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ACS Chem. Biol., **Just Accepted Manuscript** • DOI: 10.1021/acschembio.8b00693 • Publication Date (Web): 17 Aug 2018

Homo-PROTACs for the Chemical Knockdown of Cereblon

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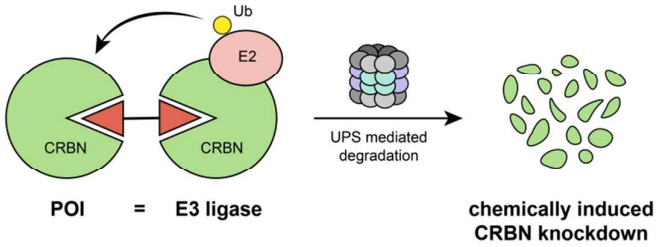
Graphical Abstract



ABSTRACT: The immunomodulatory drugs (IMiDs) thalidomide, lenalidomide, and pomalidomide, all approved for the treatment of multiple myeloma, induce targeted ubiquitination and degradation of Ikaros (IKZF1) and Aiolos (IKZF3) via the cereblon (CRBN) E3 ubiquitin ligase. IMiD-based proteolysis targeting chimeras (PROTACs) can efficiently recruit CRBN to a protein of interest leading to its ubiquitination and proteasomal degradation. By linking two pomalidomide molecules, we designed homobifunctional, so-called Homo-PROTACs and investigated their ability to induce self-directed ubiquitination and degradation. The homodimerized compound **15a** was characterized as a highly potent and efficient CRBN degrader with only minimal effects on IKZF1 and IKZF3. The cellular selectivity of **15a** for CRBN degradation was confirmed at the proteome level by quantitative mass spectrometry. Inactivation by compound **15a** did not affect proliferation of different cell lines, prevented pomalidomide-induced degradation of IKZF1 and IKZF3 and antagonized the effects of pomalidomide on multiple myeloma cells. Homobifunctional CRBN degraders will be useful tools for future biomedical investigations on CRBN-related signaling and may help to further elucidate the molecular mechanism of thalidomide analogs.

INTRODUCTION

In the past two decades, a paradigm shift from predominant inhibition of a protein target by occupancy-based drugs to the modulation of the target's intracellular protein levels has occurred. As known from natural products, endogenous ligands are able to regulate substrate recruitment to ubiquitin ligase complexes. The plant hormones auxin and jasmonate represent prominent examples of E3 ligase modulation.[1,2] The IMiDs thalidomide, lenalidomide, and pomalidomide, all approved for the treatment of multiple myeloma, were found to bind CRBN,[3–5] a substrate adaptor for the CRL4 E3 ubiquitin ligase ($CRL4^{CRBN}$), thereby facilitating the ubiquitination and the subsequent proteasomal degradation of substrates. All IMiDs enhance the binding of the lymphoid transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) to $CRL4^{CRBN}$, leading to an increased ubiquitination and degradation.[6–10] Depletion of IKZF1 and IKZF3 results in growth inhibition in multiple myeloma cells. This mode of action of IMiDs is further supported by crystal structures that provided insights into the binding mode of IMiDs to $CRL4^{CRBN}$ and the function of cereblon as a substrate adaptor.[3,11,12]

The ability of small-molecules like IMiDs to target a protein for degradation is an exciting implication for modern drug development. Another approach for chemical-induced ubiquitination and degradation of disease-causing proteins are heterobifunctional molecules, which are also known as proteolysis targeting chimeras (PROTACs).[13,14] To direct an E3 ubiquitin ligase to a protein of interest (POI) and induce the ubiquitination and subsequent proteasomal degradation of the POI, PROTACs consist of a target binding moiety and an E3 ligase recruiting molecule connected via a variable linker. Simultaneous binding to the target protein and a ubiquitin ligase leads to a transient ternary complex of the ligase, the POI, and the bifunctional PROTAC itself. The induced proximity of the target and E3 ligase enables a ubiquitin transfer to the POI, which can then be recognized and degraded by the proteasome system.[15–17] IMiDs represent an excellent starting point for the assembly of heterobifunctional compounds, which includes the CRBN-binding substructure and a linker-connected ligand of the corresponding POI.

A particular advantage of this technology is that chimeric molecules act catalytically and are therefore more potent than the ligands from which they derive. Once ubiquitin labeling of the POI is

completed, the PROTAC dissociates and is capable of forming a new ternary complex. Thus, a very low PROTAC concentration is sufficient for the degradation of the target protein.[18]

Current chemical-induced knockdown strategies are mainly focused on substrate adaptors of E3 ligase complexes, i.e. Von-Hippel-Lindau (VHL) and CRBN. By addressing VHL or CRBN, the degradation of specific targets, such as proteins of the bromo- and extra-terminal domain (BET) and non-BET families, sirtuin 2, the estrogen-related receptor alpha, the BCR-ABL fusion protein, dihydroorotate dehydrogenase, CDK9, and other kinases have been demonstrated.[19–35]

Here we describe the synthesis of homobifunctional PROTACs that utilize CRBN as the hijacked degrader and, at the same time, as the protein targeted for degradation. In such Homo-PROTACs, identical linker-connected moieties are incorporated into a single molecule. This design accomplishes a chemical-induced CRBN knockout.

RESULTS AND DISCUSSION

Synthesis of Homo-PROTACs.

The preparation of phthalimido-glutarimides (Figure 1) was performed by condensation of phthalic anhydrides **1**-**3** with Boc-protected 2-amino-glutarimide **4** in refluxing glacial acidic acid in the presence of sodium acetate.[36,37] The reaction conditions led to the successive deprotection, ring-opening, and recyclization and furnished the intermediate compounds **6** and **11** as well as thalidomide (**9**). The other monocyclic building block, compound **5**, obtained through methylation of **4**, was similarly used for the preparation of intermediates **7** and **12** and *N*-methyl-thalidomide (**10**). This was also accessible by *N*-methylation of thalidomide (**9**), and by an ultrasound-supported Mitsunobu reaction[38] of **9** and methanol. Here, sonication was advantageous to circumvent steric constraints and to account for the relatively low acidity of the imide. The mild Mitsunobu conditions appear to be generally useful for the *N*-methylation at the CRBN-binding portion of PROTACs in order to produce negative control compounds. As their structures are part of our final linker-connected dimers, pomalidomide (**13**) and *N*-methyl-pomalidomide (**14**) were required as control compounds. They were obtained by a palladium-catalyzed hydrogenation of their corresponding 4-nitro precursors **11** and **12**.

