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Molecular mechanisms of cereblon-based drugs

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ABSTRACT

Thalidomide, well known for its potent teratogenicity, has been re-evaluated as a clinically effective drug for the treatment of multiple myeloma. Although the direct target of thalidomide had been unclear until recently, we identified cereblon (CRBN) as a primary direct target of this drug by affinity purification using ferrite glycidyl methacrylate (FG) beads in 2010. CRBN functions as a unique substrate receptor of cullin-RING ligase 4 (CRL4). Various ligands including thalidomide bind to CRBN and alter substrate specificity depending on compound shape, resulting in multiple beneficial effects and/or teratogenicity. Lenalidomide, a thalidomide derivative approved by the US Food and Drug Administration (FDA), induces the degradation of onco-proteins such as Ikaros and casein kinase 1 alpha (CK1α), resulting in anti-cancer effects. Recently, novel CRBN-binding compounds have been developed and their mechanisms of action have been analyzed, including identification of CRBN-related ubiquitin conjugating enzymes (E2s). Moreover, the 3D structure of several CRBN-ligand-substrate complexes has been determined. Ligands were shown to work as a molecular glue between CRBN and its neosubstrate. In addition, investigators have been recently developing CRBN-based proteolysis-targeting chimeras to achieve degradation of proteins of interest. In this review, the molecular mechanisms of classical and new CRBN-based drugs are described, and recent advances in this field are discussed.

Contents

Abbreviations: 3D, three dimentional; ABC, activated B-cell; ALK, anaplastic lymphoma kinase; AML, acute myeloid leukemia; BCR-ABL, breakpoint cluster region-abelson murine leukemia viral oncogene homolog; BET, bromodomain and extraterminal domain; BK, big potassium; BRD4, bromodomain-containing protein 4; Cas9, CRISPR-associated protein 9; CDK9, cyclin-dependent kynase 9; CK1α, casein kinase 1 alpha; CML, chronic myeloid leukemia; COP9, consitutive photomorphogenesis 9; CRBN, cereblon; CRISPR, clustered regularly interspaced short palindromic repeats; CRL4, cullin-RING ligase 4; CSN, COP9 signalosome; CUL1, Cullin-1; Cul4A, Cullin-4A; DDB1, DNA damage-binding protein 1; DLBCL, diffuse large B-cell lymphoma; DRRS, Duane-radial ray syndrome; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; eRF1, eukaryotic release factor 1; Fbxo7, F-box only protein 7; FDA, Food and Drug Administration; FG, ferrite glycidyl; GCB, germinal cell B-cell; Gly, glycine; GS, glutamine synthetase; GSPT1, G1 to S Phase Transition 1; His, histidine; HOS, Holt-Oran syndrome; IKZF1, Ikaros family zinc finger protein 1; IKZF3, Ikaros family zinc finger protein 3; IL-2, interleukin-2; IMiDs, immunomodulatory imide drugs; K, lysine; MEIS2, myeloid ecotropic integration site 2; ORF, open reading frame; Phe, phenylalanine; POI, protein of interest; PROTAC, proteolysis-targeting chimera; pSILAC, pulsed Stable Isotope Labeling of Amino acids in Cell culture; RING, really interesting new gene; RNAi, RNA interference; Roc1, Regulator of Cullins-1; SALL4, spalt-like transcription factor 4; SCF, Skp, Cullin, F-box containing; sgRNA, single-guide RNA; SILAC, Stable Isotope Labeling of Amino acids in Cell culture; Sirt2, sirtuin 2; SUP35, suppressor gene 35; Trp, tryptophan; Tyr, tyrosine; ZFP91, zinc finger protein 91.

