act catalytically and therefore have a high potency. After ubiquitin transfer to the POI, the ternary complex dissociates and is available for the formation of new complexes. Thus, very low PROTAC concentrations are sufficient for the degradation of the target protein[23].

Here we describe the synthesis of a pomalidomide-pomalidomide conjugated homo-PROTAC (compound **8**) that recruits CRBN for the degradation of itself[24]. The E3 ubiquitin ligase CRBN serves as both the recruiter and the target at the same time (**Figure 3**). To validate our data, we also synthesized a negative binding control (compound **9**). Our data confirm that the newly synthesized homo-PROTAC is specific for CRBN degradation and has only minimal effects on other proteins.

PROTOCOL:

1. Preparation of PROTAC molecules

CAUTION: Please consult all relevant material safety data sheets (MSDS) before use. Several of the chemicals used in these syntheses are toxic and carcinogenic. Please use all appropriate safety practices and personal protective equipment.

1.1. Preparation of *tert*-butyl *N*-(2,6-dioxo-3-piperidyl)carbamate (compound 1)

1.1.1. Add 1,1'-carbonyldiimidazole (1.95 g, 12 mmol) and a catalytic amount of 4-(dimethylamino)pyridine (5 mg) to a mixture of Boc-Gln-OH (2.46 g, 10 mmol) in THF (50 mL) in 100 mL round bottom flask with a stir bar and equipped with a reflux condenser. Heat at reflux for 10 h while stirring until a clear solution is formed.

1.1.2. Remove the solvent under reduced pressure with a rotary evaporator, add EtOAc (200 mL) and transfer it to a separatory funnel. Wash the organic layer with H_2O (50 mL) and brine (50 mL) and dry it over Na_2SO_4.

1.1.3. Filter the solution through a short pad of silica gel (5 cm diameter and 5 cm height) and eluate with a further volume (200 mL) of EtOAc.

1.1.4. Evaporate the solvent and dry the obtained colorless solid in vacuo.

1.2. Preparation of *tert*-butyl *N*-(1-methyl-2,6-dioxo-3-piperidyl)carbamate (compound 2)

1.2.1. Combine compound **1** (2.28 g, 10 mmol) with milled potassium carbonate (2.76 g, 20 mmol) and DMF (25 mL) in a 100 mL round bottom flask. Add iodomethane (1.42 g, 0.62 mL, 10 mmol) drop-wise using a syringe and equip the flask with a punctured rubber septum. Place the reaction vessel into an ultrasonication bath for 2 h.

1.2.2. Dilute the red reaction mixture with EtOAc (100 mL) and transfer it to a separatory funnel. Wash the organic layer with 1 N NaOH (2x 25 mL), H_2O (25 mL), and brine (25 mL), and dry it over Na_2SO_4.

1.2.3. Filter and evaporate the solvent. Purify the product by column chromatography over silica gel (6 cm column diameter and 20 cm height) using petroleum ether/EtOAc (2:1).

1.3. Preparation of 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (compound 3)

1.3.1. Combine 3-fluorophthalic anhydride (1.25 g, 7.5 mmol), glutarimide **1** (1.14 g, 5 mmol) and a solution of sodium acetate (0.50 g, 6.0 mmol) in glacial acetic acid (20 mL) in a 50 mL round bottom flask with a stir bar and equipped with a reflux condenser. Heat the mixture at 120 °C for 6 h.

1.3.2. After cooling, pour the purple mixture onto H_2O (100 mL) and stir for 10 min. Collect the formed solid by filtration, wash with H_2O (3 × 5 mL) and petroleum ether (3 × 5 mL) and dry in vacuo.

1.4. Preparation of 4-fluoro-2-(1-methyl-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (compound 4)

1.4.1. Combine 3-fluorophthalic anhydride (1.25 g, 7.5 mmol), glutarimide **2** (1.21 g, 5 mmol) and a solution of sodium acetate (0.50 g, 6.0 mmol) in glacial acetic acid (20 mL) in a 50 mL round bottom flask with a stir bar and equipped with a reflux condenser. Heat the mixture at 120 °C for 6 h.

1.4.2. After cooling, pour the purple mixture onto H_2O (100 mL) and stir for 10 min. Collect the formed solid by filtration, wash with H_2O (3 × 5 mL) and petroleum ether (3 × 5 mL) and dry in vacuo.

1.5. Preparation of *tert*-butyl *N*-[2-[2-[2-[[2-(2,6-dioxo-3-piperidyl)-1,3-dioxo-isoindolin-4-yl]amino]ethoxy]ethoxy]ethyl]carbamate (compound 7)

1.5.1. Charge a 50 mL round bottom flask with *tert*-butyl *N*-[2-[2-(2-aminoethoxy)ethoxy]ethyl]carbamate (**5**, 0.41 g, 1.65 mmol), compound **3** (0.41 g, 1.50 mmol), dry DMF (10 mL) and DIPEA (0.39 g, 0.51 mL, 3.0 mmol). Equip with a stir bar and a reflux condenser. Heat under argon atmosphere at 90 °C for 10 h.

1.5.2. After cooling to room temperature, pour the dark green mixture onto H_2O (100 mL) and extract with EtOAc (3x 50 mL) in a separatory funnel. Wash the combined organic layers with H_2O (50 mL) and brine (50 mL), dry over Na_2SO_4, filter, and concentrate in vacuo.

1.5.3. Purify the crude product by column chromatography over silica gel (3 cm column diameter and 60 cm height) using a gradient of petroleum ether/EtOAc (1:1 to 1:2).

1.6. Preparation of homodimer (compound 8)

1.6.1. Combine the α,ω-diamine linker **6** (0.22 g, 0.22 mL, 1.50 mmol), DIPEA (1.05 mL, 6.00 mmol) and a solution of **3** (0.83 g, 3.00 mmol) in dry DMSO (20 mL) in a 50 mL round bottom flask with a stir bar and equipped with a reflux condenser. Heat under argon atmosphere at 90 °C for 18 h.

1.6.2. After cooling to room temperature, pour the dark green mixture onto H_2O (100 mL) and extract with EtOAc (3x 50 mL) in a separatory funnel. Wash the combined organic layers with H_2O (50 mL) and brine (50 mL), dry over Na_2SO_4, filter and concentrate in vacuo.

1.6.3. Purify the crude product by column chromatography over silica gel (3 cm column diameter and 50 cm height) using a gradient of petroleum ether/EtOAc (1:2) to EtOAc.

1.7. Preparation of heterodimer (compound 9)

1.7.1. Dissolve compound **7** (0.83 g, 1.65 mmol) in dry CH_2Cl_2 (10 mL). Add trifluoroacetic acid (10 mL) and stir the yellow mixture at 40 °C for 2 h in a closed 50 mL round bottom flask.

1.7.2. Remove the volatiles and coevaporate with CH_2Cl_2 (4x 5 mL). Dry the residue in vacuo for 10 h.

1.7.3. Redissolve the material in dry DMF (20 mL). Add compound **4** (0.44 g, 1.50 mmol) and DIPEA (0.78 g, 1.05 mL, 6.00 mmol) and equip the flask with a reflux condenser. Heat under argon atmosphere at 90 °C for 10 h.

1.7.4. After cooling to room temperature, pour the dark green mixture onto H_2O (100 mL) and extract with EtOAc (3x 50 mL) in a separatory funnel. Wash the combined organic layers with saturated $NaHCO_3$ (50 mL), H_2O (50 mL), 10% $KHSO_4$ (50 mL), H_2O (50 mL), and brine (50 mL), dry over Na_2SO_4, filter and concentrate in vacuo.

1.7.5. Purify the crude product by column chromatography over silica gel (3 cm column diameter and 50 cm height) using a gradient of petroleum ether/EtOAc (1:2) to EtOAc.

1.7.6. Elucidate and verify molecule structure (**Figure 5A** compound **8**, 5B compound **9**) by ^{1}H NMR and ^{13}C NMR spectra in DMSO-d_6 on a nuclear magnetic resonance (NMR) spectrometer. Check that the purity of both compounds is higher than 97% by means of liquid chromatography-mass spectrometry (LC-MS), applying a diode array detection (DAD) at 220–500 nm.

2. Functional validation of PROTAC molecules

2.1. Western blot analysis of CRBN degradation by PROTACs

NOTE: The effects of compound **8** and compound **9** on CRBN protein level were tested by western blot analysis. In addition, the impact on IKZF1 and IKZF3 levels could also be confirmed (**Figure 6**).

2.1.1. Sample preparation

2.1.1.1. Dissolve compounds **8** and **9**, lenalidomide (Len), pomalidomide (Pom), MG132, and MLN-4924 in DMSO at a concentration of 10 mM, aliquot and store at -80 °C until further usage.