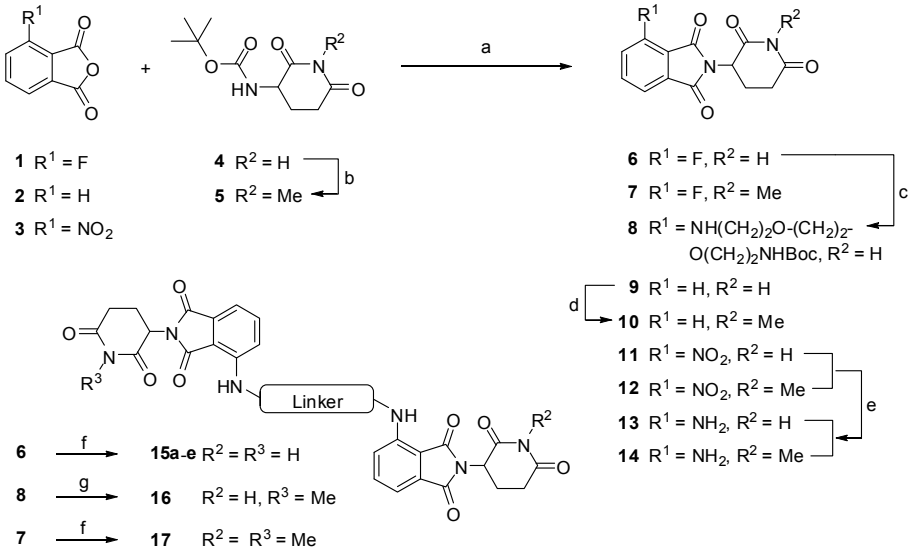
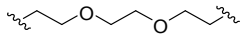
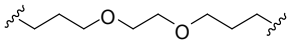
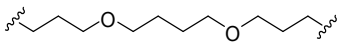
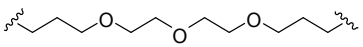
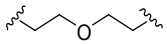
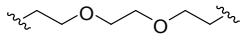
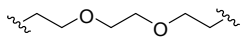
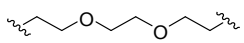
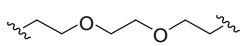


Figure 1. Synthesis of thalidomide derivatives and PROTACs. Reagents and conditions: (a) NaOAc, AcOH, reflux, 6 h; (b) MeI, K_2CO_3, DMF,))), rt, 2 h; (c) *tert*-butyl *N*-[2-[2-(2-aminoethoxy)ethoxy]ethyl] carbamate, DIPEA, DMF, 90 °C, 10 h; (d) DIAD, PPh_3, MeOH, THF,))), rt, 1 h; (e) Pd/C, H_2, DMF, rt, 8–24 h; (f) α,ω-diamine, DIPEA, DMSO, 90 °C, 18 h; (g) 1. TFA, CH_2Cl_2, 40 °C, 2 h; 2. **7**, DIPEA, DMF, 90 °C, 10 h.

4-Fluoro-thalidomide (**6**) allowed the synthetic access to introduce a linker at position 4 leading to alkylated pomalidomide derivatives. Such a linker attachment, forming a secondary amine, has been established in PROTAC design.[27,31,35] Accordingly, the synthesis of five homodimeric PROTACs was finalized by reacting **6** with different α,ω-diamines in a 2:1 molar ratio. Initially, DMF was used as a solvent but was found to be less appropriate. In the presence of a tertiary amine and at high temperature, DMF undergoes thermal decomposition to produce dimethylamine,[36] which competes in the aromatic nucleophilic substitution, generating the undesired 4-dimethylamino-thalidomide. Instead, performing the reaction in DMSO led to the final CRBN Homo-PROTACs **15a-e** (Table 1). Similar, their structures were confirmed by MS and NMR data. Due to the mirror plane of **15a-e**, symmetry-equivalent nuclei show identical resonances in ^{1}H and ^{13}C NMR spectra. PROTACs **15a-e** all contain two pomalidomide moieties connected via a linker of various lengths between 5 to 13 linear atoms, introducing varying hydrophobicity into the PROTAC molecules. It is known that the linker length is critical for the activity of PROTACs and that the nature of the linker needs to be fine-tuned over the course of their structural optimization.[26,39]

Table 1. Homo-PROTACs with the corresponding linker substructures and their biological activities.

Cmpd.	Linker	No. of linear linker atoms	CRBN degradation	IKZF1 degradation	CK1α degradation
15a		8	++	+	-
15b		10	+	++	-
15c		12	+	++	-
15d		13	+	++	-
15e		5	++	+	-
16[a]		8	-	++	n.d.[f]
17		8	-	-	n.d.
18		8	+	+	-
19		8	±	+	-
THAL[b]	n.a.[e]	-	-	+	-
10	n.a.	-	-	-	-
POM[c]	n.a.	-	-	++	-
14	n.a.	-	-	-	-
LEN[d]	n.a.	-	-	++	+

[a]Heterodimeric PROTAC. [b]Thalidomide (**9**). [c]Pomalidomide (**13**). [d]Lenalidomide. [e]not applicable. [f]not determined.

Compound **16** represents a heterodimer with pomalidomide and *N*-methyl-pomalidomide as terminal moieties. Its synthesis was not feasible by a Mitsunobu reaction or methylation of the homodimeric analog **15a**. The synthesis was accomplished in a linear approach by first performing the nucleophilic substitution of **6** with $BocNH(CH_2)_2O(CH_2)_2O(CH_2)_2NH_2$ followed by deprotection and the second nucleophilic substitution of **7**. Applying the condition to produce **15a-e**, the homodimeric compound **17** was synthesized from the methylated building block **7** and $H_2N(CH_2)_2O(CH_2)_2O(CH_2)_2NH_2$ in a 2:1 reaction. The introduction of one or two methyl group(s) at the glutarimide portion is expected to abolish the binding of two CRBN molecules at the same time and hence to form ternary complexes which are required for PROTAC-mediated self-degradation.

Pomalidomide-based Homo-PROTACs induce degradation of CRBN.

In order to determine the effect of different Homo-PROTACs, we performed western blot analyses of the multiple myeloma cell line MM1S that expresses endogenous CRBN and its IMiD-induced neo-substrates IKZF1, IKZF3, and casein kinase 1A1 (CK1α). After treatment with compounds **15a-e** at nanomolar concentrations for 16 hours, we observed a reduction of CRBN protein levels (Figure 2). In contrast, the IMiDs thalidomide, pomalidomide, and lenalidomide had no effect on CRBN protein levels, but induced degradation of IKZF1, consistent with previous observations.[6,7,9,12]

All of our CRBN Homo-PROTACs also induced a dose-dependent decrease of IKZF1 protein levels in MM1S cells. However, we observed clear differences in the impact on IKZF1 degradation: compounds **15a** and **15e** with linker sizes of 8 and 5 linear atoms, respectively, had only minor effects on IKZF1 protein levels, while compounds **15b**, **15c**, and **15d** with linker sizes of 10-13 atoms led to a pronounced degradation of IKZF1 (Figure 2A–C). None of the homodimers affected the level of CK1α, a CRBN neo-substrate that is specifically targeted for degradation by lenalidomide.[8] None of the compounds produced a change in protein levels of CUL4A, which is part of the $CRL4^{CRBN}$ E3 ligase complex (Figure 2D).