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1. Introduction

Cereblon (CRBN) was reported as a thalidomide-binding protein in 2010 (Ito et al., 2010). Thalidomide (Fig. 1a) was first developed as a sedative drug in the 1950s, but was withdrawn from the market due to its teratogenicity; its use during the early stages of pregnancy resulted in the birth of more than 10,000 deformed children (Franks, Macpherson, & Figg, 2004; Ito, Ando, & Handa, 2011; Lenz, 1988; Melchert & List, 2007; Miller & Stromland, 1999; Vargesson, 2015). Despite its side effects, studies on thalidomide continued uncovering new potentially therapeutic activities, and it was approved for the treatment of leprosy in 1998 and multiple myeloma in 2006 (Bartlett, Dredge, & Dalgleish, 2004; Singhal et al., 1999; Zeldis, Williams, Thomas, & Elsayed, 1999). The dramatic comeback of thalidomide on the medical scene inspired the development of derivatives as potentially more potent therapeutics. To this end, lenalidomide (Fig. 1b) and pomalidomide (Fig.1c) were approved by the FDA in 2006 and 2013, respectively. These thalidomide derivatives, named immunomodulatory drugs (IMiDs), are used to cure multiple myeloma. Since thalidomide has wide-ranging effects in various tissues, it was predicted to exert its activities by binding to various targets (Fig. 2a).

2. Discovery of cereblon as a primary target of thalidomide

We have been developing affinity matrices for isolating target proteins that bind to small bioactive molecular compounds (or chemicals) for more than 20 years, including magnetic nanoparticles called ferrite glycidyl methacrylate (FG) beads (Nishio et al., 2008; Sakamoto, Kabe, Hatakeyama, Yamaguchi, & Handa, 2009; Shimizu et al., 2000). We have identified target proteins of various bioactive compounds that range from not only pharmaceutical drugs but also to natural products or endocrine disrupters (Azuma et al., 2008; Gupta et al., 2013; Iizumi et al., 2007; Y. Ito et al., 2012; Kabe et al., 2016; Kabe et al., 2006; Karasawa et al., 2013; Kume et al., 2010; Kuramori et al., 2009; Masaike et al., 2010; Uga et al., 2006; Yoshida, Kabe, Wada, Asai, & Handa, 2008). We used thalidomide-immobilized FG beads to identify the long-unknown thalidomide target cereblon (CRBN) (Ito et al., 2010). Although CRBN was a protein of unknown function at the time of discovery, it became clear that CRBN forms a complex with DNA damage-binding protein 1 (DDB1), Cullin-4A (Cul4A), and Regulator of Cullins-1 (Roc1), called Cullin-RING ligase 4 ($CRL4^{CRBN}$) (Angers et al., 2006; Lee & Zhou, 2007; Petroski & Deshaies, 2005), and biochemical analysis showed that it functions as an E3 ubiquitin ligase substrate receptor. Thus, thalidomide was predicted to exert its action by target-specific proteolysis mediated by CRBN. Overexpression of thalidomide binding-deficient CRBN in chicks or zebrafish suppresses thalidomide-induced limb and ear defects, which suggested that the teratogenic effects of thalidomide are exerted through the activity of CRBN (Ito et al., 2010). After the identification of CRBN, Stewart and colleagues reported that downregulation of CRBN induces cytotoxicity in myeloma cells, and that CRBN protein levels are reduced in myeloma patients with lenalidomide resistance (Zhu et al., 2011). We collaborated with Celgene (Summit, NJ), a US biopharmaceutical company that has developed IMiDs. Celgene and our group have shown that CRBN binds not only to thalidomide but also to lenalidomide and pomalidomide; knockdown of CRBN abolished the antiproliferative effect of lenalidomide and pomalidomide (Lopez-Girona et al., 2012). This finding suggested that both the teratogenic side effects of thalidomide and the main therapeutic effects of thalidomide related compounds are mediated by CRBN protein.

3. Mechanisms of action of cereblon-binding drugs

Exactly what happens after thalidomide/IMiDs bind to CRBN remained elusive, as did the nature of the pharmacological effects. Two groups independently have shown in 2014 that IMiDs induce the breakdown of transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) (Kronke et al., 2014; Lu et al., 2014). Kronke et al. (2014) identified IKZF1 and IKZF3 by Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC)-based quantitative mass spectrometry (MS), while Lu et al. (2014) utilized open reading frame (ORF)-luciferase library. $CRL4^{CRBN}$ can recognize IKZF1 and IKZF3 as a 'neosubstrate' in the