2.1.1.2. Seed 1 x 10^6 MM1S cells in a 6-well plate with 2.5 mL media and treat cells with 100 nM or 1 µM compound **8** or **9** for 24 h.

2.1.1.3. Harvest cells after treatment and centrifuge at 700 x *g*, 5 min, 4 °C. Wash cell pellet with cold 1x PBS to remove remaining media, centrifuge at 700 x *g*, 5 min, 4 °C, and discard supernatant. Repeat this step once.

2.1.1.4. Lyse cells in lysis buffer (25 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol, 1x Protease & Phosphatase Inhibitor Cocktail) for 10 min on ice, centrifuge at 320 x *g* for 10 min, 4 °C. Harvest supernatant and determine protein concentration by a bicinchoninic acid protein assay (BCA assay) according to the manufacturer's protocol.

2.1.1.5. Denature proteins (15–30 µg/sample) with 1x LDS loading buffer (5% 2-mercaptoethanol) and boil 10 min, 75 °C.

2.1.2. SDS-PAGE

2.1.2.1. Fix gel sandwich with a 10% separating gel [4 mL 3x gel buffer (3 M Tris/HCl, 0.3% (w/v) sodium dodecyl sulfate (SDS), pH 8.45), 4 mL acrylamide 30%, 2.52 mL glycerol 50%, 1.395 mL H_2O, 75 µL 11% ammonium persulfate (APS), and 9.75 µL TEMED] and a 4% stacking gel [1.992 mL 3x gel buffer, 0.792 mL 30% acrylamide, 3.168 mL H_2O, 36 µL 11% APS, and 6 µL TEMED] in an electrode assembly unit. Remove combs, flush wells with cathode buffer (100 mM Tris/HCl, 100 mM tricine, 0.1% (w/v) SDS), and load samples.

2.1.2.2. Fill anode buffer (100 mM Tris/HCl, pH 8.9) into the tank. Load protein sample from step 2.1.1.4 and run SDS-PAGE at 70 V, 20 min, followed by 115 V, 150 min at constant voltage.

2.1.3. Immunoblotting and detection of CRBN, IKZF1 and IKZF3

2.1.3.1. Activate PVDF membrane (0.45 µm) in 100% methanol for 1 min. Equilibrate membrane and separating gel in 1x transfer buffer [10x transfer buffer (192 mM glycine, 25 mM Tris-base/HCl, 900 mL H_2O), 20% methanol, 0.1% SDS, pH 8.3].

2.1.3.2. Assemble blotting cassette according to manufacturer's protocol. Transfer gel at 180 mA for 90 min.

2.1.3.3. Wash membrane 3x in 1x TBS-T (25 mM Tris/HCl, 150 mM NaCl, pH 7.6, 0.1% Tween 20) for 5–10 min each at room temperature. Block membrane in 5% nonfat-dried milk (NFDM), TBS-T for 1 h at room temperature. Wash membrane 3x in 1X TBS-T for 5–10 min each at room temperature.

2.1.3.4. Incubate membrane with primary antibody for CRBN (1:500 in 5% BSA, TBS-T) with gentle shaking at 4 °C, overnight.

2.1.3.5. Wash membrane 3x in 1X TBS-T for 5–10 min each at room temperature. Incubate membrane with anti-mouse (1:10.000 in 5% NFDM, TBS-T) or anti-rabbit (1:5.000 in 5% NFDM, TBS-T) secondary antibody coupled to horseradish peroxidase HRP (1 h at room temperature.)

2.1.3.6. Wash membrane 2x in 1X TBS-T for 5–10 min each at room temperature. Repeat this step twice with 1x TBS.

2.1.3.7. Incubate membrane for 2 min with HRP substrate solution according to manufacturer's protocols and detect chemiluminescence in a chemiluminescence detection device.

2.1.3.8. Wash membrane 1x in 1X TBS for 5–10 min each at room temperature. For release of antibodies, strip membrane in commercially available stripping buffer for 15 min. Wash membrane 3x in 1X TBS for 5–10 min each at room temperature.

2.1.3.9. Reblock membrane in 5% nonfat-dried milk, TBS-T for 1 h at room temperature. Wash membrane 3x in 1x TBS-T for 5–10 min each at room temperature and reprobe with IKZF1, IKZF3 or tubulin according to step 2.1.3.4.

2.2. Competition experiments with MG132, MLN4942 or pomalidomide

NOTE: To confirm whether CRBN is degraded via the ubiquitin-proteasome pathway, we performed competition experiments with the proteasome inhibitor MG132 and a neddylation activating enzyme (NAE) inhibitor MLN4942 (**Figure 7**).

2.2.1. Seed 1 x 10^6 MM1S cells per well in a 6-well plate. Pretreat cells with 10 µM MG132, 10 µM MLN4942, or lenalidomide (100x), and incubate 1 h at 37 °C, 5% CO_2.

2.2.2. Add 100 nM compound **8** for 3 h at 37 °C, 5% CO_2.

2.2.3. Harvest cells for western blot according to step 2.1.1.

2.3. Cell viability assays in multiple myeloma cell lines

NOTE: This assay is used to test the impact on cell viability and additionally, antagonize the effect of IMiDs on multiple myeloma cells by pretreatment of the cells with compound **8** (**Figure 8**, **Figure 9A,B**).

2.3.1. Seed 5 x 10^4 MM1S cells per well in a 96-well plate in biological triplicates for viability assay. For western blot analysis, seed 1 x 10^6 MM1S cells per well in a 6-well plate in biological triplicates.

2.3.2. Treat cells with DMSO or 100 nM, 1 µM, or 10 µM compound **8**, compound **9** or pomalidomide and incubate for 24 h, 48 h, or 96 h at 37 °C, 5% CO_2. For rescue experiments, treat cells with 100 nM compound **8** for 3 h, before or after addition of 1 µM pomalidomide and incubate for 96 h.

2.3.3. Measure 96-well plate luminescence with a luminescent cell viability assay, according to the manufacturer's protocol on a plate reader or harvest cells from the 6-well plate for western blot analysis.

REPRESENTATIVE RESULTS:

Here we described the design, synthesis and biological evaluation of a homodimeric pomalidomide-based PROTAC for the degradation of CRBN. Our PROTAC interacts simultaneously with two CRBN molecules and forms ternary complexes that induces self-ubiquitination and proteasomal degradation of CRBN with only minimal remaining effects on pomalidomide-induced neo-substrates IKZF1 or IKZF3.

Out of a series of previously published pomalidomide-based PROTAC molecules[24], compound **8** was particularly efficient in the chemical-induced degradation of CRBN. Its synthesis can be accomplished as follows (**Figure 4**). A 1,1'-carbonyldiimidazole-promoted condensation of Boc-protected L-glutamine leads to the cyclized imide **1**. The *N*-methylated analog **2** is accessible through alkylation with methyl iodide. Both building blocks (**1** and **2**) are transformed, after *N*-deprotection under acidic conditions, to phthalimide derivatives (**3** and **4**) in the course of a ring-opening/recyclization reaction using 3-fluorophthalic anhydride. Thalidomide analogs, in general, are susceptible to hydrolytic decomposition and should only be used in the next step after sufficient drying. Compound **3** is susceptible to an aromatic nucleophilic substitution with primary aliphatic amines[25]; this conversion was found to proceed efficiently only when dry solvents are used. The design of a true homodimeric product implies the linker connection of two identical functional substructures and the application of symmetrical linker. The linker which is part of PROTAC **8** represents an *N*-to-*N*, polyethylene-based linear chain. The corresponding α,ω-diamine **6** leads to the desired final compound **8** when reacted with building block **3** in molar ration of 1:2 in DMSO at 90 °C. Among other analytical data[24], the structure of **8** was verified by NMR spectra (**Figure 5A**). Compound **9**, designed as a suitable negative control, has an only minimal, but critical structural deviations, compared to the active homo-PROTAC **8**. It is known that *N*-methylation within the glutarimide portion abolishes CRBN binding[26,27]. One pomalidomide portion of the negative control compound **9** bears an *N*-methyl residue. It can be prepared by a first nucleophilic substitution of **3** with the *N*-monoprotected linker building block **5**, followed by cleavage of the Boc protecting group and a subsequent coupling to intermediate **4**. Owing to its asymmetrical structure, some of the corresponding carbons showed distinct ^{13}C NMR signals (**Figure 5B**).

The homo-PROTAC **8** was observed to be highly potent, leading to an almost complete proteasomal degradation of CRBN. The interpretation of CRBN, IKZF1 and IKZF3 protein levels in multiple myeloma cells were confirmed by western blot analysis (**Figure 6**, **Figure 7**, **Figure 9B**), a semi-quantitative standard method, where the change in protein expression can be detected easily. The antibodies used in this paper are of good quality and the method is an optimized standard procedure in our lab.

