



Cite this: *Chem. Soc. Rev.*, 2022, 51, 6234

Discovery of CRBN as a target of thalidomide: a breakthrough for progress in the development of protein degraders

Junichi Yamamoto,^a Takumi Ito,^b Yuki Yamaguchi^a and Hiroshi Handa^{*c}

Progress in strategies aimed at breaking down therapeutic target proteins has led to a paradigm shift in drug discovery. Thalidomide and its derivatives are the only protein degraders currently used in clinical practice. Our understanding of the molecular mechanism of action of thalidomide and its derivatives has advanced dramatically since the identification of cereblon (CRBN) as their direct target. The binding of thalidomide derivatives to CRBN, a substrate recognition receptor for Cullin 4 RING E3 ubiquitin ligase (CRL4), induces the recruitment of non-native substrates to CRL4^{CRBN} and their subsequent degradation. This discovery was a breakthrough in the current rapid development of protein-degrading agents because clarification of the mechanism of action of thalidomide derivatives has demonstrated the clinical value of these compounds. This review provides an overview of the mechanism of action of thalidomide and its derivatives and describes perspectives for protein degraders.

Received 8th February 2022

DOI: 10.1039/d2cs00116k

rsc.li/chem-soc-rev

Key learning points

1. Historical overview of thalidomide and its derivatives as pharmaceuticals.
2. Mechanism of action of thalidomide and its derivatives at the molecular level.
3. The structure and function of cereblon, a direct target of thalidomide.
4. Mechanism of action of protein degraders with a mode of action different from conventional small-molecule compounds.
5. Advantages and perspectives of the two classes of protein degraders, molecular glue degraders and PROTACs.

1. Introduction

Protein degraders are compounds that selectively knock down target proteins *via* intracellular protein degradation pathways, and are regarded as a novel class of therapeutic agents that employ a different mechanism of action than conventional small molecule compounds, which lead to the breakdown of disease-related proteins. The ubiquitin-proteasome system (UPS) is a major protein degradation pathway that is responsible for the degradation of over 80% of proteins in eukaryotic cells. The UPS degrades target proteins by multiple sequential reactions (Fig. 1). First, the ubiquitin-activating enzyme (E1) forms a thioester bond with ubiquitin in an ATP-dependent

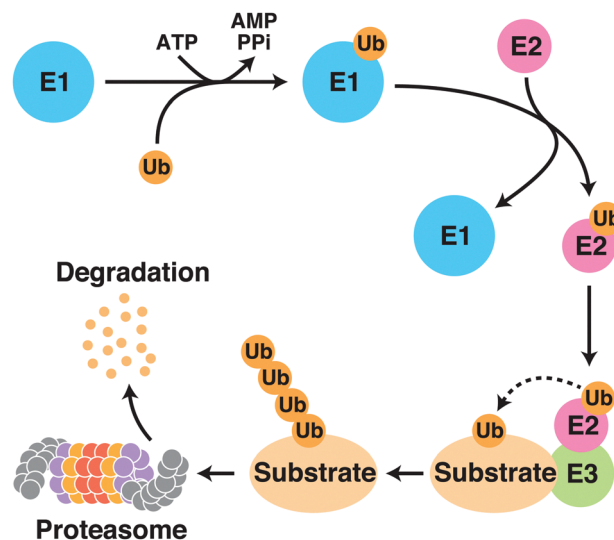


Fig. 1 Schematic representation of the ubiquitin proteasome system. Abbreviations: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; Ub, ubiquitin; ATP, adenosine triphosphate; AMP, adenosine monophosphate; PPi, pyrophosphate.

^a School of Life Science and Technology, Tokyo Institute of Technology, Yokohama 226-8501, Japan

^b Institute of Medical Science, Tokyo Medical University, Shinjuku, Tokyo 160-8402, Japan

^c Center for Future Medical Research, Tokyo Medical University, Shinjuku, Tokyo 160-8402, Japan. E-mail: hhanda@tokyo-med.ac.jp



manner. The activated ubiquitin moiety is then transferred to the ubiquitin-conjugating enzyme (E2) through a transthio-esterification reaction. Next, the E2 enzyme cooperates with a variety of ubiquitin ligases (E3) to transfer ubiquitin to lysine residues on substrate proteins. Polyubiquitinated substrate proteins are recognized, unfolded, and degraded by the proteasome. Protein degraders are small molecule compounds that induce selective degradation of target proteins by hijacking the intracellular UPS. This targeted protein degradation strategy dramatically expands the spectrum of druggable target proteins and is considered as a new promising modality for drug development, as it does not require a clear active site on target proteins.

Thalidomide derivatives, such as lenalidomide and pomalidomide, collectively referred to as immunomodulatory imide drugs (IMiDs), are mainly used to treat hematological malignancies such as multiple myeloma (Fig. 2). Multiple myeloma is a disease in which plasma cells in the bone marrow become cancerous; the development of IMiDs and other medications

have greatly improved its prognosis over the last 20 years. Thalidomide was one of the worst drug disasters in history; however, it has attracted significant attention not only because of its clinical value, but also because of its novel mechanism of action, which has great promise. Our understanding of the mechanism of action of these drugs has advanced rapidly in the last decade with the identification of cereblon (CRBN) as their direct target.¹ CRBN is a substrate recognition receptor for Cullin 4 RING E3 ubiquitin ligase (CRL4); the binding of thalidomide derivatives to CRBN triggers the recruitment of non-native substrates to CRL4^{CRBN} and their subsequent proteasomal degradation.^{2–13} Thalidomide derivatives are the first, and currently the only, protein degraders with demonstrated clinical benefit.

Protein degraders are currently under rapid development as a promising modality for drug discovery. The identification of CRBN as a primary target of thalidomide was a breakthrough that is now driving this innovative area of research. In this review, we will provide an overview of the mechanism of action



Junichi Yamamoto

Junichi Yamamoto is currently an assistant professor at Tokyo Institute of Technology. He received his PhD degree at Tokyo Institute of Technology in 2014. After postdoctoral training at Tokyo Medical University under the supervision of professor Hiroshi Handa, he joined the faculty at Tokyo Institute of Technology. His current research interest is focused on targeted protein degradation for cancer therapy.



Takumi Ito

Takumi Ito is currently an associate professor at Tokyo Medical University. He received his PhD in Engineering from Tokyo Institute of Technology (Supervisor: Hiroshi Handa). From 2008 to 2012, he was a researcher at Tokyo Institute of Technology. From 2012 to 2013, he worked as an Assistant Professor at Tokyo Institute of Technology. Then, he joined the faculty at Tokyo Medical University. His current research interests are small molecule-induced protein degradation and molecular mechanisms of cereblon-binding drugs.



Yuki Yamaguchi

RNA processing and chemical biology aimed at developing new therapeutic strategies.

Yuki Yamaguchi is currently Professor of Life Science and Technology at Tokyo Institute of Technology. He received his PhD at the same university under then-professor Hiroshi Handa. After postdoctoral training, he was appointed as Principal Investigator of JST's PRESTO program. Then, he joined the faculty at Tokyo Institute of Technology. His research has focused on the mechanisms of chromatin, transcription, and



Hiroshi Handa

Hiroshi Handa is a professor at Tokyo Medical University and a professor emeritus at Tokyo Institute of Technology. He graduated from Keio University, School of Medicine in 1972 and got his MD, PhD in 1976 from the University's Graduate School of Medicine. In 1976, he became an assistant professor at the University of Tokyo. He worked as a postdoctoral fellow in the P. Sharp Lab at MIT from 1978 to 1980. He became an associate professor in 1984 at the University of Tokyo, and a full professor from 1991 to 2012 at the Tokyo Institute of Technology.



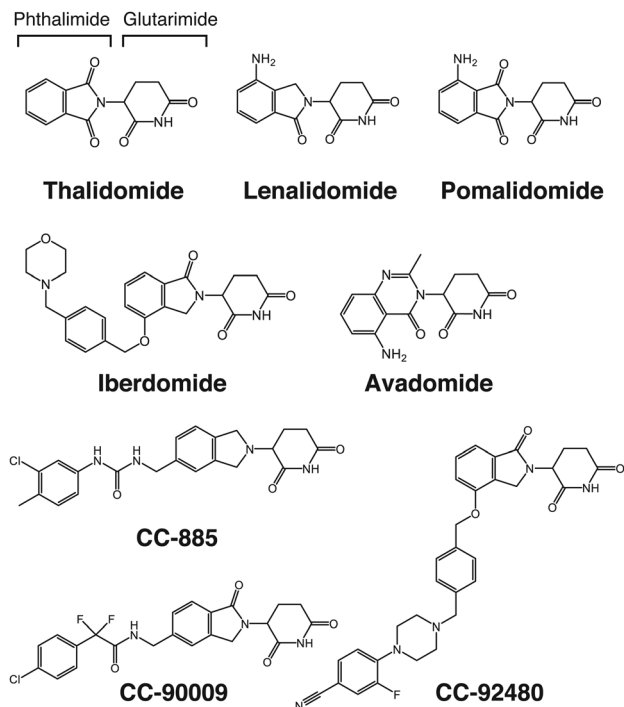


Fig. 2 Structure of thalidomide-based glue degraders. Thalidomide is composed of glutarimide and phthalimide. Thalidomide, lenalidomide, and pomalidomide are collectively referred to as IMiDs. All thalidomide-based glue degraders targeting CRBN are collectively referred to as CELMoDs.

of thalidomide and its derivatives, which will be followed by a discussion of the current status and future perspectives of protein degraders.