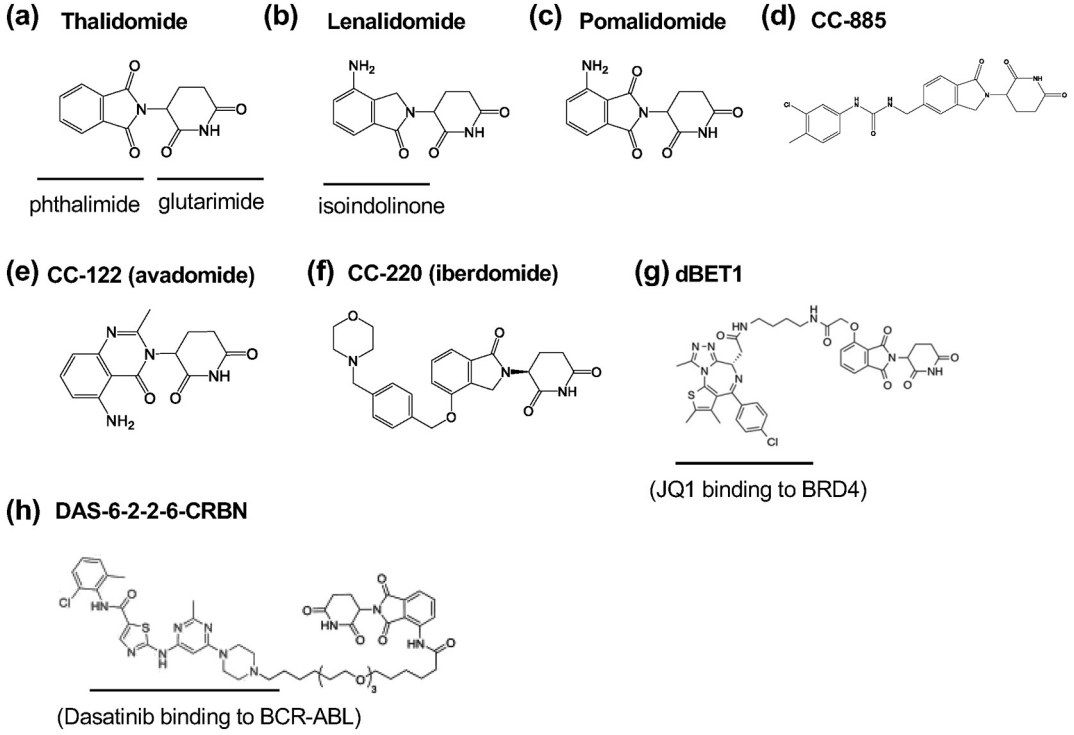

Fig. 1. Structure of CRBN-binding drugs. (a) Thalidomide. (b) Lenalidomide. (c) Pomalidomide. (d) CC-885. (e) CC-122. (f) CC-220. (g) dBET1, composed of JQ1 (a BRD4 inhibitor) and thalidomide. (h) DAS-6-2-2-6-CRBN, composed of Dasatinib (a BCR-ABL inhibitor) and pomalidomide.