In addition, degradation of CRBN by compound **8** did not affect cell viability and conferred resistance to IMiDs (**Figure 8**, **Figure 9A**), which is in line with CRISPR/Cas9-mediated knockout of *CRBN* by sgRNAs[24]. The luminescence signal in the cell viability assay was based on ATP release, which can be interpreted as dead cell count. This method can be easily performed in a short time with a high number of samples. An alternative method for the measurement of viable/dead cells is an Annexin V/ 7-AAD staining by flow cytometry.

FIGURE LEGENDS:

Figure 1: The E3 ubiquitin ligase CRBN is the main target of IMiDs. Immunomodulatory drugs bind to CRBN and recruit several neo-substrates for proteasomal degradation. IMiD-induced degradation of the lymphoid transcription factors IKZF1 and IKZF3 is responsible for the effects on multiple myeloma cells and some of the immunomodulatory properties. Casein kinase 1α is selectively degraded by lenalidomide but not the other IMiDs and contributes to the activity of lenalidomide in myelodysplastic syndrome with loss of chromosome 5q. SALL4 was recently discovered as a common target of all IMiDs that is likely linked to the teratogenicity induced by thalidomide and its analogs.

Figure 2: PROTACs degrade the protein of interest (POI). PROTACs are heterobifunctional molecules, where a linker connects a ubiquitin ligase ligand to a POI ligand. By the formation of ternary complexes, a ubiquitin ligase, such as CRBN, then ubiquitinates the POI, resulting in its proteasomal degradation.

Figure 3: **Bifunctional homo-PROTAC for the degradation of the E3 ubiquitin ligase CRBN.** In a pomalidomide-based homo-PROTAC, two ubiquitin ligase binders are connected to induce cross-ubiquitination of CRBN resulting in a chemically induced knockdown of CRBN.

Figure 4: Synthesis of homodimer 8 and heterodimer 9.

Figure 5: **^{1}H NMR (top) and ^{13}C NMR (bottom) spectra**. Spectra of compound **8** (**A**) and compound **9** (**B**) were recorded in DMSO-d_6 on an NMR spectrometer. Chemical shifts are given in parts per million (ppm).

Figure 6: Effects of compounds 8 and 9 on CRBN, IKZF1, and IKZF3. Pomalidomide-based homo-PROTAC compound **8** induces CRBN degradation with weak remaining effects of pomalidomide on IKZF1 and IKZF3. In contrast, compound **9** that contains a methyl group on one of the pomalidomide residues has no effect at the indicated concentrations (µM). MM1S cells were treated for 24 h treatment. Effects on CRBN, IKZF1, IKZF3 and tubulin (loading control) were analyzed by western blot.

Figure 7: CRBN degradation can be blocked by the proteasome inhibitor MG132 or by MLN4942 that blocks ubiquitin ligases indirectly via neddylation inhibition. The multiple myeloma cell line MM1s was pretreated with 10 µM MG132, 10 µM MLN4924 for 1 h before addition of homo-PROTAC compound **8** at 100 nM for 3 h of combined treatment.

Figure 8: Cell viability assay in MM1S multiple myeloma cells. Effects of compound **8** and negative binding control compound **9** on cell viability in the pomalidomide sensitive myeloma cell line MM1S after 24 h, 48 h and 96 h treatment. Cell viability was measured after 4 days in triplicates.

Figure 9: Compound 8 antagonizes the effect of pomalidomide in multiple myeloma cell lines. Cells were pretreated with 100 nM compound **8** for 3 h. Afterwards 1 µM pomalidomide was

added. Cell viability was measured after 4 days in triplicates. *** p <0.001 according to Student´s t-test (**A**). Western blot analysis for CRBN, IKZF1, IKZF3 and tubulin (loading control) after pretreatment of MM1S cells with 100 nM compound **8** for 3 h, before addition of 1 µM pomalidomide (**B**). Reprinted (adapted) with permission from Steinebach, C. et al. 2018[24]. Copyright 2019 American Chemical Society.

DISCUSSION:

The design of such homo-PROTACs as described here for CRBN relies on the specific affinity of pomalidomide to CRBN, which has been successfully utilized in numerous heterobifunctional PROTACs and resulted in the development of PROTAC **8** as a highly selective CRBN degrader. The specificity of our molecule has already been confirmed by proteomic analyses[24]. For genetically mediated knockout, exclusion and validation of side effects is challenging and time consuming. In addition, a chemically induced knockdown is reversible, rapid and directly applicable to a wide spectrum of cells and tissue types[28].

The IMiDs thalidomide, lenalidomide, and pomalidomide have become a mainstay in the treatment of multiple myeloma, B-cell lymphomas and myelodysplastic syndrome. IMiDs mediate their activity by modulating the specificity of the CRBN-CRL4 E3 ligase to degrade the neo-substrates IKZF1, IKZF3, or CK1α[6,11,29]. In addition, IMiDs have been shown to abrogate the chaperone function of CRBN on two other proteins, MCT-1 and BSG, that are also important for multiple myeloma growth[30]. The degradation of CRBN by the homo-bifunctional PROTAC was well tolerated by most multiple myeloma cell lines tested, implying that CRBN inactivation alone is not sufficient to cause killing of multiple myeloma cells. In contrast, pre-treatment with compound **8** abrogated the effects of IMiDs on IKZF1/3 degradation and rescued multiple myeloma cells from lenalidomide and pomalidomide. This is in line with genetic inactivation of *CRBN* and deleterious *CRBN* mutations found in lenalidomide-resistant multiple myeloma patients and highlights the essential role of CRBN in the mechanism of IMiDs[31,32]. The homo-PROTAC **8** can therefore be a useful tool to mimic a state of IMiD resistance. Other effects of IMiDs that are not fully understood yet like inhibition of angiogenicity or TNFα release may derive from an inhibition of CRBN function and our homo-PROTAC are suitable tools to investigate the inactivation of CRBN further. In addition, the chemically induced knockdown of CRBN by compound **8** may help to identify new endogenous substrates of CRBN and elucidate the physiological functions of CRBN. Given that our compound **8** had no effects on cancer cell line proliferation, CRBN inhibition alone has no anti-tumor activity. However, CRBN degraders may be clinically applicable in diseases other than cancer. In this regard, *CRBN* inactivation was recently shown to confer resistance to sepsis and to prevent high-fat-diet-induced obesity in mice [33-35].

In conclusion, we generated and validated the first chemical inhibitor of CRBN that can serve as a useful tool for future biomedical investigations on CRBN-related signaling and molecular mechanism of thalidomide and its analogs.

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DISCLOSURE:

The authors do not declare a potential financial conflict of interest.

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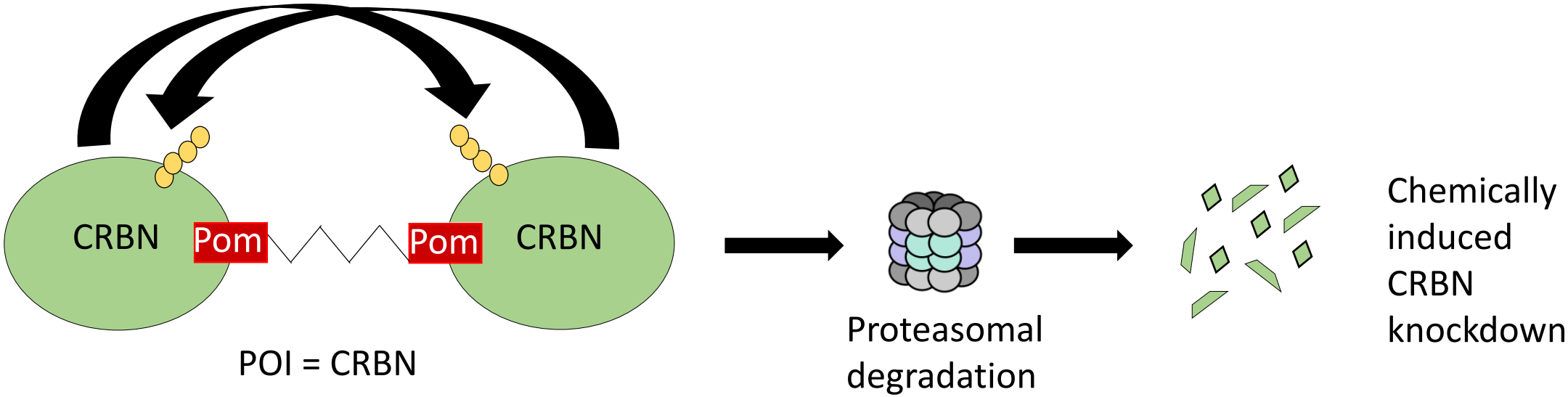