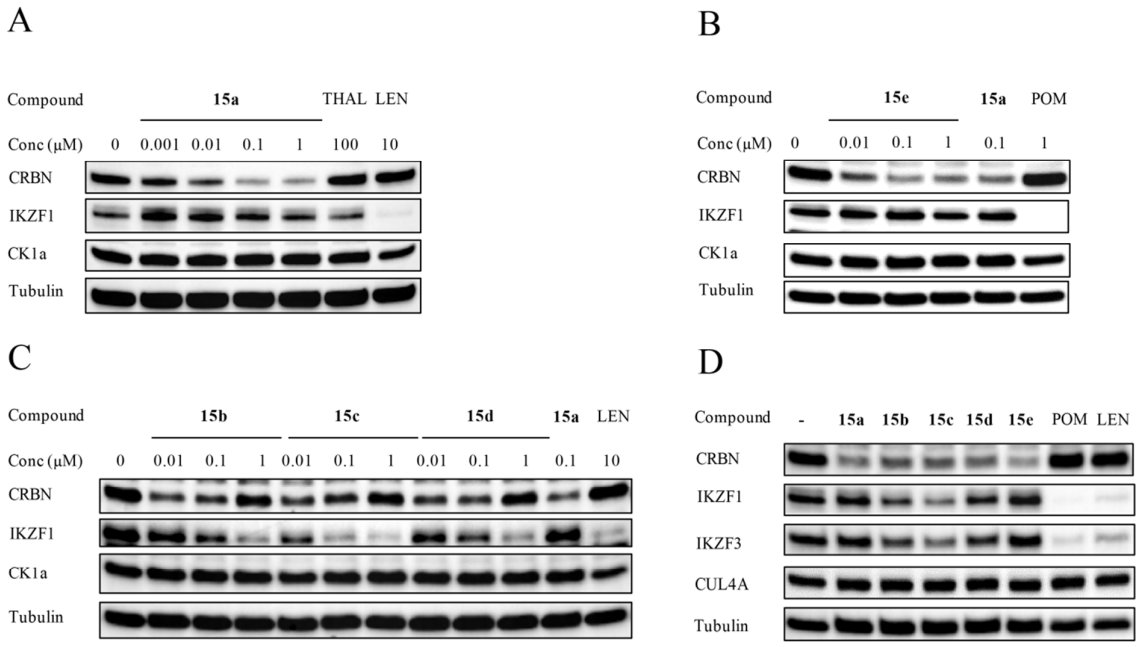


Figure 2. Pomalidomide-based Homo-PROTACs induce degradation of CRBN. (A) The multiple myeloma cell line MM1S was treated with compound **15a**, (B) compound **15e** and (C) compounds **15b**, **15c**, **15d** for 16 hours at the indicated concentrations. (D) Direct comparison of pomalidomide-derived PROTACs at a concentration of 100 nM, 1 μM pomalidomide (POM), and 10 μM lenalidomide (LEN).

Compound **15a** with a linker length of 8 atoms was identified to be the most potent CRBN degrader. Accordingly, we maintained the size of the linker, but modified the chemical attachment to the phthalimide core and included two analogues of **15a**. The structures of **18** and **19** (Figure 3A) were accessible *via* selective Mitsunobu alkylation[40] and carboxamide formation, respectively (see Supplementary Figure 9 for the synthetic routes). Both PROTACs exhibited a weaker capability for the chemically induced CRBN degradation (see Figure 3B and Supplementary Figure 1A) and were not further investigated. These data indicate in this case the advantage of a linker attachment *via* a secondary amine structure. The following biological evaluations have thus been performed with **15a**, the most advantageous Homo-PROTAC of this study.

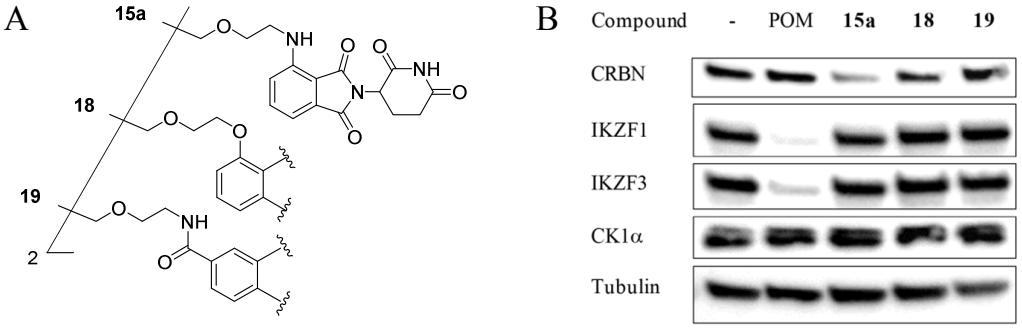


Figure 3. (A) Structures of analogous Homo-PROTACs with different attachments of the linker. (B) Comparison of the induction of CRBN, IKZF1, IKZF3 and CK1α degradation by **15a**, **18**, **19** and pomalidomide, each at a concentration of 100 nM.

Compound 15a induces CRBN-mediated ubiquitination and proteasomal degradation.

Homo-PROTAC **15a** decreased CRBN protein levels in a time- and dose-dependent manner. An effect could be observed at a concentration as low as 10 nM after incubation for 16 hours (Figure 4A). Maximum CRBN degradation was achieved with a concentration of 100 nM to 1 μM. Further increase of the concentration to 100 μM abrogated the degradation of CRBN, which is likely explained by the resulting saturation of each CRBN molecule to form binary complexes. This finding is in agreement with the "hook effect" observed for other PROTACs at higher concentrations, whereby the formation of the ternary complexes is limited due to predominant univalent saturation of the excess bifunctional ligand compared to protein partners.[41–44] At the concentration range of the "hook effect", a strong degradation of IKZF1 was observed (Figure 4A). The decrease of CRBN protein levels was observed within 15 minutes of treatment with 100 nM of compound **15a** and reached its maximum at 6 hours (Figure 4B and Supplementary Figure 1B). The CRBN protein half-life was reduced to approximately 30 minutes with **15a** as compared to more than 8 hours in control cells treated with cycloheximide (CHX) to block protein translation (see Supplementary Figure 3). The effect of PROTAC **15a** on CRBN persisted for at least 96 hours (see Supplementary Figure 2A–B). After wash out of compound **15a**, CRBN depletion persisted for several hours and slowly recovered to approximately 50% after 24 hours (see Supplementary Figure 1C).[45] Compound **15a** strongly decreased CRBN protein levels in all

multiple myeloma (U266, RPMI-8226, OPM-2) and non-multiple myeloma cell lines (HEK293T, K562, OCI-AML5) tested (see Supplementary Figure 2C).

Introduction of a methyl group at the imino nitrogen of the glutarimide ring of pomalidomide or thalidomide (leading to compounds **14** and **10**, respectively) prevented binding to CRBN and abrogated degradation of IKZF1 (see Supplementary Figure 2D). The same modification on one of the pomalidomide moieties in a homodimeric PROTAC (leading to compound **16**, Figure 1, bottom) abrogated degradation of CRBN by preventing the simultaneous binding of two CRBN molecules and the induction of their proximity (Figure 4C). Co-immunoprecipitation with anti-FLAG in HA- and FLAG-tagged CRBN expressing HEK293T cells revealed that treatment with compound **15a** induced binding of two CRBN molecules (Figure 4D). These results further support that pomalidomide-based Homo-PROTACs induce CRBN ubiquitination and degradation by the formation of ternary complexes of a 2:1 stoichiometry with two CRBNs and one PROTAC, rather than by auto-ubiquitination or binding to another ubiquitin ligase. In contrast to **15a**, compound **16** caused a strong degradation of the IKZF1 protein, suggesting that a 2:1 ternary complex, formed from **15a** and two CRBN molecules, has a reduced capability to bind IKZF1. Accordingly, compound **17** with both imino nitrogens being methylated caused neither CRBN nor IKZF1 depletion (data not shown).

We next treated HEK293T cells transfected with a vector expressing FLAG-tagged CRBN. Homodimer **15a** resulted in effective degradation of ectopically expressed CRBN. In contrast, **15a** had no effect on the ectopically expressed $CRBN^{Y384A/\ W386A}$ mutant that does not bind IMiDs (Figure 4E).[4] This finding demonstrates that binding of the pomalidomide-homodimers to the IMiD binding site is essential for induction of CRBN degradation.