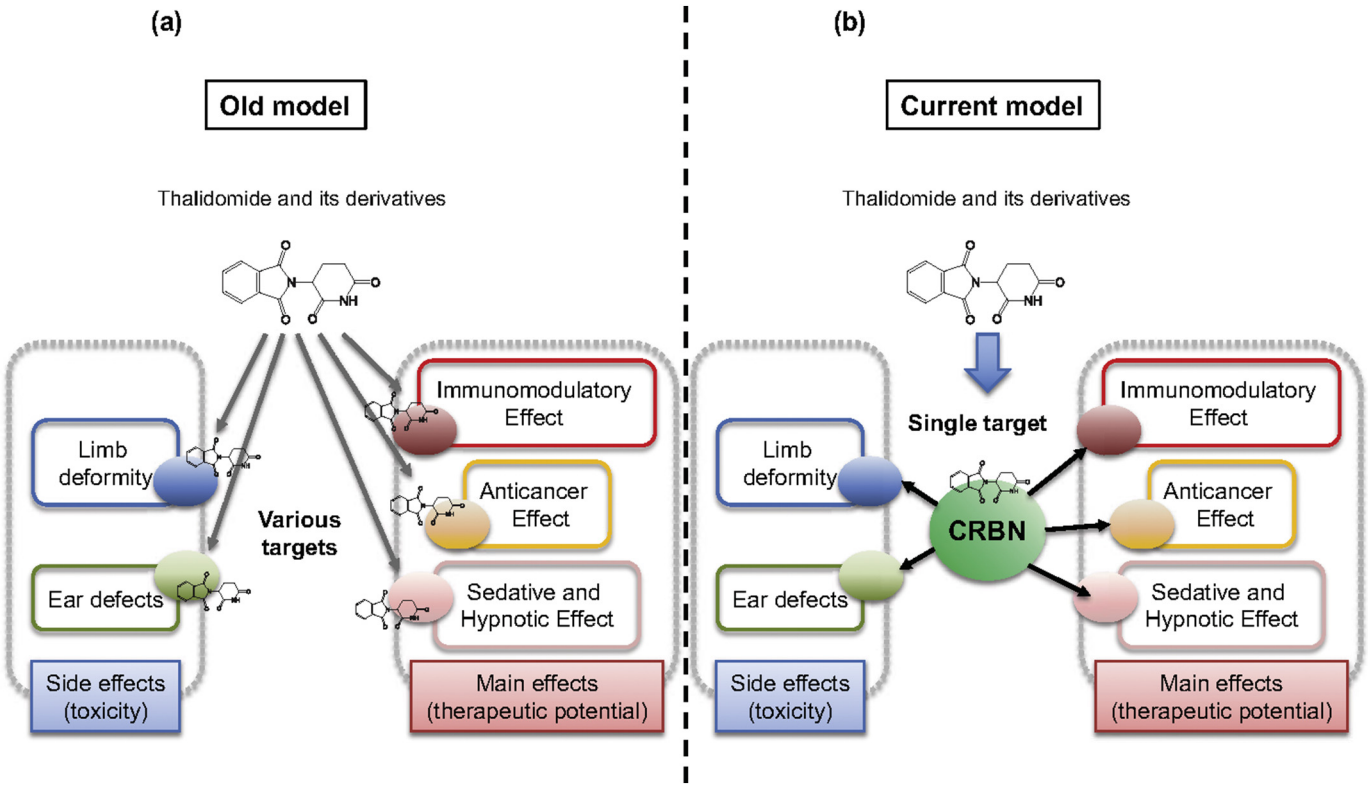

Fig. 2. The molecular target of thalidomide and derivatives. (a) Before identification of CRBN, thalidomide and its derivatives were believed to have multiple targets, explaining their pleiotropic effects (Old model). (b) However, accumulating evidence suggests that thalidomide and its derivatives bind to CRBN as a sole/primary target protein, resulting in the breakdown of multiple neosubstrates mediated by the $CRL4^{CRBN}$ ubiquitin ligase complex (Current model).

presence of IMiDs, thereby directing these transcription factors to ubiquitination and degradation. IKZF1 and IKZF3 are important for the survival of multiple myeloma. Since overexpression of a non-degradable mutant of IKZF conferred lenalidomide resistance to myeloma cells, it was concluded that the anti-multiple myeloma actions of IMiDs were due to degradation of IKZF1 and IKZF3. Celgene group including us has shown that IKZF1 and IKZF3 proteins were degraded not only by lenalidomide but also by pomalidomide, resulting in upregulation of the IL-2 expression in T-cells (Gandhi et al., 2014).

Lenalidomide is the only IMiD that has a clinical effect on improving myelodysplastic syndrome (5q-), and the mechanism has been explored (List et al., 2005; List et al., 2006). In 2015, it was found that when lenalidomide binds to CRBN, casein kinase 1α (CK1α) is also degraded along with IKZF1 and IKZF3 (Kronke et al., 2015). In 5q- syndrome, there is a deletion in the long arm of chromosome 5 (the region including CSNK1A1 gene encoding CK1α), resulting in CK1α haploinsufficiency. In such 5q- cells, CK1α degradation by lenalidomide results in effective cell death mediated by the activation of p53. The data well fit with the previous study using the CK1α inhibitor (Jaras et al., 2014). It was also found that thalidomide and pomalidomide were considerably weaker in terms of CK1α degradation activity than lenalidomide. It has since been concluded that the substrates recognized by $CRL4^{CRBN}$ can differ depending on compound shape (Fig. 3).