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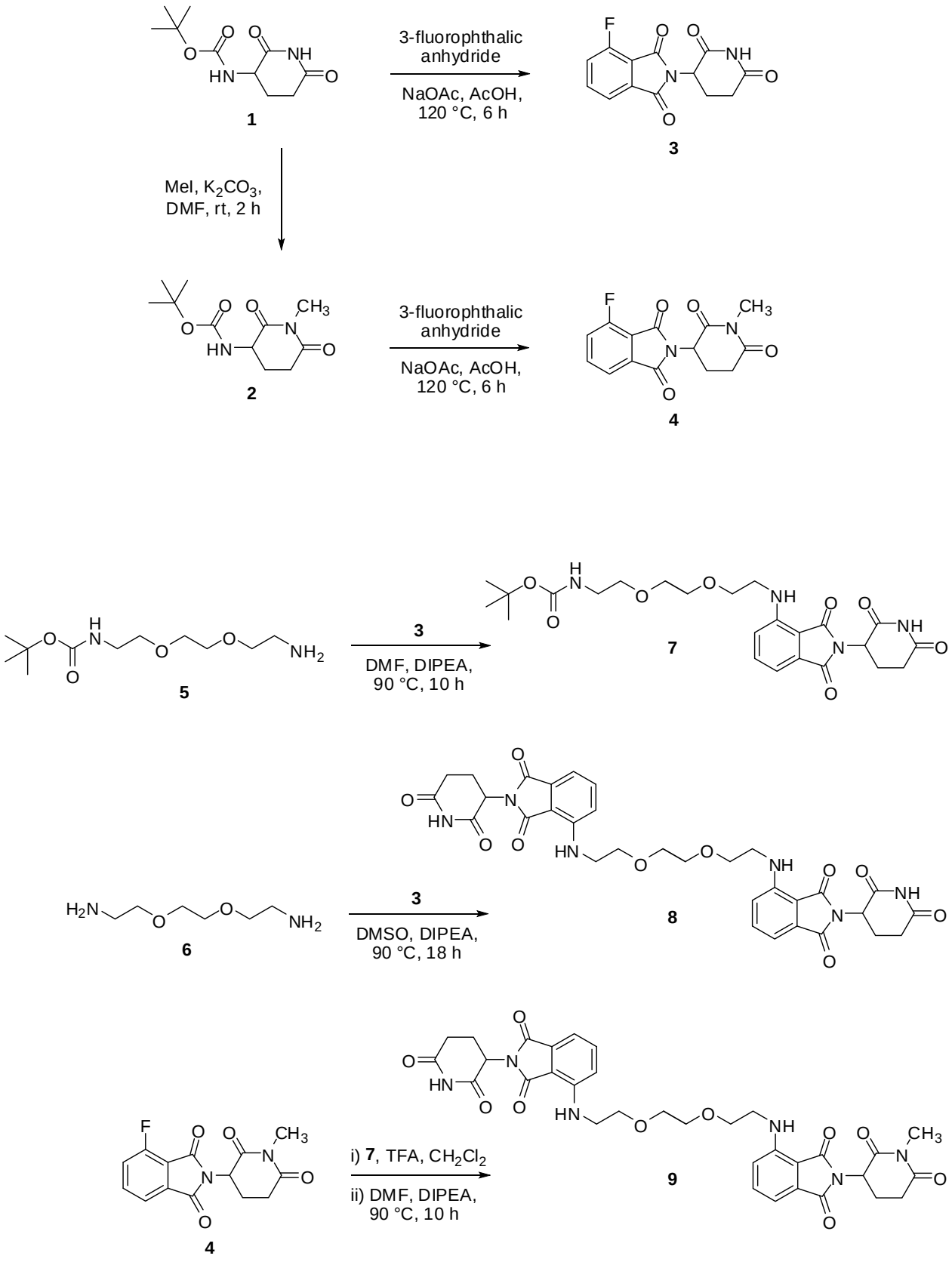

Figure 5

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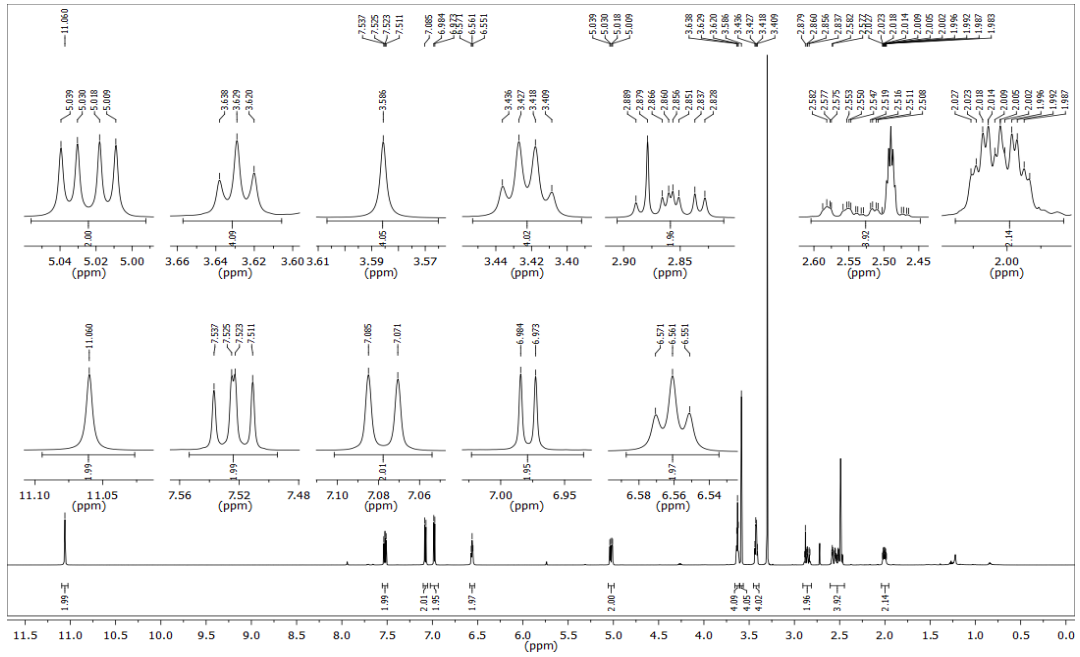
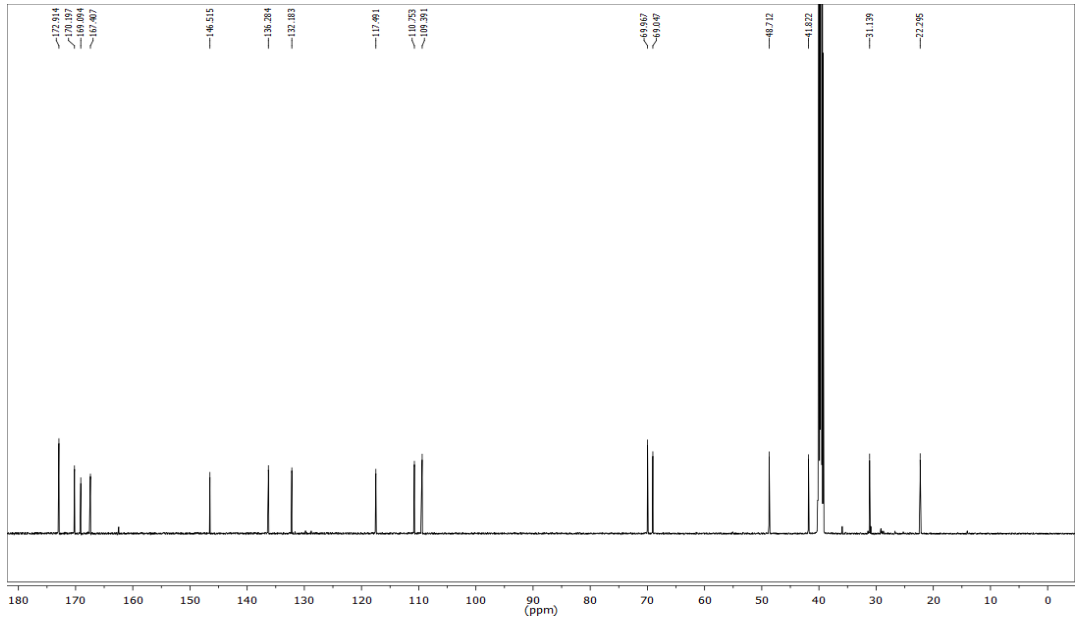
A