To determine whether compound **15a** degrades CRBN via the ubiquitin-proteasome pathway, we pre-incubated MM1S cells with the proteasome inhibitor MG132 and the neddylation activating enzyme (NAE) inhibitor MLN4924, which inhibits neddylation-dependent cullin-type ubiquitin ligases including $CRL4^{CRBN}$. Both, MG132 and MLN4924, prevented compound **15a**-mediated degradation of CRBN (Figure 4F). Pretreatment with excess thalidomide (1000×) or lenalidomide (100×) inhibited compound **15a**-mediated CRBN degradation consistent with a competition for the IMiD binding region of CRBN (Figure 4F).

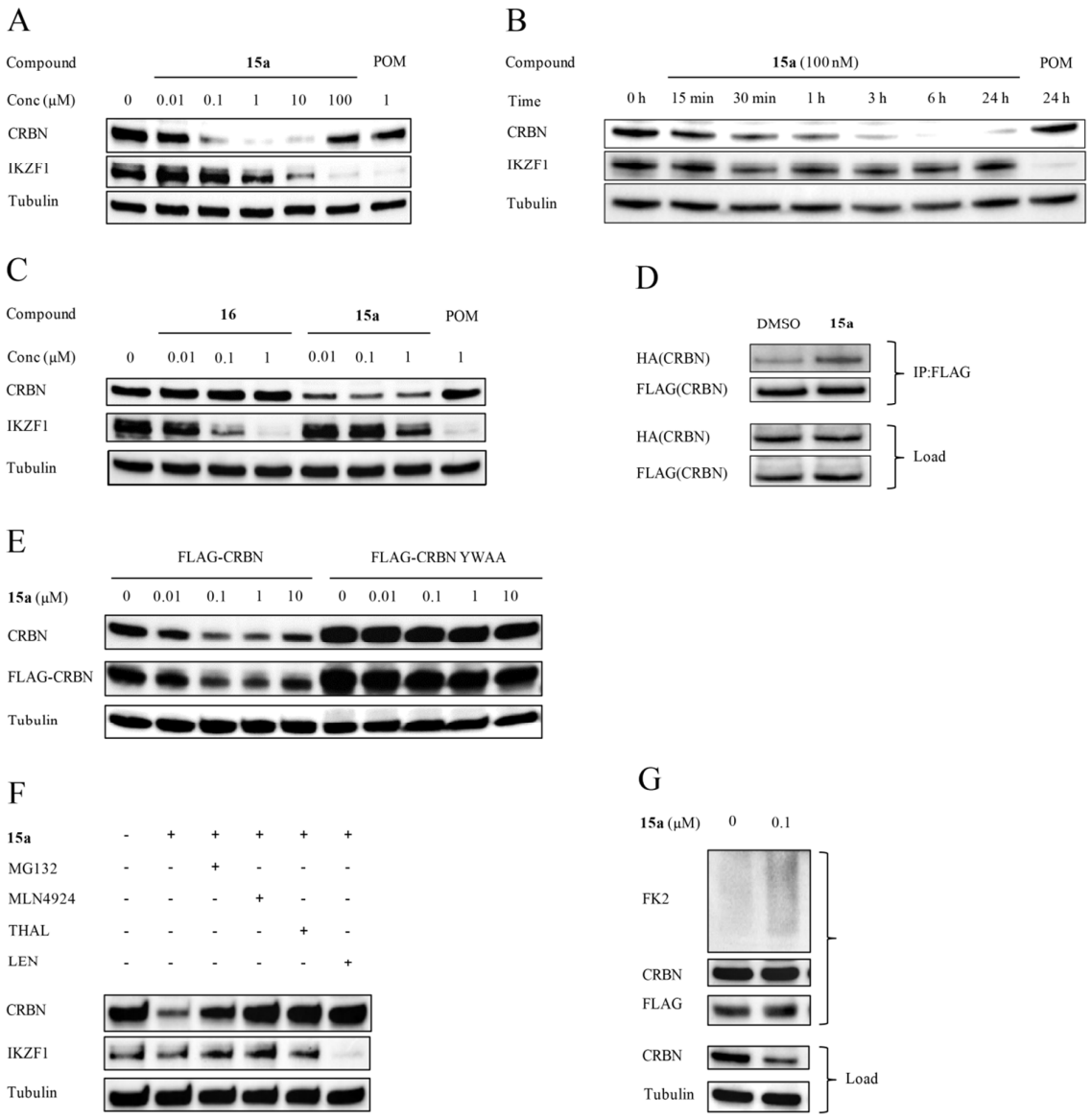


Figure 4. Compound **15a** induces ubiquitination and proteasomal degradation of CRBN. (A) The multiple myeloma cell line NCI-H929 was treated with compound **15a** for 16 hours. (B) Time course of 100 nM compound **15a** treatment in MM1S cells. (C) Effect of compound **16** that has a methyl group at one glutarimide ring predicted to abrogate binding to CRBN on MM1S cells. (D) Anti-FLAG immunoprecipitation in HEK293T cells expressing HA- and FLAG-CRBN. Cells were treated with 100 nM **15a** or control for 30 minutes. MLN4924 (10 μM) was added to prevent **15a**-mediated degradation of CRBN. (E) HEK293T cells expressing FLAG-tagged normal CRBN or the YW/AA mutation that prevents binding of IMiDs were treated with the indicated concentrations of compound **15a** for 16 hours. (F) MM1S cells were treated with vehicle, 10 μM MG132, 10 μM MLN4924, 100 μM thalidomide, or 10 μM lenalidomide 3 hours before addition of 100 nM compound **15a**. (G) Ubiquitination analysis of FLAG-tagged CRBN in HEK293T cells. Cells were treated with compound **15a** for 3 hours.

We then performed an immunoblot ubiquitination analysis in HEK293T cells expressing FLAG-CRBN. After treatment with compound **15a** for 3 hours, we observed a higher degree of CRBN ubiquitination as compared to untreated cells (Figure 4G).

The effects of **15a** on the entire proteome were assessed by applying quantitative mass spectrometry (MS) using tandem mass tag (TMT) labeling. MM1S cells were treated in two biological replicates with 100 nM of compound **15a** and pomalidomide, respectively, or DMSO as a control. In total, 9448 proteins were identified by two or more peptides and quantified across all samples. CRBN showed the highest degree of degradation in cells treated with compound **15a** for 3 hours (log_2fold-change (FC) = -0.82) and 24 hours (log_2FC = -1.0) (see Supplementary Figure 7A and 8A). In contrast, pomalidomide had no effect on CRBN protein levels (see Supplementary Figure 7B and 8B). Consistent with previous studies,[6-10,46] after pomalidomide treatment for 3 hours, the most down-regulated proteins were the IMiD-induced CRBN neo-substrates IKZF1 (log_2FC = -1.11) and IKZF3 (log_2FC = -1.24). In comparison, compound **15a** treatment for 3 hours had only weak effects on IKZF1 (log_2FC = -0.24) and IKZF3 (log_2FC = -0.26). Off-target degradation by pomalidomide-derived PROTACs was considered an undesired IMiD effect. It has been found through proteomics analysis that a heterobifunctional PROTAC, acting as a multi-kinase degrader, also depleted IKZF1 to some degree, suggesting that pomalidomide partially retains its IMiD functions despite linker attachment.[47] Compound **15a** did not cause degradation of other members of the CRL4 ligase including DDB1, CUL4A, RBX1 or any other ubiquitin ligases.

PROTAC 15a antagonizes the effects of IMiDs on multiple myeloma cells.