Celgene synthesized a large number of CRBN-binding compounds, among which CC-885 (Fig. 1d) displays antiproliferative activity against cancer cell lines ranging from blood cancer to solid tumors such as liver cancer (Matyskiela et al., 2016). Interestingly, CC-885 antiproliferative activity is especially pronounced (subnanomolar) against acute myeloid leukemia (AML) or AML-derived cell lines. Classical CRBN-binding compounds such as thalidomide, lenalidomide, or pomalidomide do not possess anti-AML activity. In addition, using cells from AML patients, it was found that the antiproliferative activity of CC-885 was more effective in leukemia cells than normal white blood cells. Thus, CC-885 was deemed a potential new therapeutic agent for AML, but its mechanism of action remained largely unknown. Since the effect of CC-885 was completely abrogated by CRBN deletion via genome editing with the CRISPR/Cas9 system in an AML strain, CC-885 was shown to function dependently of CRBN. To clarify the anti-AML functional mechanism of this drug, we performed biochemical analysis. We first examined whether CC-885-induced CRBN neosubstrates were present, and immunoprecipitation experiments identified GSPT1 (G1 to S Phase Transition 1/Eukaryotic peptide chain release factor GTP-binding subunit ERF3A) as a CC-885-dependent $CRL4^{CRBN}$ neosubstrate. GSPT1 forms a complex with eRF1 and is known to control translation termination (Cheng et al., 2009; Kikuchi, Shimatake, & Kikuchi, 1988; Malta-Vacas et al., 2009; Zhouravleva et al., 1995). GSPT1 is evolutionarily conserved from budding yeast to human, and a budding yeast strain defective in this gene undergoes G1 arrest and growth suppression. Therefore, if GSPT1 is a $CRL4^{CRBN}$ neosubstrate and is degraded by CC-885 treatment, this could explain the anti-AML action. Indeed, when various cell tumors such as AML cell lines or 293 T cells were treated with CC-885, GSPT1 was degraded, and this degradation proceeds via the ubiquitin-proteasome system. Conversely, no degradation of GSPT1 was observed in the presence of conventional CRBN-binding drugs such as thalidomide, lenalidomide, or pomalidomide. Immunoprecipitation experiments showed that binding of CRBN to GSPT1 occurs only in the presence of CC-885, but not other compounds. These results demonstrated that the anti-AML effect, as described above, was CC-885-specific.

Next, the importance of GSPT1 degradation in the anti-AML effects of CC-885 was investigated. If AML cells expressing non-degradable GSPT1 mutants are resistant to the growth inhibitory effect of CC-885, this could indicate the importance of GSPT1 degradation in its action. We found that when the GSPT1 homolog SUP35 in budding yeast was expressed in human cells, it was not degraded by CC-885. Therefore, by comparing and analyzing the amino acid sequences of human