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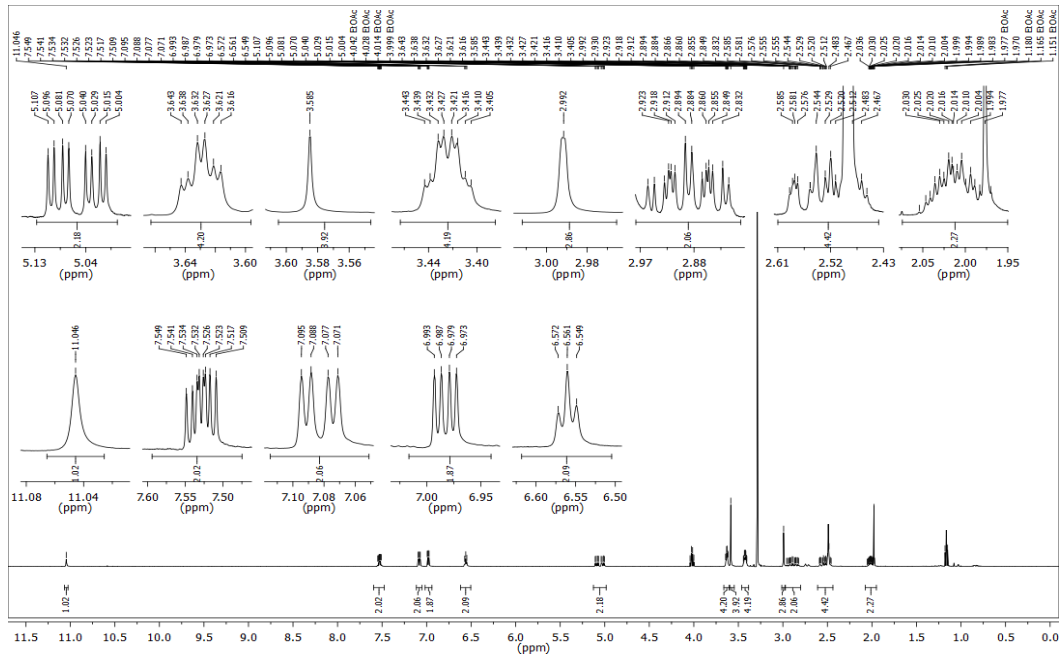


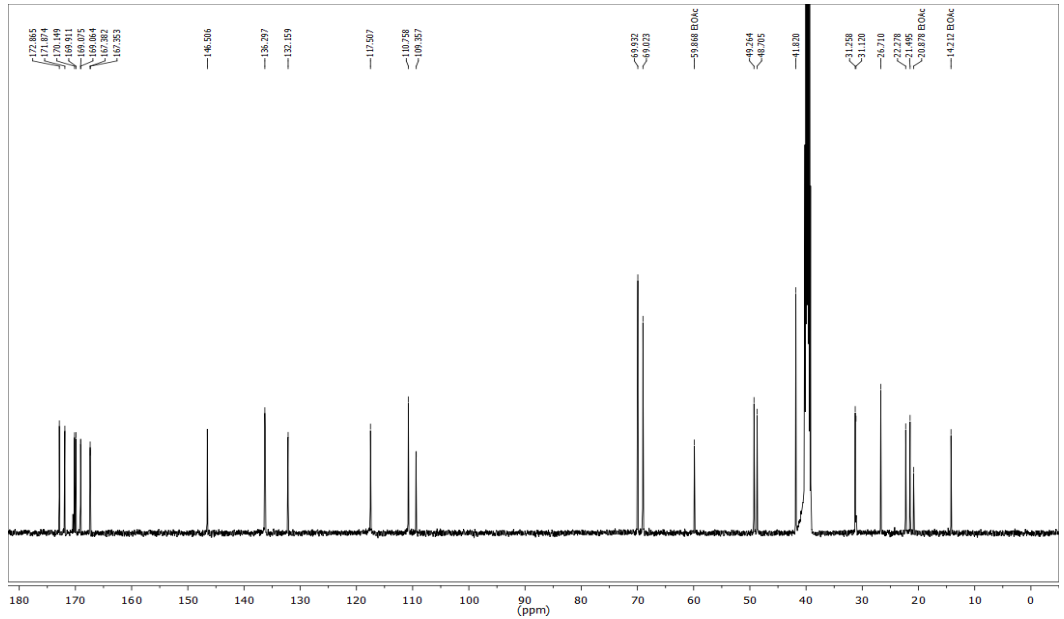
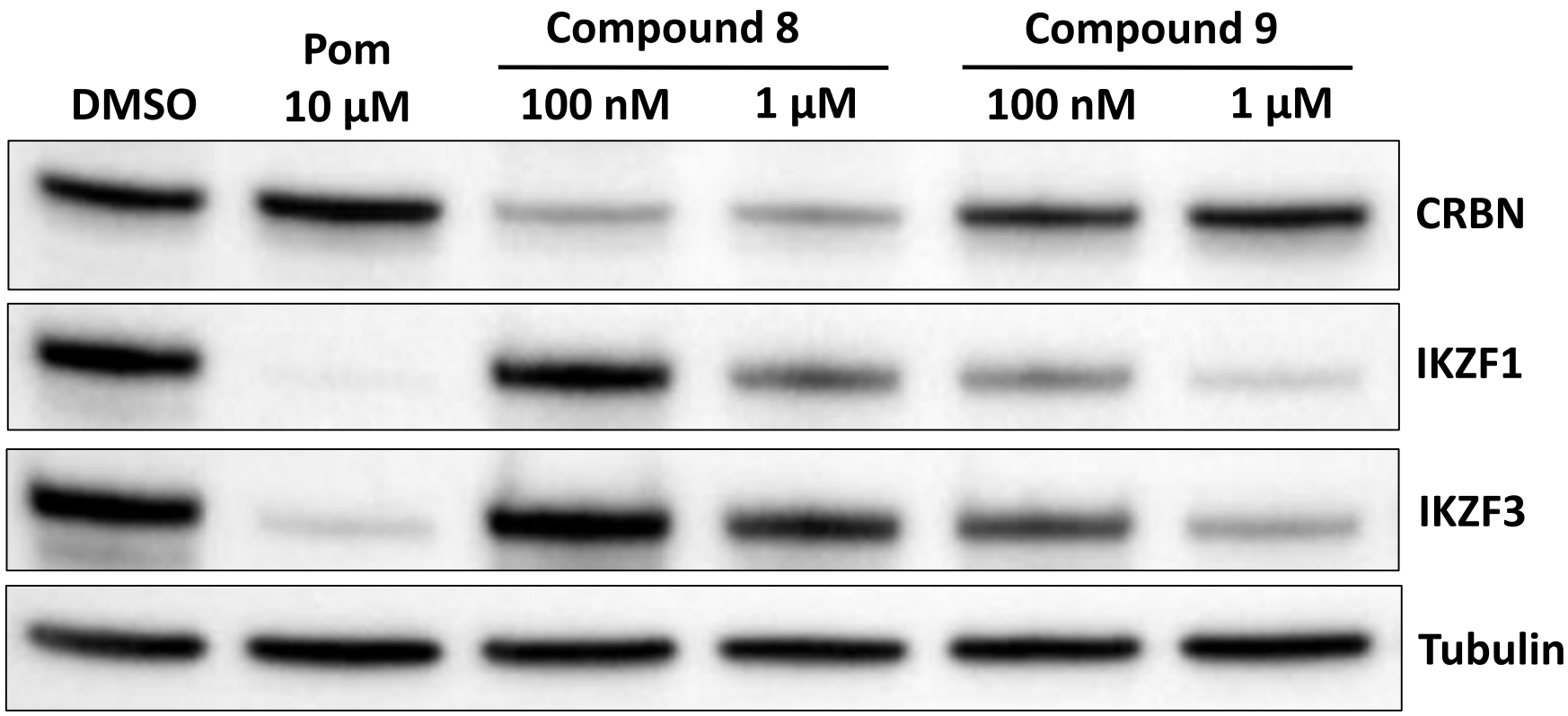