IMiDs mediate their anti-proliferative effect on multiple myeloma cells through induced degradation of the lymphoid transcription factors IKZF1 and IKZF3 via the $CRL4^{CRBN}$ E3 ubiquitin ligase.[7,9,10] In addition, IMiDs disrupt the chaperone function of CRBN for MCT-1 and BSG proteins, what contributes to the anti-proliferative effects.[48]

Since our Homo-PROTACs are potent inducers of CRBN degradation, we tested their impact on cell viability. Pomalidomide, as well as PROTAC **15a**, had no effect on the IMiD-insensitive AML cell line OCI-AML5, even at concentrations up to 10 μM (Figure 5A). In the pomalidomide-sensitive multiple myeloma cell lines MM1S, NCI-H929 (Figure 5A), LP-1, KMS27, and RPMI 8226 (see Supplementary Figure 4), compound **15a** had little or no effect on cell proliferation at concentrations of up to 1 μM, demonstrating that multiple myeloma cells tolerate degradation of CRBN to very low levels. Such low CRBN levels are achieved after treatment with nanomolar concentrations of our Homo-PROTACs (see Figure 2A, as well as 4A). Consistent with these observations, genetic inactivation of *CRBN* by CRISPR/Cas9 gene editing with lentiviral-delivered *CRBN*-specific small guide RNAs (sgRNAs) targeting either the N-terminus or the IMiD-binding site had no effect on MM1S cell proliferation, in contrast to sgRNAs targeting the essential gene RNA polymerase II subunit A (PolR2A) (see Supplementary Figure 6 and Supplementary Table 1).

At concentrations of 10 μM, compound **15a** inhibited the proliferation of MM1S cells (Figure 5A), which can be explained by the increasing degradation of IKZF1 protein compared to that of CRBN. Consistently, compounds **15c** (with an extended linker) and **16** (with one glutarimide structure methylated), which had a more pronounced effect on IKZF1 protein levels but little or no effect on CRBN, were more toxic to multiple myeloma cell lines (see Supplementary Figures 5B–C). These finding might reflect that compounds of reduced ability to form 2:1 ternary complexes tend to act as IMiDs rather than CRBN degrader.

It was then tested whether our PROTACs could antagonize the activity of pomalidomide and lenalidomide on multiple myeloma cell lines. In MM1S cells, degradation of CRBN by pretreatment with compound **15a** abrogated pomalidomide-induced degradation of IKZF1 (Figure 5B), which is consistent with the genetic inactivation of *CRBN*.[7,9,48] Since induced degradation of IKZF1 and IKZF3

by CRBN is one of the main mechanisms by which IMiDs inhibit multiple myeloma cell growth, and in the light of IMiD resistance gained by genetic *CRBN* depletion,[49] we tested whether chemical inactivation of CRBN could rescue multiple myeloma cell lines from pomalidomide and lenalidomide. For this purpose, the IMiD-sensitive multiple myeloma cell lines MM1S and NCI-H929 were pretreated for 3 hours with compound **15a**, followed by the addition of lenalidomide or pomalidomide. After treatment for 4 days, we found that the growth inhibition induced by either pomalidomide or lenalidomide was blocked (see Figure 5C, as well as Supplementary Figure 4B). Even treatment with compound **15a** after addition of lenalidomide or pomalidomide resulted in reduced activity of both IMiDs. Similarly, CRISPR/Cas9-mediated inactivation of *CRBN* by sgRNAs targeting the N-terminus or IMiD-binding region of CRBN conferred IMiD resistance to CRISPR-modified cells (see Supplementary Figure 6). This demonstrates that chemical-induced inactivation of CRBN by the Homo-PROTACs is as potent as genetic inactivation to abrogate the effect of IMiDs in multiple myeloma cells.

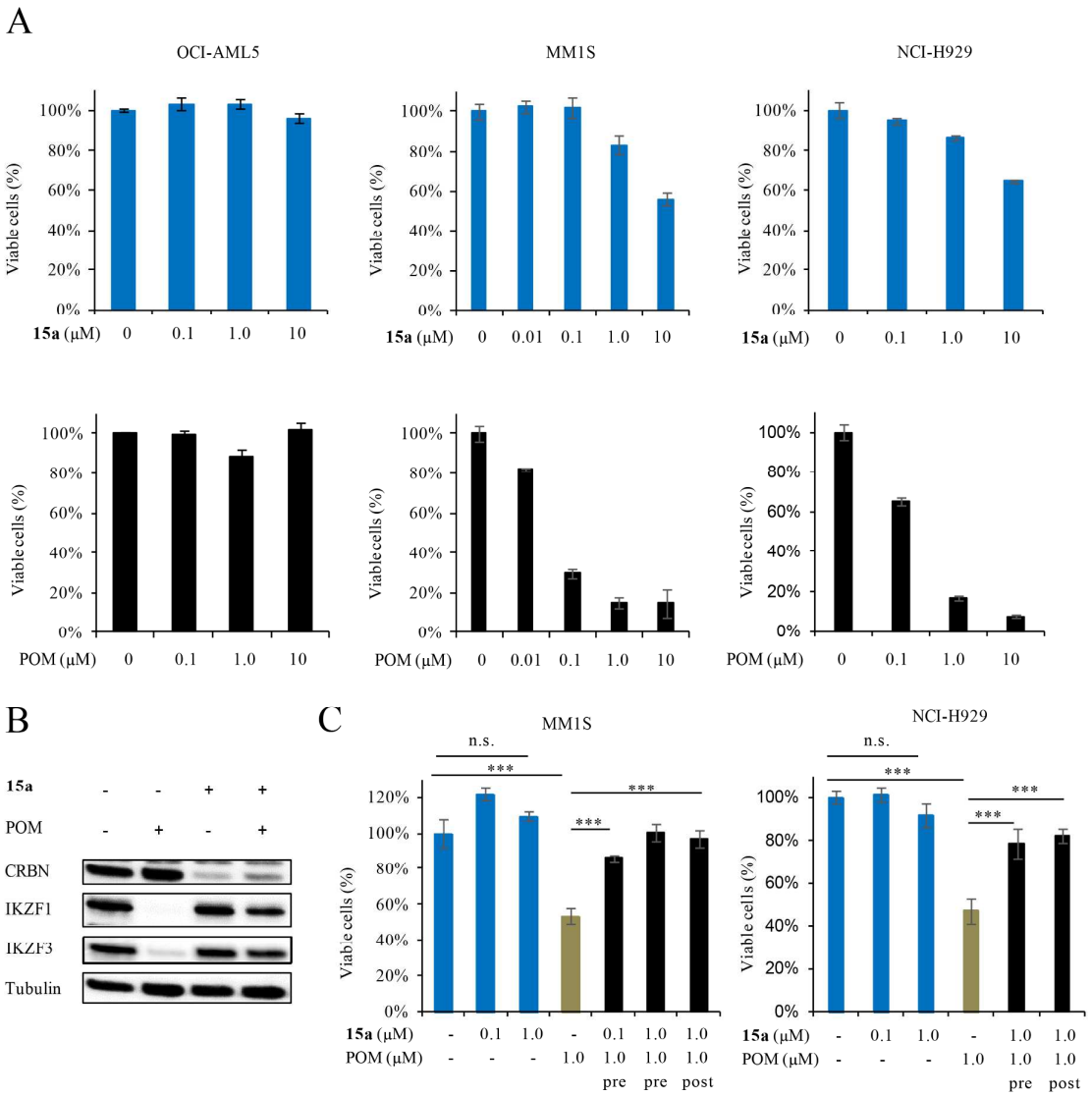


Figure 5. Impact of compound **15a** on cell proliferation and the activity of lenalidomide and pomalidomide. (A) The pomalidomide-insensitive acute myeloid leukemia cell line OCI-AML5 and the pomalidomide-sensitive multiple myeloma cell lines MM1S and NCI-H929 were treated with the indicated concentrations of compound **15a** or pomalidomide. Cell viability was analyzed after 4 days in triplicate. (B) MM1S cells were treated with 100 nM compound **15a** for 6 hours or 1 μM pomalidomide for 3 hours without or with compound **15a** pretreatment for 3 hours. (C) MM1S and NCI-H929 cells were treated with 100 nM or 1 μM compound **15a** alone, 3 hours before (pre) or after (post) addition of 1 μM pomalidomide. Cell viability was measured after 4 days. Error bars express the mean ± SEM from 3 biological replicates. *** $p<0.001$.