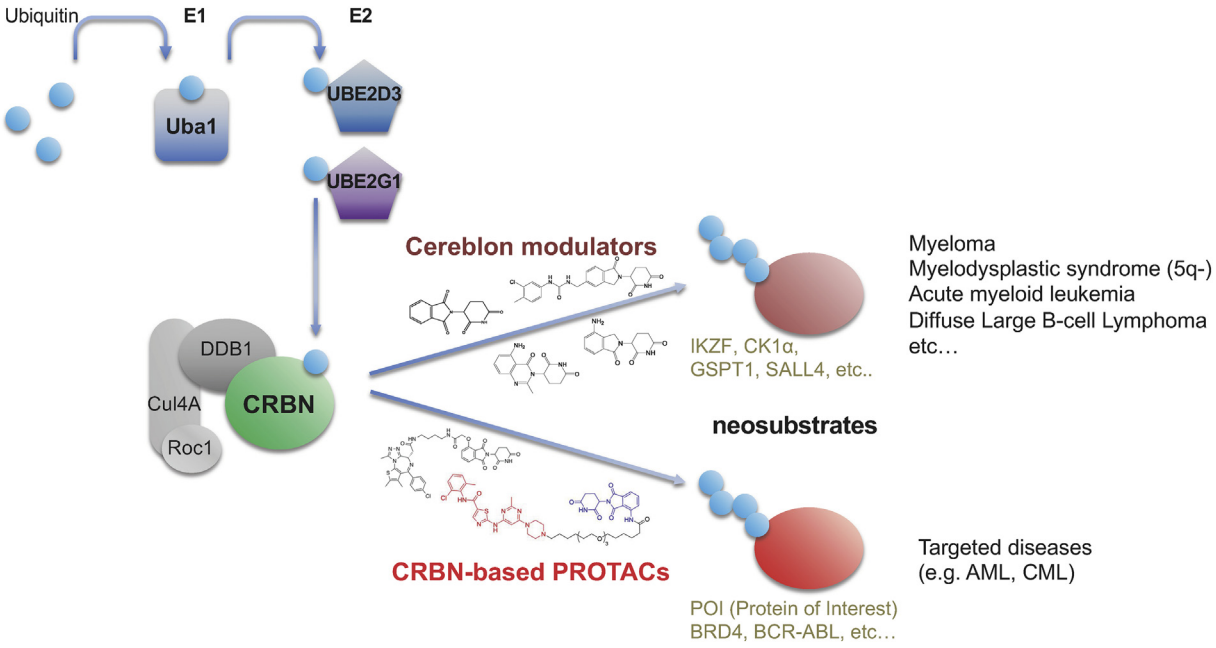

Fig. 3. The current model of mechanisms of action of thalidomide and other CRBN-binding drugs. Cereblon modulators (e.g. thalidomide and CC-885) or CRBN-based PROTACs (e.g. dBET1 and DAS-6-2-2-6-CRBN) exert their function via E3 ubiquitin ligase activity of $CRL4^{CRBN}$. $CRL4^{CRBN}$ receives ubiquitin from E2s such as UBE2D3 or UBE2G1, and ubiquitinates the neosubstrates. The therapeutic effects differ as $CRL4^{CRBN}$ target different neosubstrates depending on ligand shape.

GSPT1 and SUP35, we succeeded in narrowing down the region required for binding of GSPT1 to CRBN into a single amino acid glycine (G) 575. When GSPT1 G575 was converted to asparagine (N) (the corresponding residue in SUP35), the GSPT1 mutant lost CC-885-dependent degradation and binding to CRBN. Furthermore, when the non-degradable G575 N GSPT1 mutant was expressed in OCI-AML2 and MOLM-13 AML-derived cell lines, the inhibition of proliferation by CC-885 was mostly suppressed. As a final validation step, knockdown of GSPT1 by RNAi in OCI-AML2 cells inhibited cell growth, as occurs with CC-885. It was therefore concluded that the anti-cell proliferative effect of CC-885 was a consequence of GSPT1 breakdown via CRBN.

ZFP91 has since been identified as another IMiD-dependent $CRL4^{CRBN}$ substrate (An et al., 2017). This work aimed to identify neosubstrates other than the well-characterized IKZF1/3 that may function outside of hematology, and ZFP91 was identified in 293 T cells and HCT116 (colon cancer) cells by mass spectrometry following pulsed SILAC (pSILAC) labeling. The discovery of ZFP91 as another neosubstrate was interesting because it has a zinc-finger motif like IKZF1/3, as well as a key glycine motif as mentioned above in research on GSPT1 (Matyskiela et al., 2016).

By comparing the sequences of the identified $CRL4^{CRBN}$ neosubstrates, it was hypothesized that having a C2H2-type zinc-finger could be one of the characteristics, and a proteome-wide mass spectrometry system was used to screen a C2H2 zinc-finger library (Sievers et al., 2018). This approach identified eight new zinc-finger-containing substrate candidates. Sequential information is now considered reliable for predicting the accessibility of drug-bound $CRL4^{CRBN}$ to neosubstrates.