Figure 6



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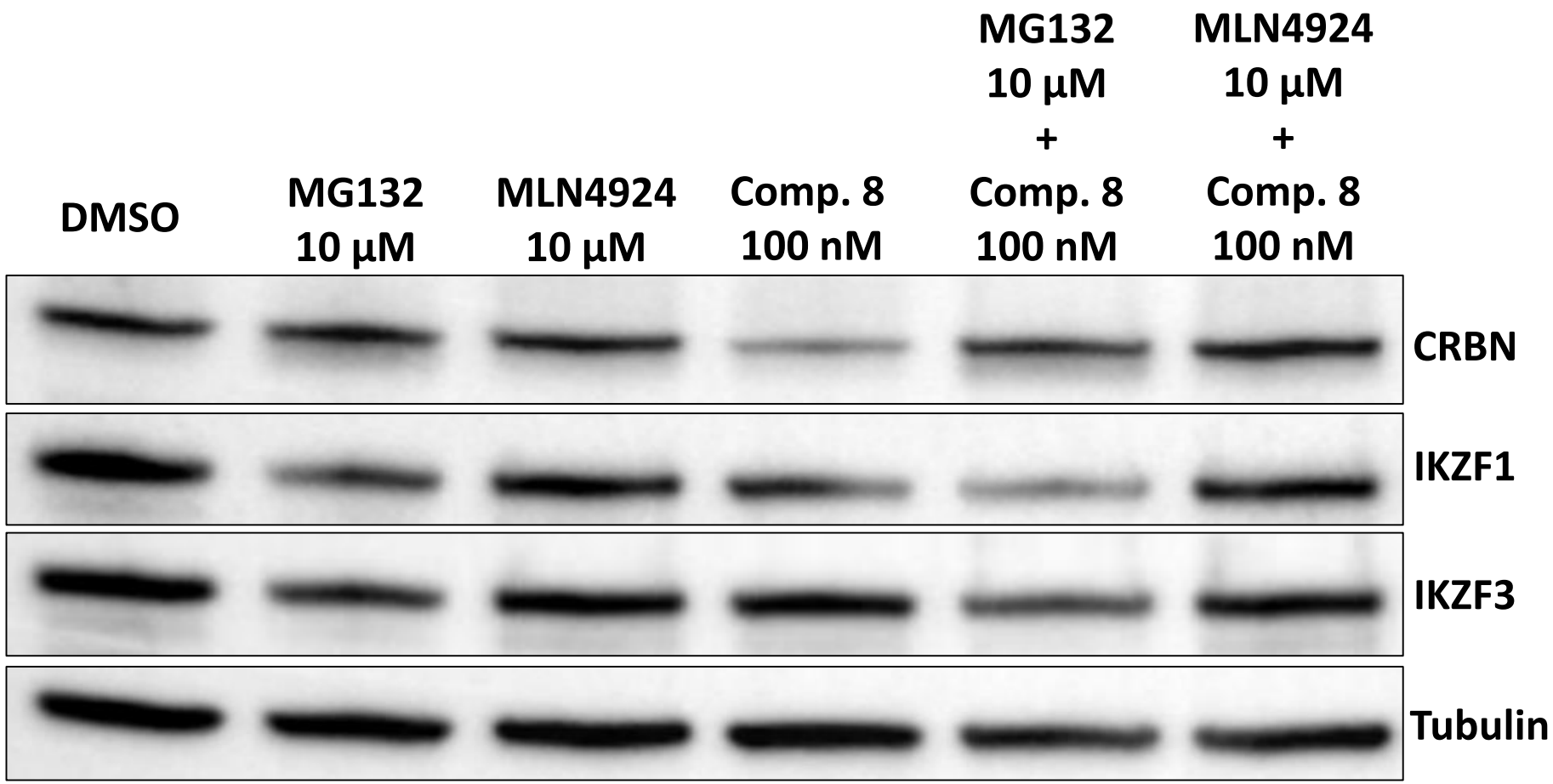

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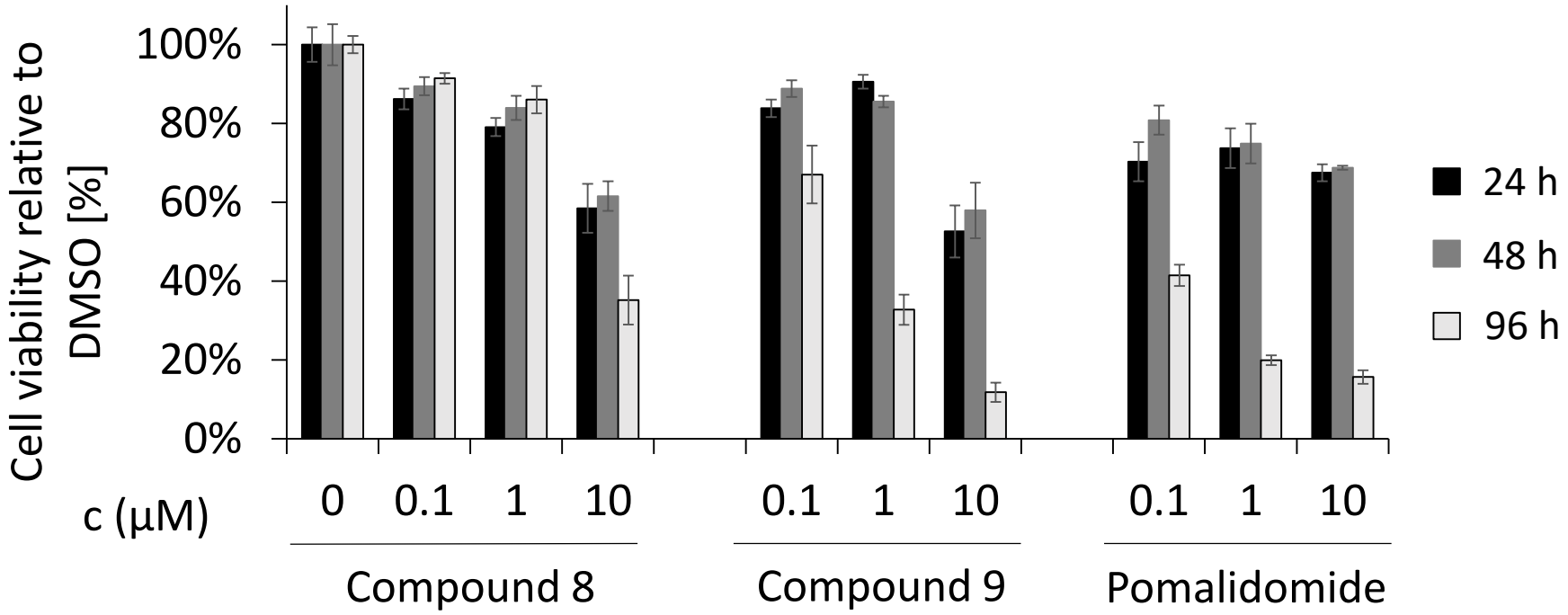
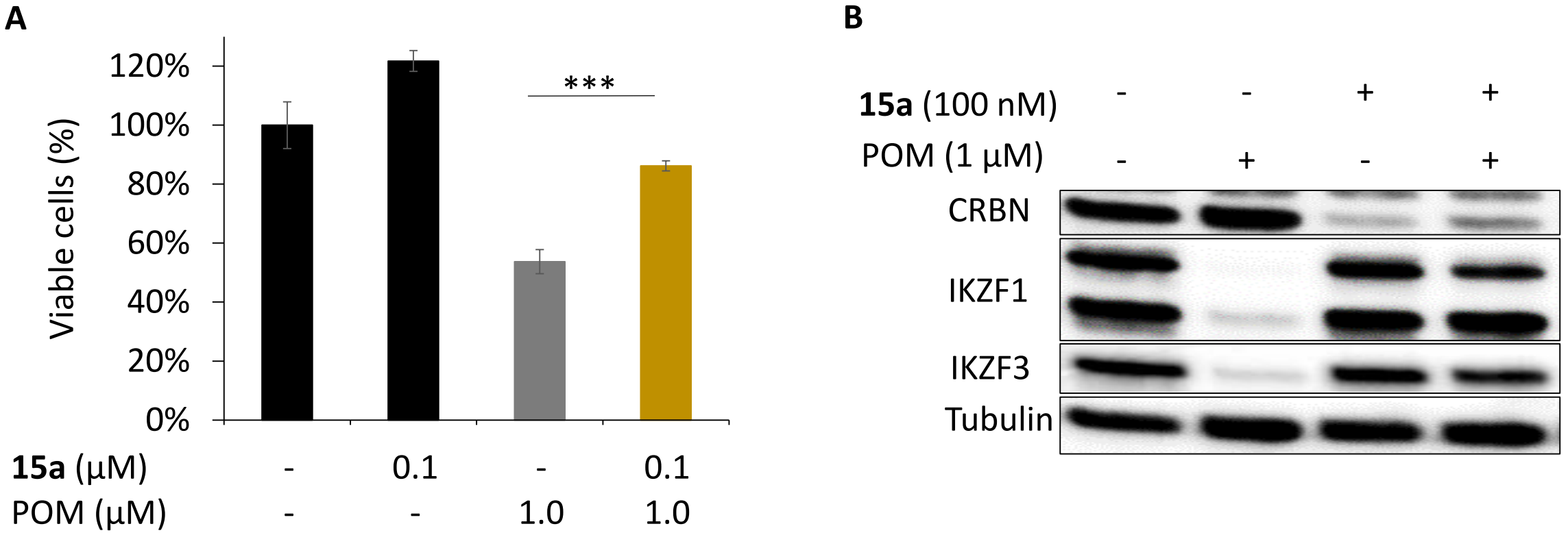