Discussion

Here we describe the design, synthesis and biological evaluation of novel, pomalidomide-based Homo-PROTACs. Since our homobifunctional PROTACs are capable of simultaneously interacting with two CRBN molecules, ternary complexes can be formed (Figure 6A). The induced homo-dimerization of CRBN results in subsequent ubiquitination and proteasomal degradation, demonstrating for the first time, a chemically induced knock-down of CRBN. In particular, the Homo-PROTAC **15a** exhibited, at low concentrations, a long-lasting effect on the intracellular CRBN level. Our mutation and competition experiments strongly indicate binding of the PROTACs at the IMiD binding site of CRBN. Immunoblotting and global proteome analyses revealed that PROTAC **15a** specifically induced degradation of CRBN and had only weak effects on pomalidomide neo-substrates IKZF1 and IKZF3 and no effect on the other members of the CRL4 ligase family, including DDB1 and CUL4A which are in close proximity to CRBN.

By investigating the influence of the incorporated linkers, we found that the extent of CRBN degradation depends on the nature of the linker and of its attachment to the E3 ligase binder. It also affected the protein levels of the pomalidomide-induced CRBN neo-substrate IKZF1. This implies that the addition of a linker to the phthalimide part does not necessarily interfere with binding of CRBN to neo-substrates such as IKZF1 and IKZF3 which has also been observed with other IMiD-based PROTACs.[24,47] Thus, the CRBN-PROTAC complexes (see Figure 6A and 6B) offer a *de novo* interface[12] for neo-substrate recruitment (see Figure 6D and 6C). For this reason, remodeling of the CRBN binding surface by small molecule binders needs to be considered as an unwanted side-effect in the development of IMiD-based PROTACs.

Direct protein-protein interactions (PPIs) of CRBN with neo-substrates were also observed in the presence of the CRBN modulators CC-885 and CC-220. Their chemo-neomorphic activities triggered by new interaction hotspots on the surface of CRBN recruit GSPT1, IKZF1, or IKZF3 to the $CRL4^{CRBN}$ ubiquitin ligase.[50-53] Moreover, recent models for PROTAC action postulate stabilizing PPIs between the ligase and the POI. Hence, a positive cooperativity of a ternary ligase-PROTAC-POI system might explain the efficacy of a bifunctional molecule.[24,54,55] The surface of CRBN is known to be amenable for PPIs.[24] In the case of our Homo-PROTACs, we hypothesized that they may facilitate

protein-protein interaction between two cereblon molecules. Consistently, the incorporated short linker in **15a** contributes to a closer proximity of two CRBN molecules, beneficial PPIs, and an efficient CRBN knockdown (Figure 2A). Thus, we were able to achieve the desired PROTAC activity at concentrations below the "hook effect" range, when the equilibria (Figure 6) are shifted to the left. Presumably, a tight ternary 2:1 complex (Figure 6A) is not capable of binding a neo-substrate (Figure 6D) and the unwanted IKZF1 degradation is prevented (see Figure 2A, as well as 4A). In contrast, the expanded pomalidomide PROTACs (**15b-d**) preserve the capacity of neo-substrate binding and degradation. In line with this, the heterodimeric PROTAC **16**, which cannot form complexes with two CRBN units (see Figure 6A and 6D), provoked a strong IKZF1 degradation. Similar effects on target degradation induced by bifunctional molecules have been reported: a VHL-addressing PROTAC with a short linker led to a much stronger degradation of HER2 than an analog with a slightly longer linker.[45,56]

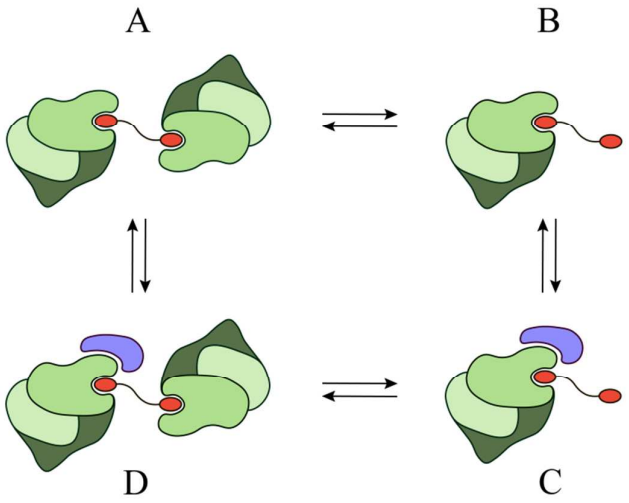


Figure 6. Interactions of CRBN with Homo-PROTACs. Equilibria between possible complexes of CRBN (green, the three distinct domains are indicated),[12] PROTACs (red) and IKAROS neo-substrates (blue) are shown

Very recently, the generation of Homo-PROTACs for the VHL E3 ligase was described.[57] These Homo-PROTACs were assembled by linking two VHL-binding molecules. Like our homobifunctional PROTACs, the VHL-Homo-PROTACs caused specific ubiquitination and proteasomal degradation at nanomolar levels. Noteworthy, only the long VHL isoform was degraded.

The short isoform, which is preferentially part of the CRL2-VHL complex, was not affected. Consequently, the VHL-Homo-PROTACs had no effect on the CRL2-VHL substrate HIF-α.

For the VHL-Homo-PROTACs, an increased linker size resulted in more effective degradation of VHL.[57] In the present study, we found that an extended linker length led to an alleviated CRBN degradation, for reasons that have been discussed above. These data confirm that the appropriate choice of the linker remains one of the most important aspects of PROTAC design and more research into the nature of the linker is required since it largely affects ternary complex formation and stability required for potent degradation.[24,55,58]

CRBN is a multifunctional protein that is involved in different cellular processes and diseases. For example, a CRBN mutation is associated with human autosomal recessive nonsyndromic mental retardation.[59] Genetic inactivation of *CRBN* confers resistance to sepsis and prevents high-fat-diet-induced obesity and insulin resistance in mice,[60–62] implying that pharmacological inhibition of CRBN may have clinical applications. The multiprotein structure of E3 ligases and the lack of pharmaceutically targetable regions on the protein surface have so far prevented the generation of specific inhibitors for these enzymatic complexes. Our homodimeric pomalidomide-PROTACs operate as potent inhibitors of the $CRL4^{CRBN}$ E3 ligase, insofar as they initiate the self-degradation of the adapter protein CRBN.