2. Pharmacological activities of thalidomide and its derivatives

Thalidomide was first developed in the 1950s by the German pharmaceutical company Grünenthal as a sedative or antiemetic for morning sickness in pregnant women. Thalidomide was marketed in over 40 countries worldwide but was later withdrawn from the market because it causes a wide range of serious teratogenic effects in the fetus, including characteristic limb defects.^{14,15} By 1962, thalidomide was discontinued in most countries. It is estimated that thalidomide caused serious birth defects in over 10 000 newborns; however, the total number of fetuses actually affected by thalidomide is unknown because the rate of miscarriage increased during this period, and because thalidomide causes damage to a wide variety of tissues that can lead to miscarriage.¹⁶

Subsequent studies, however, showed that thalidomide has unexpected clinical benefits, allowing it to re-enter the market. Thalidomide is effective for treating erythema nodosum leprosum (ENL), an infectious inflammatory disease characteristic of leprosy.¹⁷ Moreover, thalidomide has immunomodulatory properties, modulating the expression of inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukins.^{18,19}

Research on the pharmacological effects of thalidomide has continued, demonstrating its unique properties with diverse pharmacological activities such as inhibition of angiogenesis²⁰ and anti-myeloma²¹ and immunomodulatory effects. The U.S. Food and Drug Administration (FDA) approved thalidomide for use in ENL and multiple myeloma in 1998 and 2006, respectively. The use of thalidomide is strictly controlled under the program called the Thalidomide Risk Evaluation and Mitigation Strategy. Perhaps, the most important clinical benefit of thalidomide and its derivatives is their anti-cancer activity against hematological malignancies including multiple myeloma. Lenalidomide and pomalidomide are thalidomide derivatives that were developed by Celgene Corporation (currently part of Bristol-Myers Squibb) and collectively referred to as IMiDs because of their potent immunomodulatory effects (Fig. 2).^{22,23} These derivatives have considerably higher anti-myeloma activity than thalidomide. Lenalidomide is now the first-line treatment for multiple myeloma and is also FDA-approved for treating mantle lymphoma, follicular lymphoma, and myelodysplastic syndromes (MDS) with deletion of chromosome 5q (del(5q)). Pomalidomide is approved for treating patients with relapsed/refractory multiple myeloma because it is also effective in patients with multiple myeloma refractory to lenalidomide. The prognosis of multiple myeloma has been greatly improved with the advent of IMiDs and proteasome inhibitors. Continued research efforts have uncovered many pharmacologically beneficial effects of thalidomide; the molecular mechanisms underlying its clinical benefits and side effects, however, were unclear until we identified CRBN as a direct target of thalidomide.

3. CRBN as a direct target of thalidomide

This section briefly outlines how CRBN was identified as a direct molecular target of thalidomide by affinity chromatography using nano-magnetic beads technology.¹ Ferrite glycidyl methacrylate (FG) beads used for this identification are nano-sized magnetic particles composed of ferrite nanoparticles coated with a copolymer of glycidyl methacrylate (GMA) and styrene as a core and poly(GMA) as an outer surface (Fig. 3(a) and (b)).²⁴ FG beads have a high binding capacity because of their small diameter of approximately 200 nm; poly(GMA) on the surface reduces non-specific protein binding. Target proteins of a wide variety of compounds, including pharmaceuticals,²⁵ metabolites,²⁶ and natural products,²⁷ have been isolated from cell lysates in a single-step with FG beads. When FR259625, a carboxyl derivative of thalidomide, was immobilized to FG beads and used for affinity purification from HeLa cell extracts or other complex samples, CRBN and damage specific DNA binding protein 1 (DDB1) were specifically purified and identified as thalidomide-binding proteins (Fig. 3(c)). Further analysis revealed that CRBN is the protein that binds directly to thalidomide, and that DDB1 binds indirectly to thalidomide through CRBN.¹

Thalidomide binds to the highly conserved C-terminal region of CRBN; alanine substitutions of the conserved tyrosine



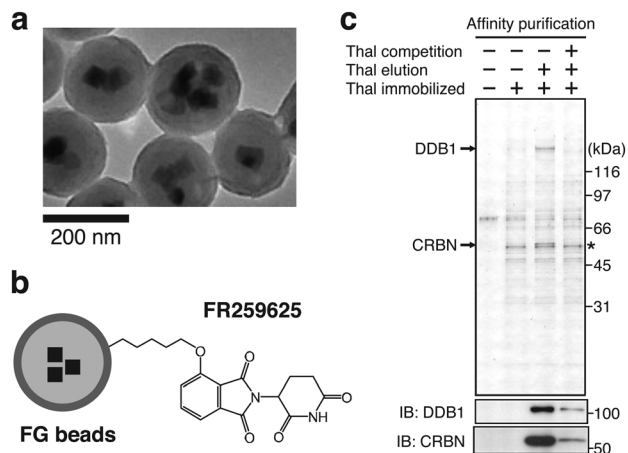


Fig. 3 Identification of CRBN as a direct target of thalidomide using FG beads. (a) Electron microscope image of FG beads. Reproduced from ref. 24 with author right. (b) Schematic representation of thalidomide-immobilized FG beads. FR259625, a carboxy derivative of thalidomide, was immobilized to the beads. (c) Affinity purification of CRBN and DDB1 from HeLa cell extract using thalidomide-immobilized FG beads. Bound fractions were analyzed by silver staining (top) and immunoblotting (IB) (bottom). The asterisk indicates non-specific signal. From ref. 1. Reprinted with permission from AAAS.

residue at position 384 and the tryptophan residue at position 386 of the 442 amino acid protein remove the ability to bind thalidomide while maintaining the ability to form CRL4^{CRBN}. Animal experiments with zebrafish and chickens showed that overexpression of the YW/AA mutant of CRBN conferred resistance to thalidomide-induced limb defects in these embryos (Fig. 4). CRBN is, therefore, a direct target of thalidomide, mediating its teratogenicity.¹ CRBN and DDB1 together with Cullin 4 (CUL4A or CUL4B) and Ring-Box 1 (RBX1) form the E3 ubiquitin ligase complex CRL4^{CRBN}. In fact, a large number of proteins such as DDB2, CSA, COP1, VprBP, and CDT2 are known, or suspected, to serve as substrate recognition receptors of CRL4, and CRBN is a recent addition to this growing list. Subsequent studies have revealed that most, if not all, of the

diverse pharmacological effects of thalidomide and its derivatives are mediated by their binding to and modulation of CRL4^{CRBN}.

4. Molecular mechanisms of action of thalidomide and its derivatives

CRBN was considered as a substrate recognition receptor for CRL4^{CRBN}, but its actual substrates were unknown. Identification of its substrates, therefore, has been a key focus for researchers trying to elucidate the molecular mechanism of action of thalidomide. To date, a number of substrates that are recognized by CRL4^{CRBN} only in the presence of thalidomide or its derivatives have been identified; these are collectively referred to as neosubstrates (Table 1). Thalidomide and its derivatives largely exert their pharmacological activities by binding to CRBN and altering its substrate specificity, leading to the recognition and degradation of neosubstrates (Fig. 5). In other words, thalidomide and its derivatives act as molecular glues between CRBN and neosubstrates. Protein degraders have attracted interest as a new modality for drug discovery; in general, they are compounds that promote the binding of an E3 ubiquitin ligase to a specific target protein and lead to its degradation. Many thalidomide-based degraders that are clinically used or under development reportedly have multiple neosubstrates, suggesting that their diverse pharmacological effects are caused by the degradation of diverse target proteins by CRL4^{CRBN}.

CRISPR library screens have recently identified two E2 enzymes that are recruited to RBX1 and are involved in the ubiquitination process by CRL4^{CRBN}.^{28,29} These enzymes play distinct roles in the ubiquitination reaction: UBE2D3 triggers mono-ubiquitination of neosubstrates, whereas UBE2G1 elongates the polyubiquitin chain. In the following section, we describe detailed mechanisms by which thalidomide and its derivatives exert pharmacological and teratogenic effects.