Figure 9



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Name of Reagent/Material

1,1'-Carbonyldiimidazole
2,2′-(Ethylenedioxy)-bis(ethylamine)
2-Mercaptoethanol
3-Fluorophthalic anhydride, 98 %
4-Dimethylaminopyridine, 99 %
Acrylamidstammlösung/ Bisacrylamid (30%/0,8%)
Aiolos (D1C1E) mAB
Anti-CRBN antibody produced in rabbit
Anti-rabbit IgG HRP-linked antibody
Ammonium Persulfate
Boc-Gln-OH
Bovine Serum Albumin
CellTiter-Glo Luminescent Cell Viability Assay
ChemiDoc XRS+
DMF, anhydrous, 99.8 %
DMSO, anhydrous, 99.7 %
Glycine
Goat anti-mouse (HRP conjugated)
Halt Protease & Phosphatase Inhibitor Single-use Cocktail (100X)
Ikaros (D6N9Y) Mab
ImmobilonP Transfer Membrane (0,45µm)
Iodomethane, 99 %
Methanol
Mg132
Mini Trans-Blot electrophoretic transfer cell
Mini-PROTEAN Tetra Vertical Electrophoresis Cell
MLN4942
Monoclonal Anti-α-Tubulin antibody produced in mouse (B512)
N-Ethyldiisopropylamine, 99 %
Nonfat dried milk powder
Nunc F96 MicroWell White Polystyrene Plate
NuPAGE LDS Sample Buffer (4X)
Pierce BCA Protein Assay kit
Pomalidomide
RestoreTM Western Blot Stripping Buffer
sodium dodecyl sulfate
Sodium Chloride
TEMED
tert-Butyl N-[2-[2-(2-aminoethoxy)ethoxy]ethyl]carbamate
Tricin
Trizma base
Tween-20
WesternBright ECL spray

Company	Catalog Number	Comments
TCI chemicals	C0119	
Sigma-Aldrich	385506	Compound 6
Sigma-Aldrich	M6250	
Alfa Aesar	A12275	
Acros	148270250	Toxic
Carl Roth	3029.1	
Cell signaling	15103S	
Sigma	HPA045910	
Sigma	7074S	
Roth	9592.2	
TCI chemicals	B1649	
Sigma-Aldrich	A7906-100G	
Promega	G7571	
Bio-Rad	1708265	
Acros	348435000	Extra Dry over Molecular Sieve
Acros	348445000	Extra Dry over Molecular Sieve
Sigma-Aldrich	15523-1L-R	
Santa Cruz biotechnology	sc-2005	
Thermo Scientific	1861280	
Cell signaling	14859S	
Merck	IPVH000010	
Sigma-Aldrich	I8507	Highly toxic
Sigma-Aldrich	32213-2.5L	
Selleckchem	S2619	
Bio-Rad	1703930	
Bio-Rad	1658004	
biomol (cayman)	Cay15217-1	
Sigma	T5168	
Alfa Aesar	A11801	
PanReac AppliChem	A0830,0500	
Thermo Scientific	136101	
Thermo Scientific	NP0008	
Thermo Scientific	23225	
Selleckchem	S1567	
Thermo Scientific	46430	
Carl Roth	183.1	
Sigma-Aldrich	A9539-500g	
Carl Roth	2367.3	
Sigma-Aldrich	89761	Compound 5
Carl Roth	6977.4	
Sigma-Aldrich	T1503-1kg	
Sigma-Aldrich	P7949-500ml	
Advansta	K-12049-D50	

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Name:	Jan Kränke
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Institution:	University Hospital Ulm
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Yours sincerely,

Jan Krönke

Department of Internal Medicine III
University Hospital Ulm
Albert-Einstein-Allee 23
89081 Ulm
Germany

jan.kroenke@uni-ulm.de
Tel. +49 731 500 45718

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We carefully proofread the manuscript.

2. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

ALA has been signed and uploaded with the revised manuscript.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The permission to reuse the adapted figures in this journal is granted by ACS Chemical Biology and indicated in the manuscript on page 11, Figure legend 7:" Reprinted (adapted) with permission from Steinebach, C. et al. Homo-PROTACs for the Chemical Knockdown of Cereblon. ACS Chem Biol. **13** (9), 2771-2782, 2018. Copyright 2019 American Chemical Society."

4. Figure 1: Please number different panels as separate/individual movies/figure.

All panels of figure 1 were number as separate figures.

5. Figure 2: Please delete "))),".

This was deleted.

6. Figure 3: Please define panels A and B in the figure legend.

Panels have been defined as requested.

7. Figure 6 and Figure 7B: Please include a space between numbers and their units (24 h, 48 h, 100 nM, etc.).

We have corrected the figures accordingly.

8. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

We uploaded all figures separately as .pdf file.

9. Please revise lines 41-44 and 71-73 to avoid previously published text.

Lines revised.

10. Affiliations: Please provide an email address for each author.

Email addresses for each author have been provided within the submission process.

11. Please define all abbreviations before use.

We defined all abbreviations.

12. Please use centrifugal force (x g) for centrifuge speeds.

We specified all centrifugation steps in units x g.

13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed?
Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
14. 1.1.6: Please describe how this step is done.
15. 1.2.3: Is this done at room temperature?
16. 2.1.1.1: Please describe how the treatment is done and specify the treatment time.
17. 2.1.1.2: What happens after centrifugation, the supernatant discarded?
18. 2.1.1.3: What is washed?
19. 2.1.3.8, 2.1.3.10: Please specify the primary/secondary antibody used in this step.
20. 2.1.3.13: Please specify incubation conditions.

We read carefully through the protocol section and tried to specify each step.

21. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We read carefully through the protocol section and shortened the protocol.

22. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

We corrected the manuscript accordingly.

23. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

As reviewer #3 proposed, we would like to visualize the syntheses of the compounds as well as one validation experiment and marked these sections accordingly (yellow).

24. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

25. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

26. Please remove commercial language (ChemiDoc, Bruker Avance, etc.).

All commercial language has been removed and only listed in the table of Materials.

27. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.

We described all results and referenced to the figures at the beginning of each experimental setup and in the discussion.

28. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We added the following comment.
Thalidomide analogs, in general, are susceptible to hydrolytic decomposition and should only be used in the next step after sufficient drying. Compound **3** is susceptible to an aromatic nucleophilic substitution with primary aliphatic amines (Ref. 25). This conversion was found to proceed efficiently only when dry solvents are used.

29. Table of Materials: Please remove trademark (™) and registered (®) symbols and sort the items in alphabetical order according to the name of material/equipment.

We removed all trademarks in the table of Materials.

30. References: Please do not abbreviate journal titles.

We edited the references by Endnote using JoVE´s suggested style. We hope the references are in the right style now.

Reviewer #1:
Manuscript Summary:
In the manuscript the authors describe the synthesis and application of a pomalidomide-based PROTAC and analyze its potential to selectively induce the auto ubiquitination and degradation of CRBN using PROTACS. The authors describe the synthesis of a novel pomalidomide pomalidomide compound that specifically targets CRBN

Major Concerns:
No major concerns

Thank you for the strong vote of support!

Reviewer #2:

Manuscript Summary:

The authors have done expert chemical synthesis to create a symmetrical small molecule that selectively promotes autodegradation of CRBN.

Major Concerns:

1) The Introduction does not adequately state the goal of this research, i.e., the reason why compound 8 has been made and the experiments performed. It appears to be stated at the end of the Abstract: "Homobifunctional CRBN degraders will be useful tools for future biomedical investigations on CRBN-related signaling and may help to understand the molecular mechanisms of thalidomide analogs." However, this needs to be emphasized more and better. It is already known that thalidomide and its analogs (lenalidomide and pomalidomide) work through CRBN, so there is little to be gained from demonstrating that cells are rescued from them by CRBN degradation via the homo-PROTAC compound 8. Compound 8 provides proof of the general principle that an E3 ubiquitin ligase can be selectively eliminated by a small molecule, potentially opening a new path in drug development. In the particular case of compound 8, it may be useful to have a CRBN degrader for studies of the biology and proteome regulated by CRBN, as stated at the end of the Abstract. However, as a research tool, there is substantial competition from genetic approaches, or chemical genetic approaches (e.g., fusion to a ligand-controlled degron). The relative merits of ProTACS vis-à-vis those other approaches should be discussed.