In the past years, CRBN has drawn attention as the target of the IMiDs thalidomide, lenalidomide, and pomalidomide that are a mainstay in the treatment of multiple myeloma. These drugs mediate their activity in cancer by modulating the specificity of the $CRL4^{CRBN}$ E3 ligase to degrade the neo-substrates IKZF1, IKZF3, and CK1α.[3,6–12] Genetic inactivation of *CRBN* has been shown to cause resistance to IMiDs in cell lines and inactivating *CRBN* mutations were detected in IMiD-resistant multiple myeloma patients.[49,63] Both, the chemical-induced CRBN degradation and approaches of genetic knockdown of CRBN confer resistance to IMiDs. We show that chemical knockdown of CRBN by our Homo-PROTAC **15a** did not affect multiple myeloma cell proliferation, abrogated the degradation of IKZF1 and IKZF3 and conferred pomalidomide and lenalidomide resistance in different multiple myeloma cells. Our data therefore highlight the essential role of CRBN in IMiD activity and further support that the main effect of IMiDs in multiple myeloma relies in

induced degradation of neo-substrates by CRBN rather than blocking the physiological function of CRBN. In the light of the clinical relevance of CRBN as a target for IMiDs and PROTACs, our compounds represent valuable tools to induce a cellular state of inactivated CRBN. The low toxicity of **15a** also implies that this homodimeric PROTAC seems not to have a profound global effect on the ubiquitin-proteasome system since multiple myeloma cells are strongly dependent on cellular protein quality control mechanisms.[64,65]

Unlike the anti-cancer properties of IMiDs, the exact mechanism for many of their pleiotropic effects, including immune modulation, teratogenicity, and anti-angiogenesis, are not completely understood. These effects may result from degradation of neo-substrates but alternatively also from inhibition of the endogenous function of CRBN. Hence, our tool compounds will be useful to discriminate whether an IMiD effect depends on CRBN-mediated targeted degradation of neo-substrates or from blocking CRBN activity. Recently, IMiDs gained increased importance due to the establishment of the PROTAC technology.[31,34] Some of the IMiD-based PROTACs have promising preclinical anti-tumor activity but may have unwanted effects similar to those of their parent IMiDs. Thus, further knowledge on the function of CRBN and the identification of its downstream protein substrates is needed.[66]

Chemical inactivation of a target protein offers several advantages over classical genetics, such as reversibility, the rapid and direct impact on the posttranslational protein level, as well as the general applicability to a broad range of cell and tissue types.[67] The typically low selectivity of chemical probes can be overcome by a sophisticated design of such compounds. The Homo-PROTACs reported herein provide a further example and will be useful tools to induce CRBN degradation, to unravel endogenous substrates and physiological roles of CRBN and to provide additional insights into the molecular mechanism of IMiDs.

METHODS

Chemistry. Chemical synthesis and physicochemical data for all compounds are provided in the Supporting Information.

Cell lines. Cell lines MM1S, U266, KMS27, LP-1, NCI-H929, OPM-2, K562, OCI-AML5, and HEK293T were obtained from American Type Culture Collection (ATCC) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Cells were cultured in RPMI 1640 (Biochrom) or DMEM (Thermo Scientific) supplemented with 10-20% heat-inactivated fetal bovine serum (FBS) (Biochrom) and 1% penicillin, streptomycin, and L-humidified incubator under 5% CO_2. Cells have been authenticated by STR profile analyses and tested for mycoplasma contamination.

Ectopic CRBN expression. For overexpression of CRBN, an RSF91 expression vector (gift of C. Baum, Hannover Medical School) containing normal FLAG-tagged CRBN or mutant CRBN Y384A/W386A (YW/AA) that cannot bind IMiDs was used.[7] HEK293T cells were seeded in a 6-well plate and after 24 h cells were transfected with 1 µg plasmid DNA using TransIT-LT1 transfection reagent (Mobitec).

Immunoblotting. After designated treatment, cells were lysed in IP lysis buffer (Pierce) containing HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Protein content was quantified with a BCA assay (Pierce) and equal amounts of proteins were separated by SDS-PAGE at a constant voltage and transferred onto Immobilon-P transfer membrane (Millipore). The following antibodies were used in the study for western blot analyses: rabbit anti-CRBN (Sigma, HPA045910), rabbit anti-Ikaros (Cell Signaling, D6N9Y), rabbit anti-IKZF3 (Cell Signaling, D1C1E), goat anti-Casein kinase 1A1 (Santa Cruz, C-19), mouse anti-Tubulin (Sigma, DM1A), rabbit anti-DYKDDDDK (Cell Signaling), HRP-linked anti-HA (Miltenyi, 130-091-972), FK2 (HRP-conjugate, Enzo Lifescience), rabbit anti-CUL4A (Cell Signaling, #2699S). Western blots for drug treatments were performed 2-5 times per experiment. Quantification was performed using ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016).

Co-immunoprecipitation. HEK239T cells were co-transfected with an HA-CRBN and FLAG-CRBN expressing vector. After 48 hours, cells were treated with DMSO or 100 nM **15a** for 30 minutes. 10

μM MLN4924 was added to block **15a**-induced degradation of CRBN. Cells was lysed in IP lysis buffer (Pierce) and immunoprecipitation was performed with anti-FLAG sepharose beads (Sigma) for 2 hours in the presence of the drugs. After 3 washes with IP lysis buffer, FLAG-CRBN was eluted with FLAG peptide and the samples were analyzed by western blot.

Ubiquitination analysis. HEK293T cells were transfected with a FLAG-CRBN expressing plasmid. 48 hours after transfection cells were treated with DMSO or compound **15a** for 3 hours. Cells were then washed twice with ice-cold PBS and lysed under denaturing conditions using 2% SDS-containing lysis buffer and boiled for 10 minutes at 95 °C. The SDS was diluted with the addition of 10 × IP lysis buffer (Pierce) containing HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), MG132 (10 μM) and PR619 (50 nM) and incubated at 4 °C for 30 minutes. Immunoprecipitation was performed using anti-FLAG M2 Affinity Gel (Sigma-Aldrich) according to the manufacturer's protocol and protein was eluted with FLAG peptide (Sigma-Aldrich). CRBN was detected by a FLAG-specific antibody and ubiquitinated CRBN by the FK2 antibody.

Cell viability assay. Cells were seeded in a 96- or 384 well plate and treated with the respective concentrations of the test compounds. To determine the number of viable cells, CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was performed according to the manufacturer´s protocol. Luminescence was detected on a PolarStar plate reader (BMG labtech).

CRISPR-mediated knockout of CRBN. For CRISPR gene editing, single guide RNAs were cloned into the lentiviral SGL40C.EFS.dTomato vector (a gift from D. Heckl, Hannover).[68] Guide RNA sequences are listed in the Supporting Information. A luciferase-specific sgRNA was used as a negative control and a sgRNA targeting the essential gene RNA polymerase II subunit A (PolR2A) as a positive control. MM1S cells stably expressing Cas9 were seeded in a 96-well plate and infected with sgRNA-expressing lentiviral vectors. For drug treatment, cells were split 4 days post infection 1:1:1, seeded in 96 well plates and treated with 1 μM lenalidomide, 1 μM pomalidomide or DMSO as a control. After 7, 11 and 14 days the percentage of infected cells was measured by flow cytometry.