4.1. Anti-myeloma effects

Thalidomide derivatives of the IMiDs class, such as lenalidomide and pomalidomide, are promising anti-myeloma agents. Lenalidomide affects the expression of a variety of cytokines and associated factors.²³ Prior to the discovery of CRBN, *interferon regulatory factor 4* (IRF4) was identified as an important downstream gene that mediates the anti-myeloma effects of lenalidomide. IRF4 is a hematopoietic cell-specific gene that forms an autoregulatory circuit with another oncogene MYC; IRF4 and MYC play important roles in myeloma cell survival, and their downregulation by lenalidomide causes growth inhibition.³⁰ The loss of CRBN confers lenalidomide and pomalidomide resistance in multiple myeloma cell lines.³¹ Lenalidomide and pomalidomide bind directly to CRBN more strongly than thalidomide, and their binding to CRBN results in IRF4 downregulation and inhibition of myeloma cell growth.³² In IMiD-treated multiple myeloma patients, CRBN is a favorable prognostic factor,^{33,34}

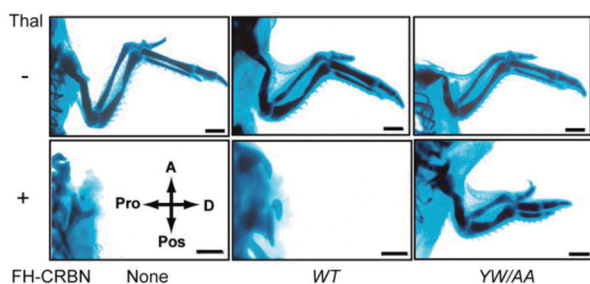
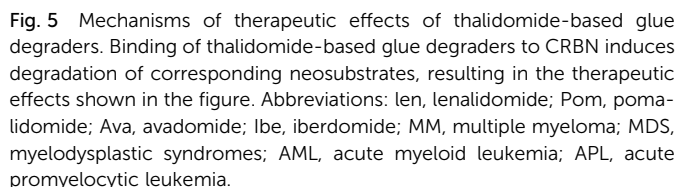


Fig. 4 CRBN mediates thalidomide teratogenicity. The plasmids expressing wild-type (WT) CRBN or its thalidomide binding-deficient mutant (YW/AA) were electroporated as indicated into the forelimb field of stage 14 chicken embryos. Forelimbs were then treated with thalidomide or vehicle and stained with Victoria blue at stage 36. Abbreviations: thal, thalidomide; A, anterior; Pos, posterior; Pro, proximal; D, distal; FH, Flag and HA tag. The scale bar indicates 1 mm. From ref. 1. Reprinted with permission from AAAS.



Genes	Compounds	Binding motifs	Ref.
Ikaros Aiolos	Lenalidomide Pomalidomide Iberdomide CC-885 CC-92480	C2H2 zinc finger	2 and 3
CK1 α	Lenalidomide		4
GSPT1	CC-885 CC-90009		5
SALL4	Thalidomide Pomalidomide	C2H2 zinc finger	6 and 7
TAP63 Δ NP63	Thalidomide		8
ARID2	Pomalidomide		9
PLZF	Pomalidomide	C2H2 zinc finger	10 and 11
ZMYM2	Pomalidomide Avadomide	MYM zinc finger	12 and 13



In 2014, two groups independently reported Ikaros (IKZF1) and Aiolos (IKZF3) as neosubstrates that are involved in the anti-myeloma effects of lenalidomide;^{2,3} these were the first reported CRBN neosubstrates. To identify proteins whose ubiquitination levels are affected by lenalidomide, Ebert and colleagues performed ubiquitin-modified proteome analysis by enriching peptides containing di-glycine (K-ε-GG) remnants, which remain in ubiquitinated lysine residues after trypsin digestion.² Meanwhile, Kaelin and colleagues used a dual reporter vector carrying an ORF library to investigate proteins whose stability is affected by lenalidomide.³ Ikaros and Aiolos are members of the Ikaros family of transcription factors that contain multiple C2H2 zinc finger motifs. Ikaros and Aiolos are specifically expressed in the hematopoietic lineage and play important roles in regulating lymphocyte differentiation and

Early IMiDs such as lenalidomide and pomalidomide were produced by subtle modifications to thalidomide. The more recently developed thalidomide analogs, however, are structurally more diverse (Fig. 2). These compounds are collectively named cereblon E3 ligase modulators (CELMoDs) because their efficacy is no longer limited to immunomodulation. A number of new CELMoDs have been developed by Celgene Corporation in an effort to identify compounds inducing degradation of Ikaros and Aiolos with greater activity. Iberdomide (Fig. 2), a CELMoD with a higher binding affinity for CRBN than lenalidomide and pomalidomide, induces more efficient degradation of known neosubstrates including Ikaros and Aiolos.³⁵ This compound is thus expected to have a more potent effect against myelomas, and clinical trials for treating relapsed/refractory multiple myeloma with iberdomide are currently underway. CC-92480, another CELMoD, is also in clinical trials for relapsed/refractory multiple myeloma. Lenalidomide-refractory multiple myeloma patients frequently exhibit downregulation of CRBN expression. CC-92480 has a much higher potency for degrading Aiolos than lenalidomide; this compound has high anti-myeloma activity even in lenalidomide-resistant multiple myeloma cell lines with low CRBN expression.³⁶

Ikaros and Aiolos play critical roles in mediating the anti-myeloma effects of lenalidomide and pomalidomide. Given that both compounds induce the degradation of Ikaros and Aiolos, an unresolved question is why pomalidomide shows efficacy in lenalidomide-resistant multiple myeloma. There is

Unlike thalidomide and pomalidomide, lenalidomide is approved for del(5q) syndrome. Casein kinase 1 α (CK1 α) is a lenalidomide-dependent neosubstrate involved in this anti-MDS effect.⁴ Lenalidomide is much more potent in degrading CK1 α than thalidomide or pomalidomide; at clinically relevant concentrations, only lenalidomide promotes its degradation. CK1 α is a multifunctional serine/threonine kinase involved in many biological processes, including p53-mediated apoptosis, regulation of the WNT signaling pathway, cell cycle regulation, and the immune response.^{40–42} The *CSNK1A1* gene, which encodes CK1 α , is located in the 5q region and is haploinsufficient in del(5q) syndrome. Therefore, del(5q) cells are more sensitive than normal cells to lenalidomide-mediated CK1 α degradation; this explains why lenalidomide is particularly effective in MDS with del(5q).^{43,44} Lenalidomide treatment activates the p53-mediated apoptotic pathway and inhibits proliferation of *CSNK1A1* haploinsufficient cells. Concordantly, CK1 α overexpression confers lenalidomide resistance to patient-derived MDS del(5q) cells *in vitro*.⁴

The CELMoD CC-885 exhibits anti-cancer activity against a wide range of cell types *via* CRL4^{CRBN}; it is particularly effective for acute myeloid leukemia (AML), making it a potential candidate for AML treatment.⁵ In 2016, G1 to S phase transition 1 (GSPT1) was identified as a CC-885-dependent neosubstrate. GSPT1 is a component of the translation termination complex that recognizes the termination codon and induces translation termination; its deletion causes G1 arrest. GSPT1 specifically associates with CC-885-bound CRBN. Expression of a CRBN-binding deficient mutant of GSPT1 confers resistance to CC-885, demonstrating that the anti-AML effect of CC-885 is caused by the

degradation of GSPT1 by CRL4^{CRBN}.⁵ CC-885 possesses a novel therapeutic property against AML; however, its clinical development is difficult because, in addition to GSPT1, CC-885 also induces degradation of Ikaros, Aiolos, and CK1 α and has a complicated mechanism of action. CC-90009 was instead developed as a first-in-class, GSPT1-selective CELMoD and is currently being tested in Phase I and II trials for the treatment of AML. CC-90009 exhibits reduced general cytotoxicity but maintains its anti-AML activity. Global proteomic analysis with AML cell lines suggest that, with the exception of GSPT1, CC-90009 has little effect on the proteome.⁴⁵

Chromosomal translocations are characteristic features of many types of cancers including leukemias and lymphomas, and are used as diagnostic markers and therapeutic targets. In a chromosomal translocation, two different chromosomes are broken and fused together, and the resulting chimeric fusion gene drives oncogenic transformation. Thalidomide derivatives lead to the degradation of multiple leukemogenic fusion proteins. The *zinc finger MYM-type containing 2* (ZMYM2) gene generates a fusion gene with *fibroblast growth factor receptor 1* (FGFR1) through the chromosomal translocation t(8;13)(p11;q12), which is involved in the transformation to AML (Fig. 6(a)). ZMYM2–FGFR1 is a pomalidomide- and avadomide-dependent neosubstrate of CRL4^{CRBN}^{12,13}. The binding mode of ZMYM2 to CRBN is similar to that of Ikaros and Aiolos, but its drug specificity is different, with ZMYM2 showing strong preference to avadomide. Concordantly, avadomide exhibits antiproliferative activity *in vitro* in bone marrow samples derived from patients with hematological malignancies harboring the t(8;13)(p11;q12) translocation.¹²

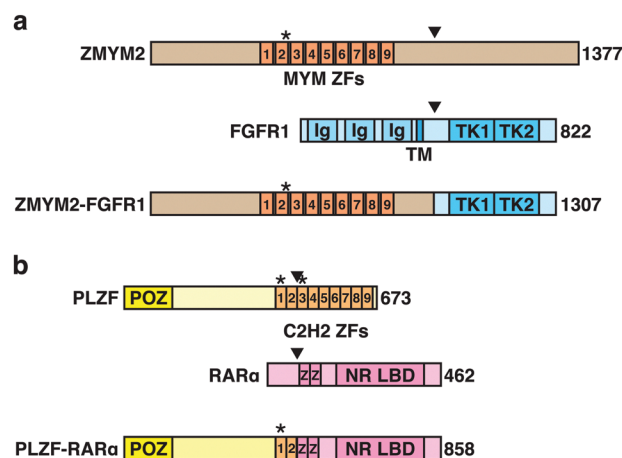


Fig. 6 Schematic representation of fusion gene products targeted by thalidomide-based glue degraders. (a) Schematic representation of ZMYM2, FGFR1, and the fusion protein ZMYM2-FGFR1. (b) Schematic representation of PLZF, RAR α , and the fusion protein PLZF-RAR α . Abbreviations: ZF, zinc finger motif; Ig, immunoglobulin-like domain; TM, transmembrane domain; TK, tyrosine kinase domain; POZ, BTB/POZ domain; NR LBD, nuclear receptor ligand-binding domain. The asterisks indicate degrons recognized by CRBN. The arrowheads indicate breakpoints.