We thank reviewer #2 for the comment. Here, we describe the synthesis of highly selective and potent homodimeric CRBN degraders. We agree that genetic approaches like CRISPR-mediated knockout of CRBN are likewise potent and specific and also confer resistance to IMiDs, as already mentioned in a previous publication (Ref.24 S6A). However, the major advantage of a chemical degrader is that it is reversible, rapid and can be direct to a wide spectrum of target cells and tissue types (Ref:28). We discussed this aspect in our manuscript.

2) Another deficiency of the presentation is that the authors do not provide a chemical mechanism, even theoretical, as to how compound 8 works, and/or how they designed their ultimate approach. The ImIDs were not originally conceived of as ProTACs; instead, after long and brilliant research, it was elucidated that they had certain protein targets (IKZF1, IKZF3, CK1α, and SALL4) that were directed to CRBN for ubiquitination and degradation. I was not aware that the structural determinants of the IMiDs responsible for these two functions (targeting and CRBN binding) had been determined, but it would be logical to link two copies of the CRBN binding moiety, without the other parts, to make a homo-ProTAC for CRBN degradation, which also does not affect original IMiD targets such as IKZF1 and IKZF3. None of that is explained; it is merely suggested by Figure 1C.

We thank reviewer #2 for this comment. We agree that a crystal structure of our PROTAC may underline the mechanism of action, but previously described crystal structures of CRBN-DDB1 complexes with IMiDs showed the precise mechanism of action and binding moieties of the molecules (Ref. 3, 8). It is a good idea to synthesize our compounds with only the glutarimide rings as the binding moiety, but already described PROTACs also contain all parts of the IMiD molecule.

Minor Concerns:
1) For the benefit of readers unfamiliar with the JoVE format, it should be stated at the outset of the Protocol section that there is a Table of all materials needed.

We included that information at the beginning of the Protocol section: "All reagents and materials used in this paper are listed in a table of materials attached. "

2) Page 8, line 215: "2.1.1.1) Treat 1x106 MM1S cells with compound 8 or 9 for indicated time points." What is/are the dose(s) to be used?

We included the doses of the compounds:
2.1.1.1) Seed 1x 10^6 MM1S cells in a 6-well plate with 2.5 ml media and treat cells with 100 nM or 1 µM compound **8** or **9** for 24h.

3) Page 8, line 217: "2.1.1.2) Harvest cells after treatments and centrifuged at 2800 RPM". Should be present tense, "centrifuge". More important, the centrifuge to use needs needs to be specified, or stated in units x g.

We corrected the tense and specified all centrifugation steps in units x g.

4) Page 8, line 225: "2.1.1.5) Measure protein concentration by a BCA assay according to the manufacturer's protocol." "BCA" is an example of an undefined acronym.

We corrected the abbreviation BCA:
2.1.1.5) Measure protein concentration by a bicinchoninic acid protein assay (BCA assay) according to the manufacturer's protocol.

5) Page 8, line 226: "Load 15-30 µg of protein." Presumably this refers to how much protein to load per well for protein electrophoresis. This is done after the next step, of course: "2.1.1.6) Denature proteins with 1x LDS loading buffer (5% 2-Mercaptoethanol) and boil 10 min, 75 °C."

We added the sentence "Load protein sample from 2.1.1.4." to section 2.1.2.2.

6) Page 9, line 260: "2.1.3.8) Incubate membrane in primary antibody solution (in 5% BSA, TBS-T) with gentle shaking at 4°C, over night." The source of primary antibody needs to be specified.

We specified every antibody used in our protocol. All details are included in the table of materials.

7) Page 9, line 278: "2.1.3.16) Strip membrane in stripping buffer for 15 min." The stripping buffer needs to be specified. Also, why is this being done? There is no subsequent reprobing of the blot specified. Does the stripping merely need to be enough to inactivate the horseradish peroxidase, or do the antibodies need to be removed?

We thank reviewer #2 for this observation and totally agree that the protocol needs to be specified in regard of stripping and reprobing. We changed this section accordingly:

2.1.3.7) Incubate membrane for 2 min with HRP substrate solution according to manufacturers' protocol and detect chemiluminescence in a chemiluminescence detection device.

2.1.3.8) Wash membrane 1x in 1X TBS for 5-10 min each at room temperature. For release of antibodies, strip membrane in commercial available stripping buffer for 15 min. Wash membrane 3x in 1X TBS for 5-10 min each at room temperature.

2.1.3.9) Reblock membrane in in 5% nonfat-dried milk, TBS-T for 1 h at room temperature. Wash membrane 3x in 1X TBS-T for 5-10 min each at room temperature and reprobe with IKZF1, IKZF3 or tubulin according to 2.1.3.4.

8) Page 10, line 304: "2.3.1) Seed 5x105 MM1S cells per well in a 96-well plate and in 1x106 MM1S cells per well in a 6-well plate biological triplicates." Presumably this means "in biological triplicates." 5x105 MM1S cells per well in a 96-well plate is likely to produce a much higher cell concentration than would 1x106 MM1S cells per well in a 6-well plate. Most likely, what is meant is "5x104 MM1S cells per well in a 96-well plate". In any case, the concentration should be specified, and should be the same regarding of the well size.

Again, we thank the reviewer #2 for this very thoughtful observation. We corrected this mistake in the revised manuscript.

9) Animated Figure 1B could not execute on my computer.

We apologize for this and tested the video by running it on various computers.

Reviewer #3:

Notes to the authors:

1. While the authors discuss the potential future use of homo-PROTACs, the purpose of this study remains vague. A quantitative mass-spec analysis of MM1s cells with/without treatment would have been a valuable addition to identify the native protein targets of CRBN.

We thank reviewer #3 for that comment. The reviewer raises the importance of the identification of new proteins that may be endogenous CRBN substrates and may possess pharmacological relevance. We totally agree with this aspect and already performed TMT-labeling MS proteomics experiments in our previous publication (Ref. 24, Homo-PROTACs for the Chemical Knockdown of Cereblon) to characterize potential off-target effects in an unbiased fashion at the global proteome level. Our proteomic analyses also revealed several candidates that may represent endogenous substrates of the CRBN E3 ligase whose levels increase after treatment with our compounds or pomalidomide. However, functional validation and biological evaluation of potential new substrates will require a multitude of experiments and time. Therefore, we believe that this is beyond the scope of our manuscript. The focus of our present manuscript lies on the preparation and functional validation of the compounds. Quantitative mass spectrometry is a

highly sophisticated and complex analyses and inclusion would have extended the manuscript dramatically what is the reason that we did not include it but referred to our previous manuscript. We totally agree that further analyses should be performed to identify additional CRBN substrates.

2. It is unclear what purity the dimers 8 and 9 have. Any contamination with monomeric pomalidomide would have confounding effects. In this regard, how would the authors argue that compound 9, the methylated negative control, induces IKZF degradation at higher concentrations (Fig. 4)? The same for compound 8?

We added the following notation with respect to the purity of **8** and **9**.
1.7.8) Elucidate and verify molecule structure (Figure 5A compound **8**, 5B compound **9**) by ^{1}H NMR and ^{13}C NMR spectra in DMSO-d_6 on a nuclear magnetic resonance (NMR) spectrometer. Check the purity of both compounds to be higher than 97% by means of liquid chromatography-mass spectrometry (LC-MS) applying a diode array detection (DAD) at 220 – 500 nm.

Traces of pomalidomide are not responsible for neo-substrate degradation because (i) the purity of the PROTACs was checked by LC-MS and NMR as noted in the revised version and (ii) pomalidomide is not an intermediate in these syntheses. Also, remaining degradation of neo-substrates has been observed in other IMiD-based PROTACs (Ref. 12-21).

3. The power of JOVE is the visualization of the actual experiments or reagent generation. A video showing the PROTAC preparation would have been valuable.

We thank the Reviewer for this suggestion and agree that a major cohesive aspect of our manuscript is the synthesis of the PROTAC. We included steps of the molecule preparation for visualization.

4. The manuscript is overall well written. However, Fig. 7b (and possibly 7a) were taken from a previous publication (Ref. 32, Fig. 5b), which violates accepted standards of (self)plagiarism.

We agree with Reviewer #3 that we already published part of the figures in a previous publication (Ref.24 in review manuscript). The permission to reuse the adapted figures in the JoVE journal is granted by ACS Chemical Biology and indicated in the manuscript on page 7, Figure legend 9:" Reprinted (adapted) with permission from Steinebach, C. et al. Homo-PROTACs for the Chemical Knockdown of Cereblon. ACS *Chem Biol*. **13** (9), 2771-2782, 2018. Copyright 2019 American Chemical Society." According to the JoVE editors the inclusion of previously published figures is accepted.