Accumulating knowledge on drug-dependent $CRL4^{CRBN}$ neosubstrates has allowed us to conclude that CRBN is the only target of thalidomide (or its analogs), which appears to exert its various activities by degrading numerous substrates, depending on drugs (Fig. 2b).

Next-generation CRBN-binding drugs are being developed by Celgene and possibly others. CC-122 (avadomide; Fig. 1e) induces the degradation of IKZF1 and IKZF3, and has antitumor and immunomodulatory activities against diffuse large B-cell lymphoma (DLBCL) (Hagner et al., 2015). Avadomide has broader activity than lenalidomide, since it is active not only against activated B-cell (ABC) DLBCL, but also germinal center B-cell (GCB) DLBCL, and it has been tested in phase I trials in patients with advanced solid tumors, non-Hodgkin lymphoma, and multiple myeloma (Rasco et al., 2019). Another CRBN-binding drug, CC-220 (iberdomide; Fig. 1f), has a higher affinity for CRBN, induces degradation of IKZF1 and IKZF3 more strongly than lenalidomide or pomalidomide, and has been studied for treating systemic lupus erythematosus (Matyskiela et al., 2018; Nakayama et al., 2017; Schafer et al., 2018). Currently, researchers are investigating identification of CC-122 or CC-220-specific neosubstrates of CRBN. These unidentified neosubstrates might be new therapeutic targets.

4. Structural biology of CRBN

Many identified neosubstrates possess at least one specific C2H2 zinc-finger motif. However, neither CK1α nor GSPT1 has a zinc-finger motif. To reveal the structural basis of recognition of neosubstrates by CRBN in the presence of drugs, structural analyses including X-ray crystallography have been conducted.

The crystal structure of the CRBN-DDB1 complex was first determined using human DDB1 and chicken CRBN, bound to thalidomide, lenalidomide, and pomalidomide (Chamberlain et al., 2014; Fischer et al., 2014). These studies revealed the amino acids of CRBN required for binding of IMiDs; His380, Trp382, Trp388, Trp402, and Phe404 are involved in IMiD binding, via hydrogen bonds and van der Waals contacts. In particular, in vitro, Tyr386 of CRBN affects the integrity of the binding pocket, and Trp388 is directly involved in the compound binding. Mutation of these amino acids to alanine ablated binding of IMiDs to CRBN.

Moreover, in 2016, the crystal structure of CRBN-DDB1 in complex with CK1α and lenalidomide was reported (Petzold, Fischer, & Thoma, 2016). This study showed how CK1α binds to the CRBN-lenalidomide surface via a beta-hairpin loop in CK1α consisting of residues 35–41. In fact, this study was the first to identify the specific glycine (Gly40 of CK1α) that is reportedly required in the surface-turn of IMiD-dependent $CRL4^{CRBN}$ substrates.

Next, we attempted structural biological analysis of CRBN-CC-885-GSPT1 since the X-ray crystallography structure had already been determined for both CRBN and GSPT1 (Chamberlain et al., 2014; Cheng et al.,

2009). Crystal structures of full-length DDB1 and CRBN (residues 40–442), CC-885, and domains 2 and 3 of GSPT1 (amino acids 437–633) were solved at a resolution of 3.6 Å. DDB1 is contained in the complex because the CRBN structure is stabilized by forming a heterodimer with DDB1. Domains 2 and 3 in the C-terminal part of GSPT1 were already known to bind CRBN in the presence of CC-885 based on previous biochemical experiments (Matyskiela et al., 2016).