SALL4, TP63, and PLZF are all plausible as neosubstrates involved in thalidomide embryopathy (Fig. 7), but as mentioned above, its actual phenotypes are extremely diverse. It is unclear whether thalidomide-induced degradation of these neosubstrates, alone or in combination, accounts for such a diverse spectrum of defects, particularly given that previous studies used simplified animal models. It is also possible that neosubstrates responsible for the teratogenic effects of thalidomide differ among species. Further validation with primate models may offer more definitive answers as to which neosubstrates are involved in thalidomide embryopathy.

The mode of action of thalidomide-based degraders is to physically link CRBN and neosubstrates as molecular glues. Structural knowledge of how CRL4^{CRBN} interacts with a neosubstrate in the presence of a molecular glue is thus important for understanding its specific mechanism of action and developing more effective drugs. Since the discovery of CRBN, determining the structure of complexes that contain CRL4^{CRBN}, thalidomide derivatives, and neosubstrates has been a major focus in this field of research. Human CRBN is a 441- or 442-amino acid protein that is evolutionarily conserved from plants to humans; it was first reported as a gene product associated with people with an intellectual disability.⁶⁵ CRBN consists of the N-terminal LON-like domain (LLD), which is similar to the bacterial Lon protease, and the C-terminal thalidomide-binding domain (TBD) (Fig. 8(a)). DDB1 binds to the central region of CRBN, and three tryptophan residues (tri-trp) in the TBD are required for thalidomide binding. The glutarimide moiety of thalidomide is critical for binding to CRBN.¹

In 2014, two groups independently determined by X-ray crystallography the structure of CRBN-DDB1 bound to IMiDs. Thomä and colleagues reported three-dimensional structures using human DDB1, chicken CRBN, and thalidomide, lenalidomide, or pomalidomide,⁶⁶ while Cathers and colleagues reported three-dimensional structures using human DDB1, human CRBN, and lenalidomide,⁶⁷ with consistent results. The DDB1-binding motif of CRBN consists of a series of α -helices, located at residues 188–248 of the LLD. DDB1 is composed of three β -propeller domains (BPA, BPB, and BPC);⁶⁸ CRBN binds to a cavity between BPA and BPC (Fig. 8(b)).^{66,67} The glutarimide ring of IMiDs inserts into a hydrophobic pocket consisting of three tryptophan residues (tri-Trp; W380, W386, and W400) and one phenylalanine residue (F402) in the TBD; the phthalimide or isoindolinone ring protrudes outward and is exposed on the surface of CRBN (Fig. 8(c)). Within the tri-Trp pocket, two hydrogen bonds are formed between the glutarimide ring and the backbone of the H378 and W380 residues; a further hydrogen bond is formed with the side chain of H378. This provides a structural explanation as to why the thalidomide-binding deficient mutant of CRBN (YW/AA) discovered in a previous study¹ has lost its ability to bind thalidomide. The TBD also has a C4-type zinc finger motif consisting of C323, C326, C391, and C394. Its role is not well understood, but a missense mutation (C391R) in this motif was found in five individuals with a severe intellectual disability from the same Saudi family.⁶⁹

The tertiary structure of the complex containing a neo-substrate, an IMiD, and CRBN was first reported in 2016. Thomä and colleagues determined the crystal structure of human DDB1-CRBN-lenalidomide-CK1 α .⁷⁰ The β -hairpin loop (residues 35–41) of CK1 α was bound to the surface provided by CRBN and lenalidomide (Fig. 8(d)). In the same year, the crystal structure of the DDB1-CRBN-CC-885-GSPT1 complex was also

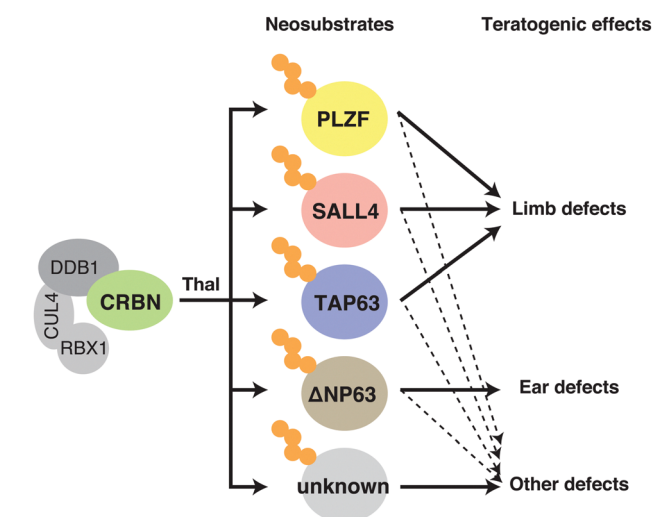
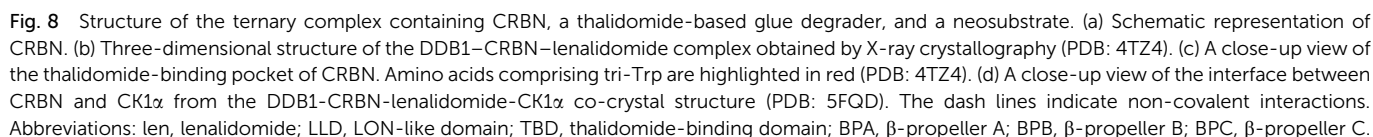


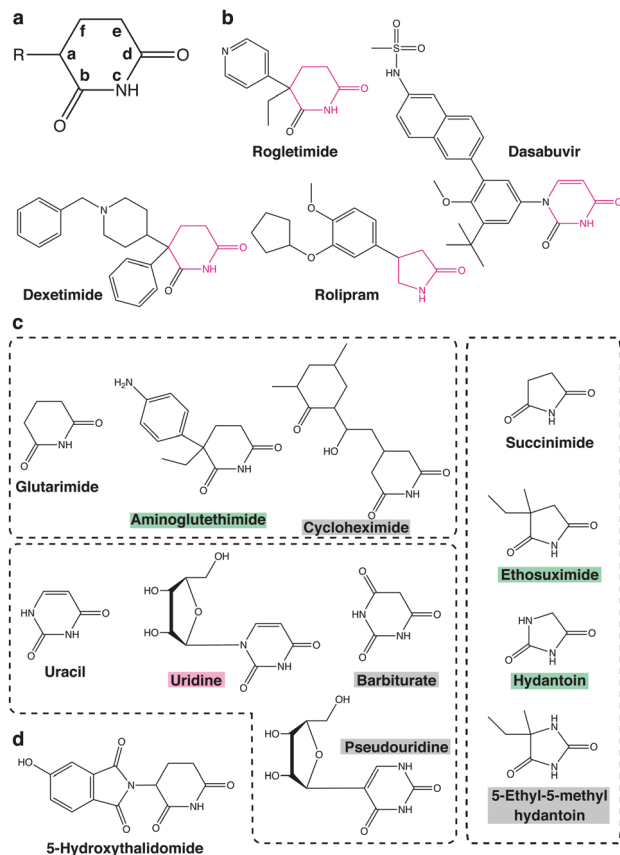
Fig. 7 Mechanisms of teratogenic action of thalidomide. Binding of thalidomide to CRBN in fetal development induces degradation of SALL4, TP63, PLZF, and possibly other unknown neosubstrates, resulting in the various defects shown in the figure. Abbreviation: thal, thalidomide.



containing a key glycine residue has been confirmed and extended.^{71,72}

The glutarimide moiety of thalidomide-based degraders is mainly involved in the binding to CRBN, while the other part contributes to the recognition of neosubstrates. SARs for CRBN binding have been investigated in detail by a series of studies using the thalidomide-binding domain of cereblon isoform 4 from *Magnetospirillum gryphiswaldense* (MsCt4).^{73–76} In an early study, the uracil moiety of uridine was shown to fit in the thalidomide-binding pocket of MsCt4, albeit of lower affinity than thalidomide, suggesting that the minimal structure for CRBN binding is a cyclic imide.⁷³ Subsequently, detailed SARs were studied by fluorescence resonance energy transfer using