Proteome analysis using liquid chromatography mass spectrometry. *Cell lysis and peptide digestion.* Cell pellets were lysed for 30 minutes with ice cold urea lysis buffer containing 8 M urea, 75 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 μg/mL aprotinin (Sigma, A6103), 10 μg/mL

leupeptin (Roche #11017101001), and 1 mM PMSF (Sigma, 78830). The samples were spun at 20,000 *g* for 10 minutes and a BCA assay was used to determine the concentration of protein in each cleared lysate. Lysis buffer was used to bring the protein concentration of each lysate to 1 mg/mL. Protein disulfide bonds were reduced with 5 mM dithiothreitol at 25 °C for 45 minutes. The proteins were alkylated in the dark using 10 mM iodoacetamide at 25 °C for 45 minutes. Lysates were then diluted 1:4 using 50 mM Tris, pH 8.0, to lower the urea concentration to 2 M. LysC was added to each lysate at a 1:50 enzyme-to-substrate ratio and samples were digested at 25 °C for 2 hours. Afterwards, trypsin was added at a 1:50 enzyme-to-substrate ratio and the samples were digested at 25 °C overnight. The digestion was quenched with 100% formic acid to reach a volumetric concentration of 1% formic acid. Samples were spun at 5000 *g* for 5 minutes in order remove precipitated urea and desalted using Sep-Pak C18 columns (Waters, 100 mg WAT023590). Columns were conditioned with 1 × 1 mL 100% acetonitrile, 1 × 1 mL 50% acetonitrile/0.1% formic acid, and 4 × 1 mL 0.1% trifluoroacetic acid. Each sample was loaded onto a column and washed with 3 × 1 mL 0.1% trifluoroacetic acid and 1 × 1 mL 1% formic acid. Peptides were eluted off the column with 2 × 0.6 mL 50% acetonitrile/0.1% formic acid and dried down.

TMT labeling and basic reverse phase fractionation. For TMT labeling, 60 μg of peptides from each sample were labeled with a TMT10 reagent according to manufacturer instructions (Thermo Scientific). The peptides were each reconstituted in 60 μL of 100 mM HEPES and labeled for 1 hour at 25 °C with 0.48 mg TMT10 tag in 24.6 μL of 100% acetonitrile. Labeling efficiency was checked to ensure proper and complete labeling. The labeling reaction was quenched with 4.8 μL of 5% hydroxylamine for 15 minutes at 25 °C. Afterwards, all peptides were combined and desalted using a Sep-Pak C18 column (Waters, 100 mg WAT036790) following a similar procedure as described above. The sample reconstituted in 3% acetonitrile/0.1% formic acid and fractionated using basic reverse phase fractionation as reported previously (ubi, ptm, cptac). A 4.6 mm Zorbax column (Agilent) was used to fractionate the sample into 96 fractions that were concatenated in a noncontiguous manner into 25 fractions. Each fraction was dried down and resuspended at 1 μg/μL concentration in 3% acetonitrile/0.1% formic acid.

LC-MS/MS and spectra analysis. All samples were analyzed using nanoflow HPLC-HCD-MS/MS using an Orbitrap Fusion Lumos mass spectrometer and an Easy-nLC 1260 system. 1 μL of each sample was injected at a flow rate of 500 nL/min onto a Picofrit column self-packed with 1.9 μm C-18 beads that was heated to 50 °C. The LC-MS/MS gradient and flow rate were used as previously described.[69] Spectra were acquired for 110 minutes. MS1 scans were acquired at 60 K resolution at a scan range of 350-1800 m/z and a maximum injection time of 50 ms. Ions were fragmented with a collision energy of 38%. MS2 scans were acquired at 50 K resolution with a maximum injection time of 105 ms in a 0.7 isolation window. Spectra were searched using Spectrum Mill software (Agilent). For peptide identification, MS/MS spectra were searched against the Uniprot human database with 150 contaminants.

Statistical analysis. Statistical analysis was performed using Prism v5.0 (GraphPad Software). Data are expressed as mean ± SEM. Statistical differences between two data sets were assessed using a two-sided Student *t*-test. *P* values less than 0.05 were considered statistically significant. *P* values greater than 0.05 were indicated as not significant (n.s.) and the statistical significance is also reported in the Figure Legends.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS publications website at DOI:

Supplementary Figures 1–9, Supplementary Table 1, as well as synthetic procedures (PDF)

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Author Contributions

#These authors contributed equally to this work. C.S. and M.G. designed homodimeric PROTACs. C.S. synthesized the compounds. S.L., H.K., and S.K. performed drug treatments, western blot, and cell viability analyses. N.D.U., D.M., and S.A.C. performed proteomic analyses. C.S., S.L., M.G., and J.K. analyzed data and wrote the manuscript. M.G. and J.K. supervised the project.

Notes

The authors declare that they have no conflicts of interest with the contents of this article. This work was supported by the Deutsche Forschungsgemeinschaft (Emmy-Noether Program Kr-3886/2-1 and SFB-1074 to J.K.; FOR2372 to M.G.).

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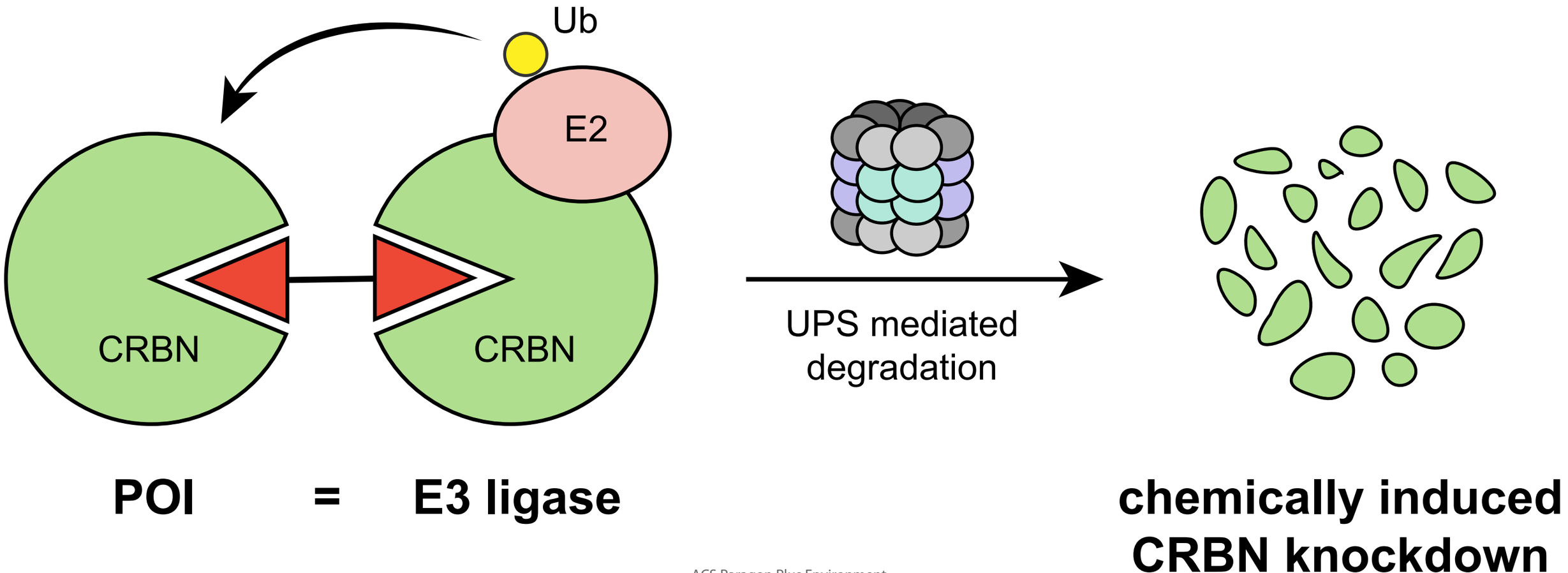

POI = E3 ligase

chemically induced CRBN knockdown