Tutorial Review



MsCI4 and various cyclic imides and lactams.⁷⁵ In order to discuss structural requirements of various, five- to eight-membered cyclic compounds for CRBN binding, a simplified nomenclature shown in Fig. 9(a) will be used below. The carbon linked to the phthalimide group of thalidomide is designated position (a), while positions (b), (c), and (d) correspond to the proximal carbonyl group, the amino group, and the distal carbonyl group, respectively. The SAR study indicated that the carbonyl in position (d) is essential, whereas the other carbonyl in position (b) increases affinity for MsCI4 in both five- and six-membered cyclic compounds; the amino group in position (c) is also required for binding. Four-, five-, and six-membered lactams show measurable affinity, but the seven- and eight-membered lactams do not, suggesting a size limitation of the ligand-binding pocket. These results indicated that the minimal structure for CRBN binding is a lactam with an appropriate size (Fig. 9(b)).⁷⁴ Interestingly, there are several compounds in existing pharmaceutical products that fulfill this minimal structure. Examples include the anticholinergic drug dexetamide, the aromatase inhibitor rogletimide, the hepatitis C virus

Only positions (a) and (b) of imides seem to have potential for structural diversification. Substitutions at position (a) affect the affinity of five- and six-membered cyclic compounds for MsCl4 binding. Thalidomide shows higher affinity than glutarimide; similarly, uridine shows higher affinity than uracil, suggesting that compounds containing a substituent at position (a) bind to MsCl4 more strongly than the corresponding compounds without it. On the other hand, aminogluthethimide, which carries two substituents at position (a), shows slightly lower affinity than glutarimide. Likewise, ethosuximide containing methyl and ethyl groups at position (a) shows much weaker affinity than succinimide (Fig. 9(c)), suggesting that imide compounds containing a quaternary carbon at position (a) may not be good CRBN binders. Meanwhile, the carbonyl at position (b) contributes to the binding but is not essential, and its substitution affects affinity for MsCl4. For example, substitution of the oxygen atom linked to the carbon at position (b) of succinimide with a methyl group decreases affinity for MsCl4, while substitution of the same oxygen atom of hydantoin to sulfur conversely increases the affinity. However, the amino group of position (c) and the carbonyl group of position (d) are critical for MsCl4 binding and cannot be derivatized. Replacement of the carbon atom at position (e) with nitrogen also substantially reduces affinity for MsCl4 in both five- and six-membered rings, suggesting that a polar group at this position disrupts the interaction (Fig. 9(c)). With regard to position (f), cycloheximide, a glutarimide-based six-membered ring with a branched carbon at this position, and barbiturate, a uracil-based six-membered ring with a carbonyl group at the same position, do not bind to MsCl4, suggesting that a large substituent at position (f) causes steric clash with the pocket (Fig. 9(c)).⁷⁴ Taken together, these results indicate that only positions (a) and (b) can be exploited for structural diversification to modulate affinity for CRBN. According to a recent study using microscale thermophoresis assays, the affinities of these potential CRBN-binders for MsCl4 are generally consistent with those for the TBD of human CRBN with a few exceptions.⁷⁷

Establishing SARs of thalidomide-based degraders using degradation of intracellular neosubstrates as a measure of activity is more challenging due to concurrent factors, such as membrane permeability and competitive binding to other neosubstrates. Binding affinities of thalidomide-based degraders for both CRBN and neosubstrates are critical for targeted degradation of neosubstrates. The phthalimide moiety is important for ligand-induced degradation of IKZF1, and substitutions on the aromatic ring or deviations from isoindoline structure inhibit its degradation.⁷⁸ Moreover, the crystal structure of DDB1-CRBN-pomalidomide binding to the zinc finger domain of IKZF1 revealed how lenalidomide and pomalidomide bind to IKZF1 more strongly than thalidomide. The additional amino group present in the phthalimide moiety of lenalidomide and pomalidomide forms a water-mediated

Thalidomide is teratogenic in humans, non-human primates, rabbits, chickens, and zebrafish, but not in rodents including mice.⁸¹ Animal experiments on mice did not show any serious side effects,⁸² which is considered to be one of the reasons behind the thalidomide disaster. The anti-myeloma effects of IMiDs are, furthermore, also not observed in mice.⁸³ This species specificity of thalidomide has been a long-standing question, but recent structural studies have now provided important insights. Despite the 94% amino acid sequence identity between human and mouse CRBN, Ikaros and Aiolos were not degraded when mouse Crbn, instead of human CRBN, was expressed in human cell lines.⁶⁷ The V388 in human CRBN was substituted with isoleucine in mice (I391) (Fig. 10(a)); structural analysis shows that the bulky side chain of isoleucine is sterically hindered to prevent neosubstrate access to mouse Crbn.⁴ The expression of humanized CRBN, in which I391 of mouse Crbn is substituted with valine (I391V), was sufficient for the degradation of Ikaros and Ck1 α in mouse cell lines.

Fig. 10 Partial sequences of CRBN TBDS and SALL4 zinc fingers from 6 vertebrate species. Multiple sequence alignment of the CRBN TBD (a) and the SALL4 zinc finger motif containing a degron (b) from 6 vertebrate species. Substitutions from the human orthologs are highlighted in magenta. Arrows denote key residues partially responsible for species specificity of thalidomide and its derivatives. Amino acids comprising the tri-Trp pocket of CRBN (a) and those comprising the C2H2 motif and the degron (CXXCG) of SALL4 (b) are shown in gray background.

In past decades, the mainstay of pharmaceuticals has been small molecule compounds that exert their effects by binding to the active site of a target protein, temporarily inhibiting its enzymatic activity. Therefore, proteins that lack a clear active site, such as transcription factors, are considered undruggable; it is estimated that these comprise approximately 80% of the human proteome. Thalidomide-based degraders have a different mode of action, acting as molecular glues that alter the substrate specificity of CRBN, leading to the degradation of new target proteins. Such protein degraders with a new mode of action, *i.e.* hijacking the UPS, causing the degradation of target proteins, are attracting attention as a new modality with the potential to target undruggable proteins. Protein degraders have a catalytic mechanism of action in which a single compound induces the degradation of multiple target proteins; this is superior to conventional small molecules with the stoichiometric mode of action.

One class of protein degraders called “molecular glue degraders” includes thalidomide and its derivatives. The term was first proposed in the study of the plant hormone auxin. Auxin acts by physically linking TIR1, an F-box protein of the Skp, Cullin, F-box containing complex (SCF), to Aux/IAA, leading to its proteasomal degradation.⁸⁶ The only protein degraders currently used in clinical practice are thalidomide-based glue degraders such as thalidomide, lenalidomide, and pomalidomide. Although many neosubstrates have already been identified, the number is expected to rise substantially in

the future. This is because numerous C2H2 zinc finger proteins contain a potential degron(s) that may be recognized by CRL4^{CRBN}; this largest family of transcription factors may be targeted by appropriate modification of the compound.

Recently, new classes of molecular glue degraders have been reported. A series of sulfonamides, such as E7820, Indisulam, and chloroquinoxaline sulfonamides, act as molecular glues; they bind to the DCAF15 subunit of CRL4^{DCAF15}, causing the degradation of the splicing factor RBM39.^{87,88} The cyclin-dependent kinase (CDK) inhibitors CR8 and HQ461 also function as molecular glue degraders that cause the degradation cyclin K *via* its binding partner CDK12. This occurs in the absence of substrate recognition receptors because CR8 directly binds to the DDB1 subunit of CRL4 and recruits the CDK-cyclin complex *via* CDK12.^{89,90} These findings suggest that molecular glue degraders are more diverse with a broader potential than anticipated as a therapeutic modality.

The other class of protein degraders is the PROteolysis-Targeting Chimeras (PROTACs), which are composed of two functional groups targeting E3 and protein of interest (POI), respectively, and a spacer linking them. The benefit of this concept is that any desired protein can be targeted for degradation as long as there is a compound that binds to it. On the other hand, due to their tripartite nature, they have large molecular weights and may have unfavorable pharmacokinetics. Moreover, the so-called hook effect of PROTACs may limit the efficiency of POI degradation. Since two ligands bind to their targets independently, high concentrations of PROTACs favor the formation of two binary complexes over the formation of the ternary complex. PROTACs were first reported in 2001; in that study, methionine aminopeptidase MetAP-2 was successfully recruited to the E3 ubiquitin ligase SCF ^{β -TRCP} for ubiquitination.⁹¹ The compound, however, contains a membrane-impermeable phosphopeptide moiety and cannot target intracellular MetAP-2 for degradation. Later, revealing that the mechanism of action of thalidomide is molecular glue fueled the rapid development of PROTACs. A variety of E3s have been targeted by PROTACs, including CRBN, Von Hippel-Lindau (VHL), IAP, and MDM2,^{92–95} among which CRBN is perhaps most extensively studied as a target of PROTACs. For CRBN-based PROTACs, thalidomide or its derivative is used for the E3 binding site (Fig. 11). Although we will not go into the details in this review, CRBN-based PROTACs targeting BRD4, FKBP12, BCR-ABL, SIRT2, CDK6, and BTK have been reported,^{92,96–100} and the number of successful PROTACs is steadily increasing. Click-formed proteolysis-targeting chimeras (CLIPTACs) are proposed as a new strategy to improve the poor pharmacokinetics of PROTACs caused by their large molecular weights; in this strategy, PROTACs are generated in cells by click chemistry from two precursors with good pharmacokinetics.¹⁰¹ In addition, light-controllable PROTACs that use light to activate or deactivate PROTACs have also been developed to solve the on-target-off-tissue toxicity, which also limits their clinical application.^{102,103} PROTAC technology has received particular attention in the field of anti-cancer drugs, and several PROTACs are currently in clinical trials.

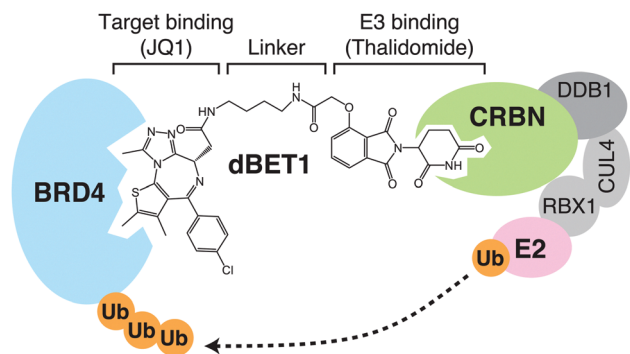


Fig. 11 CRBN-based PROTACs. dBET1, one of the CRBN-based PROTACs, consists of a linker connecting thalidomide and JQ1, the latter of which binds to its target BRD4. Formation of the ternary complex shown in the figure leads to the degradation of BRD4 via CRL4^{CRBN}.

10. Conclusions and perspectives

About 70 years have passed since the development of thalidomide. However, a major advance in our understanding of its mechanism of action has been made in the past decade. The breakthrough that drove this advance was the discovery of CRBN as a direct target of thalidomide. A series of studies focusing on the function of CRBN showed that thalidomide and its derivatives act as molecular glues that alter its substrate specificity, leading to the degradation of various neosubstrates. This finding achieved a molecular understanding of the diverse pharmacological effects of thalidomide and its derivatives, including anti-myeloma and teratogenic effects. Furthermore, demonstration of the clinical usefulness of molecular glue degraders has triggered rapid progress in the development of protein degraders, including PROTACs.

In the past decade, research on CRBN has focused mainly on the mechanism of action of thalidomide and its derivatives, but there are still unknowns concerning the physiological functions of CRBN. Understanding physiological functions of CRBN will be important in promoting the clinical application of thalidomide-based protein degraders. *CRBN* was initially identified as a gene associated with people with an intellectual disability, and its function was predicted to be related to brain function. Subsequent studies have shown that it is also involved in the regulation of ion channels and energy metabolism. It seems that the physiological function of CRBN are, at least in part, mediated by its native substrates, which are targeted for ubiquitination and degradation in the absence of drugs. Previous studies have identified SLO1, MEIS2, glutamine synthetase, AMPK α , and CLC-1 as native substrates of CRL4^{CRBN}.^{66,104–107} SLO1, a subunit of BK channels, is a protein identified in early studies as an interactor of CRBN,¹⁰⁸ and subsequent studies have shown that it is targeted for ubiquitination by CRL4^{CRBN}. Reportedly, polyubiquitination of SLO1 does not cause its degradation, but is important for its retention in the endoplasmic reticulum, which in turn regulates BK channel activity.¹⁰⁴ In addition, the nonsense mutation (E419X) of *CRBN* associated with intellectual disability causes a loss of interaction with BK channels; the resulting destabilization of

BK channels affects learning and memory.¹⁰⁹ With regard to AMPK, it is a master regulator of energy metabolism, and its dysfunction is responsible for a wide range of metabolic diseases, including obesity, diabetes, and hypertension. *Crbn* knockout mice were used to show that *Crbn* is a negative modulator of AMPK; compared to wild-type mice, feeding a normal diet to *Crbn*-deficient mice produced constitutive activation of AMPK. The knockout mice also showed significantly less weight gain on a high-fat diet and greatly improved insulin sensitivity.¹¹⁰ AMPK α , one of the subunits of AMPK, is ubiquitinated by CRL4^{CRBN},¹⁰⁶ providing a molecular explanation for the regulation of AMPK by CRBN. CRBN is, furthermore, involved in the homeostatic regulation of glutamine. Glutamine synthesis is negatively feedback-regulated by its product, glutamine. The mechanism is that glutamine induces acetylation of glutamine synthetase via p300/CBP, which then induces the degradation of glutamine synthetase via CRL4^{CRBN}, thereby maintaining glutamine homeostasis.¹⁰⁵ These findings have provided us with an understanding of the diverse physiological roles of CRBN, but unanswered questions remain. How is CRBN function regulated? How many native substrates are there, in total, for CRL4^{CRBN}? Does any molecule occupy the thalidomide-binding pocket of CRBN when CRL4^{CRBN} catalyzes ubiquitination of a native substrate? With regard to the last question, the observation that thalidomide and its derivatives inhibit the ubiquitination and degradation of MEIS2⁶⁶ and AMPK α ,¹⁰⁶ which are considered to be native substrates, implies the presence of a native ligand. As mentioned above, common metabolites such as uridine and succinimide can bind to the tri-Trp pocket of CRBN.^{73,74} These metabolites may serve as native ligands of CRBN, but further research is needed to reach definitive conclusions.

Development of therapeutic agents has not kept pace with the accumulation of basic knowledge, such as the identification of disease-causing genes. Challenges in developing therapeutics are manifold, but one of the difficulties is that most proteins are undruggable as targets of small molecule compounds. Protein degraders have shown great promise as a new modality that may overcome this difficulty. Although molecular glue degraders and PROTACs aim to achieve the same outcome, each strategy is better suited for a different type of targets and requires different considerations in molecular design because of their different target-binding modes and pharmacological properties. Thalidomide-based glue degraders target a wide variety of proteins with similar structural motifs. The design of new molecular glue degraders is limited by the fact that a compound-bound E3 ubiquitin ligase and a neosubstrate must exhibit excellent surface complementarity. However, its better pharmacokinetics and potential to target the large family of zinc finger-containing transcription factors is a major advantage over PROTACs. Since high sequence homology is not required for neosubstrates, the proteome that can be approached by thalidomide-based glue degraders may be broader than expected. Moreover, thalidomide-based glue degraders are already in use in clinical practice. Discovery of novel neosubstrates for these drugs may lead to the discovery of new therapeutic properties of the



Author contributions

All authors conceived and designed the review. J. Y. and Y. Y. mainly wrote the manuscript. J. Y. and T. I. prepared figures and table. H. H. supervised the review.

Conflicts of interest

H. H. received research support from Bristol-Meyers Squibb.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Numbers 20K08741 (to J. Y.), 20H03182 (to Y. Y.), 18H05502 (to T. I.), and 21H02955 (to T. I.).

References

- 1 S. Schreiber, S. Carr and B. Ebert, *Science*, 2014, **343**, 301–305.
- 2 G. Lu, R. Middleton, H. Sun, M. Naniong, C. Ott, C. Mitsiades, K. Wong, J. Bradner and W. Kaelin, *Science*, 2014, **343**, 305–309.
- 3 J. Krönke, E. Fink, P. Hollenbach, K. MacBeth, S. Hurst, N. Udeshi, P. Chamberlain, D. Mani, H. Man, A. Gandhi, T. Svinkina, R. Schneider, M. McConkey, M. Järås, E. Griffiths, M. Wetzler, L. Bullinger, B. Cathers, S. Carr, R. Chopra and B. Ebert, *Nature*, 2015, **523**, 183–188.
- 4 M. Matyskiela, G. Lu, T. Ito, B. Pagarigan, C. Lu, K. Miller, W. Fang, N. Wang, D. Nguyen, J. Houston, G. Carmel, T. Tran, M. Riley, L. Nosaka, G. Lander, S. Gaidarova, S. Xu, A. Ruchelman, H. Handa, J. Carmichael, T. Daniel, B. Cathers, A. Lopez-Girona and P. Chamberlain, *Nature*, 2016, **535**, 252–257.
- 5 K. Donovan, J. An, R. Nowak, J. Yuan, E. Fink, B. Berry, B. Ebert and E. Fischer, *eLife*, 2018, **7**, e38430.
- 6 M. Matyskiela, S. Couto, X. Zheng, G. Lu, J. Hui, K. Stamp, C. Drew, Y. Ren, M. Wang, A. Carpenter, C. Lee, T. Clayton, W. Fang, C. Lu, M. Riley, P. Abdubek, K. Blease, J. Hartke, G. Kumar, R. Vessey, M. Rolfe, L. Hamann and P. Chamberlain, *Nat. Chem. Biol.*, 2018, **14**, 981–987.
- 7 T. Asatsuma-Okumura, H. Ando, M. De Simone, J. Yamamoto, T. Sato, N. Shimizu, K. Asakawa, Y. Yamaguchi, T. Ito, L. Guerrini and H. Handa, *Nat. Chem. Biol.*, 2019, **15**, 1077–1084.
- 8 J. Yamamoto, T. Suwa, Y. Murase, S. Tateno, H. Mizutome, T. Asatsuma-Okumura, N. Shimizu, T. Kishi, S. Momose, M. Kizaki, T. Ito, Y. Yamaguchi and H. Handa, *Nat. Chem. Biol.*, 2020, **16**, 1208–1217.
- 9 S. Yamanaka, H. Murai, D. Saito, G. Abe, E. Tokunaga, T. Iwasaki, H. Takahashi, H. Takeda, T. Suzuki, N. Shibata, K. Tamura and T. Sawasaki, *EMBO J.*, 2021, **40**, e105375.
- 10 N. Shimizu, T. Asatsuma-Okumura, J. Yamamoto, Y. Yamaguchi, H. Handa and T. Ito, *Commun. Biol.*, 2021, **4**, 277.
- 11 A. Renneville, J. Gasser, D. Grinshpun, P. Jean Beltran, N. Udeshi, M. Matyskiela, T. Clayton, M. McConkey, K. Viswanathan, A. Tepper, A. Guirguis, R. Sellar, S. Cotteret, C. Marzac, V. Saada, S. De Botton, J. Kiladjian, J. Cayuela, M. Rolfe, P. Chamberlain, S. Carr and B. Ebert, *Blood Cancer Discovery*, 2021, **2**, 250–265.
- 12 S. Yamanaka, Y. Horiuchi, S. Matsuo, K. Kido, K. Nishino, M. Maeno, N. Shibata, H. Kosako and T. Sawasaki, *Nat. Commun.*, 2022, **13**, 183.
- 13 W. McBride, *Lancet*, 1961, **278**, 1358.
- 14 D. Burley and W. Lenz, *Lancet*, 1962, **279**, 271–272.
- 15 W. Lenz, *Teratology*, 1988, **38**, 203–215.
- 16 J. Sheskin, *Clin. Pharm. Ther.*, 1965, **6**, 303–306.
- 17 E. Sampaio, E. Sarno, R. Galilly, Z. Cohn and G. Kaplan, *J. Exp. Med.*, 1991, **173**, 699–703.
- 18 S. McHugh, I. Rifkin, J. Deighton, A. Wilson, P. Lachmann, C. Lockwood and P. Ewan, *Clin. Exp. Immunol.*, 1995, **99**, 160–167.
- 19 R. D'Amato, M. Loughnan, E. Flynn and J. Folkman, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 4082–4085.

- This journal is © The Royal Society of Chemistry 2022

- 51 P. Schafer, Y. Ye, L. Wu, J. Kosek, G. Ringheim, Z. Yang, L. Liu, M. Thomas, M. Palmisano and R. Chopra, *Ann. Rheum. Dis.*, 2018, **77**, 1516–1523.
- 52 T. Parman, M. Wiley and P. Wells, *Nat. Med.*, 1999, **5**, 582–585.
- 53 V. Jacques, A. Czarnik, T. Judge, L. Van der Ploeg and S. DeWitt, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, E1471–E1479.
- 54 T. Mori, T. Ito, S. Liu, H. Ando, S. Sakamoto, Y. Yamaguchi, E. Tokunaga, N. Shibata, H. Handa and T. Hakoshima, *Sci. Rep.*, 2018, **8**, 1294.
- 55 Y. Hirose, T. Kitazono, M. Sezaki, M. Abe, K. Sakimura, H. Funato, H. Handa, K. Vogt and M. Yanagisawa, *Proc. Natl. Acad. Sci. U. S. A.*, 2020, **117**, 23106–23112.
- 56 R. Al-Baradie, K. Yamada, C. Hilaire, W. Chan, C. Andrews, N. McIntosh, M. Nakano, E. Martonyi, W. Raymond, S. Okumura, M. Okihira and E. Engle, *Am. J. Hum. Genet.*, 2002, **71**, 1195–1199.
- 57 J. Kohlhase, *Hum. Mol. Genet.*, 2002, **11**, 2979–2987.
- 58 J. Kohlhase and L. Holmes, *Birth Defects Res., Part A*, 2004, **70**, 550–551.
- 59 D. Belair, G. Lu, L. Waller, J. Gustin, N. Collins and K. Kolaja, *Sci. Rep.*, 2020, **10**, 2864.
- 60 A. Mills, B. Zheng, X. Wang, H. Vogel, D. Roop and A. Bradley, *Nature*, 1999, **398**, 708–713.
- 61 A. Yang, M. Kaghad, D. Caput and F. McKeon, *Trends Genet.*, 2002, **18**, 90–95.
- 62 A. Yang, R. Schweitzer, D. Sun, M. Kaghad, N. Walker, R. Bronson, C. Tabin, A. Sharpe, D. Caput, C. Crum and F. McKeon, *Nature*, 1999, **398**, 714–718.
- 63 H. Chen, A. Beasley, Y. Hu and X. Chen, *PLoS One*, 2015, **10**, e0143878.
- 64 M. Barna, N. Hawe, L. Niswander and P. Pandolfi, *Nat. Genet.*, 2000, **25**, 166–172.
- 65 J. Higgins, J. Pucilowska, R. Lombardi and J. Rooney, *Neurology*, 2004, **63**, 1927–1931.
- 66 E. Fischer, K. Böhm, J. Lydeard, H. Yang, M. Stadler, S. Cavadini, J. Nagel, F. Serluca, V. Acker, G. Lingaraju, R. Tichkule, M. Schebesta, W. Forrester, M. Schirle, U. Hassiepen, J. Ottl, M. Hild, R. Beckwith, J. Harper, J. Jenkins and N. Thomä, *Nature*, 2014, **512**, 49–53.
- 67 P. Chamberlain, A. Lopez-Girona, K. Miller, G. Carmel, B. Pagarigan, B. Chie-Leon, E. Rychak, L. Corral, Y. Ren, M. Wang, M. Riley, S. Delker, T. Ito, H. Ando, T. Mori, Y. Hirano, H. Handa, T. Hakoshima, T. Daniel and B. Cathers, *Nat. Struct. Mol. Biol.*, 2014, **21**, 803–809.
- 68 T. Li, X. Chen, K. Garbutt, P. Zhou and N. Zheng, *Cell*, 2006, **124**, 105–117.
- 69 A. Sheereen, M. Alaamery, S. Bawazeer, Y. Al Yafee, S. Massadeh and W. Eyaid, *J. Med. Genet.*, 2017, **54**, 236–240.
- 70 G. Petzold, E. Fischer and N. Thomä, *Nature*, 2016, **532**, 127–130.
- 71 M. Matyskiela, T. Clayton, X. Zheng, C. Mayne, E. Tran, A. Carpenter, B. Pagarigan, J. McDonald, M. Rolfe, L. Hamann, G. Lu and P. Chamberlain, *Nat. Struct. Mol. Biol.*, 2020, **27**, 319–322.
- 72 Q. Sievers, G. Petzold, R. Bunker, A. Renneville, M. Slabicki, B. Liddicoat, W. Abdulrahman, T. Mikkelsen, B. Ebert and N. Thomä, *Science*, 2018, **362**, eaat0572.
- 73 M. Hartmann, I. Boichenko, M. Coles, F. Zanini, A. Lupas and B. Hernandez Alvarez, *J. Struct. Biol.*, 2014, **188**, 225–232.
- 74 I. Boichenko, K. Bär, S. Deiss, C. Heim, R. Albrecht, A. Lupas, B. Hernandez Alvarez and M. Hartmann, *ACS Omega*, 2018, **3**, 11163–11171.
- 75 C. Heim, D. Pliatsika, F. Mousavizadeh, K. Bär, B. Hernandez Alvarez, A. Giannis and M. Hartmann, *J. Med. Chem.*, 2019, **62**, 6615–6629.
- 76 C. Heim and M. Hartmann, *Acta Crystallogr., Sect. D: Struct. Biol.*, 2022, **78**, 290–298.
- 77 S. Maiwald, C. Heim, B. Hernandez Alvarez and M. Hartmann, *ACS Med. Chem. Lett.*, 2020, **12**, 74–81.
- 78 G. Burslem, P. Ottis, S. Jaime-Figueroa, A. Morgan, P. Cromm, M. Toure and C. Crews, *ChemMedChem*, 2018, **13**, 1508–1512.
- 79 H. Furihata, S. Yamanaka, T. Honda, Y. Miyauchi, A. Asano, N. Shibata, M. Tanokura, T. Sawasaki and T. Miyakawa, *Nat. Commun.*, 2020, **11**, 4578.
- 80 J. An, C. Ponthier, R. Sack, J. Seebacher, M. Stadler, K. Donovan and E. Fischer, *Nat. Commun.*, 2017, **8**, 15398.
- 81 N. Vargesson, *Birth Defects Res., Part C*, 2015, **105**, 140–156.
- 82 R. Brent, *J. Pediatr.*, 1964, **64**, 762–770.
- 83 M. Chesi, G. Matthews, V. Garbitt, S. Palmer, J. Shortt, M. Lefebure, A. Stewart, R. Johnstone and P. Bergsagel, *Blood*, 2012, **120**, 376–385.
- 84 E. Fink, M. McConkey, D. Adams, S. Haldar, J. Kennedy, A. Guirguis, N. Udeshi, D. Mani, M. Chen, B. Liddicoat, T. Svinkina, A. Nguyen, S. Carr and B. Ebert, *Blood*, 2018, **132**, 1535–1544.
- 85 Y. Gemechu, D. Millrine, S. Hashimoto, J. Prakash, K. Sanchenkova, H. Metwally, P. Gyanu, S. Kang and T. Kishimoto, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**, 11802–11807.
- 86 X. Tan, L. Calderon-Villalobos, M. Sharon, C. Zheng, C. Robinson, M. Estelle and N. Zheng, *Nature*, 2007, **446**, 640–645.
- 87 T. Uehara, Y. Minoshima, K. Sagane, N. Sugi, K. Mitsuhashi, N. Yamamoto, H. Kamiyama, K. Takahashi, Y. Kotake, M. Uesugi, A. Yokoi, A. Inoue, T. Yoshida, M. Mabuchi, A. Tanaka and T. Owa, *Nat. Chem. Biol.*, 2017, **13**, 675–680.
- 88 T. Han, M. Goralski, N. Gaskill, E. Capota, J. Kim, T. Ting, Y. Xie, N. Williams and D. Nijhawan, *Science*, 2017, **356**, eaal3755.
- 89 M. Slabicki, Z. Kozicka, G. Petzold, Y. Li, M. Manojkumar, R. Bunker, K. Donovan, Q. Sievers, J. Koepfel, D. Suchyta, A. Sperling, E. Fink, J. Gasser, L. Wang, S. Corsello, R. Sellar, M. Jan, D. Gillingham, C. Scholl, S. Fröhling, T. Golub, E. Fischer, N. Thomä and B. Ebert, *Nature*, 2020, **585**, 293–297.
- 90 L. Lv, P. Chen, L. Cao, Y. Li, Z. Zeng, Y. Cui, Q. Wu, J. Li, J. Wang, M. Dong, X. Qi and T. Han, *eLife*, 2020, **9**, e59994.



- 91 K. Sakamoto, K. Kim, A. Kumagai, F. Mercurio, C. Crews and R. Deshaies, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 8554–8559.
- 92 G. Winter, D. Buckley, J. Paulk, J. Roberts, A. Souza, S. Dhe-Paganon and J. Bradner, *Science*, 2015, **348**, 1376–1381.
- 93 W. Hon, M. Wilson, K. Harlos, T. Claridge, C. Schofield, C. Pugh, P. Maxwell, P. Ratcliffe, D. Stuart and E. Jones, *Nature*, 2002, **417**, 975–978.
- 94 Y. Itoh, M. Ishikawa, M. Naito and Y. Hashimoto, *J. Am. Chem. Soc.*, 2010, **132**, 5820–5826.
- 95 A. Schneekloth, M. Pucheault, H. Tae and C. Crews, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 5904–5908.
- 96 G. Winter, D. Buckley, J. Paulk, J. Roberts, A. Souza, S. Dhe-Paganon and J. Bradner, *Science*, 2015, **348**, 1376–1381.
- 97 A. Lai, M. Toure, D. Hellerschmied, J. Salami, S. Jaime-Figueroa, E. Ko, J. Hines and C. Crews, *Angew. Chem., Int. Ed.*, 2015, **55**, 807–810.
- 98 M. Schiedel, D. Herp, S. Hammelmann, S. Swyter, A. Lehotzky, D. Robaa, J. Oláh, J. Ovádi, W. Sippl and M. Jung, *J. Med. Chem.*, 2017, **61**, 482–491.
- 99 S. Su, Z. Yang, H. Gao, H. Yang, S. Zhu, Z. An, J. Wang, Q. Li, S. Chandarlapaty, H. Deng, W. Wu and Y. Rao, *J. Med. Chem.*, 2019, **62**, 7575–7582.
- 100 A. Zorba, C. Nguyen, Y. Xu, J. Starr, K. Borzilleri, J. Smith, H. Zhu, K. Farley, W. Ding, J. Schiemer, X. Feng, J. Chang, D. Uccello, J. Young, C. Garcia-Irrizary, L. Czabaniuk, B. Schuff, R. Oliver, J. Montgomery, M. Hayward, J. Coe, J. Chen, M. Niosi, S. Luthra, J. Shah, A. El-Kattan, X. Qiu, G. West, M. Noe, V. Shanmugasundaram, A. Gilbert, M. Brown and M. Calabrese, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**, E7285–E7292.
- 101 H. Lebraud, D. Wright, C. Johnson and T. Heightman, *ACS Cent. Sci.*, 2016, **2**, 927–934.
- 102 J. Liu, H. Chen, L. Ma, Z. He, D. Wang, Y. Liu, Q. Lin, T. Zhang, N. Gray, H. Kaniskan, J. Jin and W. Wei, *Sci. Adv.*, 2020, **6**, eaay5154.
- 103 M. Reynders, B. Matsuura, M. Bérouti, D. Simoneschi, A. Marzio, M. Pagano and D. Trauner, *Sci. Adv.*, 2020, **6**, eaay5064.
- 104 J. Liu, J. Ye, X. Zou, Z. Xu, Y. Feng, X. Zou, Z. Chen, Y. Li and Y. Cang, *Nat. Commun.*, 2014, **5**, 3924.
- 105 T. Van Nguyen, J. Lee, M. Sweredoski, S. Yang, S. Jeon, J. Harrison, J. Yim, S. Lee, H. Handa, B. Kuhlman, J. Jeong, J. Reitsma, C. Park, S. Hess and R. Deshaies, *Mol. Cell*, 2016, **61**, 809–820.
- 106 E. Kwon, X. Li, Y. Deng, H. Chang and D. Kim, *FASEB J.*, 2019, **33**, 6539–6550.
- 107 Y. Chen, Y. Peng, M. Hu, J. Huang, Y. Chien, J. Wu, T. Chen and C. Tang, *Sci. Rep.*, 2015, **5**, 10667.
- 108 S. Jo, K. Lee, S. Song, Y. Jung and C. Park, *J. Neurochem.*, 2005, **94**, 1212–1224.
- 109 T. Song, S. Liang, J. Liu, T. Zhang, Y. Yin, C. Geng, S. Gao, Y. Feng, H. Xu, D. Guo, A. Roberts, Y. Gu and Y. Cang, *PLoS Genet.*, 2018, **14**, e1007165.
- 110 K. Lee, S. Yang, Y. Kim, Y. Choi, J. Nam, C. Choi, H. Choi and C. Park, *Diabetes*, 2013, **62**, 1855–1864.
- 111 M. Gadd, A. Testa, X. Lucas, K. Chan, W. Chen, D. Lamont, M. Zengerle and A. Ciulli, *Nat. Chem. Biol.*, 2017, **13**, 514–521.
- 112 R. Nowak, S. DeAngelo, D. Buckley, Z. He, K. Donovan, J. An, N. Safaee, M. Jedrychowski, C. Ponthier, M. Ishoe, T. Zhang, J. Mancias, N. Gray, J. Bradner and E. Fischer, *Nat. Chem. Biol.*, 2018, **14**, 706–714.
- 113 B. Smith, S. Wang, S. Jaime-Figueroa, A. Harbin, J. Wang, B. Hamman and C. Crews, *Nat. Commun.*, 2019, **10**, 131.
- 114 W. Farnaby, M. Koegl, M. Roy, C. Whitworth, E. Diers, N. Trainor, D. Zollman, S. Steurer, J. Karolyi-Oezguer, C. Riedmueller, T. Gmaschitz, J. Wachter, C. Dank, M. Galant, B. Sharps, K. Rumpel, E. Traxler, T. Gerstberger, R. Schnitzer, O. Petermann, P. Greb, H. Weinstabl, G. Bader, A. Zoephel, A. Weiss-Puxbaum, K. Ehrenhöfer-Wölfer, S. Wöhrle, G. Boehmelt, J. Rinnenthal, H. Arnhof, N. Wiechens, M. Wu, T. Owen-Hughes, P. Ettmayer, M. Pearson, D. McConnell and A. Ciulli, *Nat. Chem. Biol.*, 2019, **15**, 672–680.
- 115 J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Židek, A. Potapenko, A. Bridgland, C. Meyer, S. Kohl, A. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A. Senior, K. Kavukcuoglu, P. Kohli and D. Hassabis, *Nature*, 2021, **596**, 583–589.