X-ray crystallographic analysis revealed that CRBN binds to domain 3 of GSPT1 in the presence of CC-885, and CC-885 binds to both CRBN and GSPT1, hence it was concluded that CC-885 forms an interaction hotspot in CRBN, and GSPT1 was found to have a specific folded structure around G575 that binds to both the drug and CRBN. When the unique G575 is replaced by any other amino acid, the ability to bind CRBN is lost, as discussed above. Glycine is the smallest amino acid, suggesting that steric hindrance occurs when any other amino acid is present at this location in the structure. This distinct folded structure was also found in IKZF1, and was suggested to be important for binding between pomalidomide and CRBN. In the folded structure of IKZF1, there is a glycine similar to that in GSPT1, and CRBN binding was indeed lost when this glycine was mutated. Meanwhile, the crystal structure of CRBN, lenalidomide, and CK1α complex also revealed a specific glycine present in CK1α (Petzold et al., 2016). As expected, substitution of this residue with other amino acids led to the loss of CRBN- and CK1α-binding activity, further demonstrating the importance of the specific glycine.

Determining the crystal structure of $CRL4^{CRBN}$ and the mechanism of drug binding also clarified why activity is dependent on the thalidomide enantiomer. Thalidomide possesses one chiral center at the C3 carbon atom of the glutarimide ring. The two optical isomers, (*R*)- and (*S*)-enantiomers, were thought to exert different activities resulting in sedative and teratogenic effects, respectively. In 2018, we clarified the molecular differences between the two isomers by synthesizing each thalidomide enantiomer separately (Mori et al., 2018). Firstly, biochemical approaches showed that the (*S*)-enantiomer binds more strongly (~10-fold) to CRBN, and this isomer is therefore more potent inhibitor of CRBN auto-ubiquitination and IKZF1 recruitment to CRBN. Crystal structure analysis showed that while the (*S*)-enantiomer binds to CRBN in a 'relaxed' manner, the (*R*)-enantiomer must be twisted for CRBN binding, resulting in a clear difference in binding ability. We examined the effect of each enantiomer on the development of zebrafish, and (*S*)-thalidomide effectively inhibited fin outgrowth while (*R*)-thalidomide had a much weaker effect. Taken together, evidence suggests that (*S*)-thalidomide preferentially binds to CRBN, and the remaining (*R*)-thalidomide will be readily racemized, thereby increasing the supply of (*S*)-thalidomide.

5. Physiological functions of CRBN

We have described the functions of CRBN (in the form of the CRL4 E3 complex) exerted by the drug-dependent degradation of neosubstrates. Additionally, various other physiological functions of CRBN have been investigated. It was originally reported in 2004 that mutation of CRBN may lead to mental retardation or intellectual disability (Higgins, Pucilowska, Lombardi, & Rooney, 2004), and in 2005, CRBN was shown to interact directly with the cytosolic carboxy terminus of the BK_{Ca} channel α subunit (Slo) (Jo, Lee, Song, Jung, & Park, 2005). Since forebrain-specific conditional CRBN knockout mice splayed learning disabilities (Rajadhyaksha et al., 2012), CRBN was suggested to be related to brain (hippocampus) function, but the molecular mechanism and association with $CRL4^{CRBN}$ E3 activity was unknown. A report showed biochemically that the BK channel interacts with and is ubiquitinated by $CRL4^{CRBN}$ (Liu et al., 2014). Interestingly, ubiquitination of the BK channel by $CRL4^{CRBN}$ did not lead to protein degradation, but was a regulatory mark of cellular localization. BK channel ubiquitination by $CRL4^{CRBN}$ results in its retention at the endoplasmic reticulum and inhibition of channel function because it does not reach the cell surface. By contrast, a CRBN binding-deficient BK channel mutant failed to be ubiquitinated and was expressed at the cell surface. These findings suggested that $CRL4A^{CRBN}$ performs an important function as a gatekeeper for epileptogenesis by suppressing active BK channels, since increased macroscopic current and neuronal excitability caused by a gain-of-function point mutation in BK channels can lead to generalized epilepsy in humans (N'Gouemo, 2011). Thalidomide treatment inhibits the ubiquitination of BK channels, hence thalidomide may work as an antagonist for this substrate.

In another study, MEIS2 was found to be an endogenous substrate of $CRL4^{CRBN}$ (Fischer et al., 2014), and thalidomide or pomalidomide treatment inhibited the ubiquitination of MEIS2 by CRBN. MEIS2 is a homeodomain-containing transcription factor believed to be involved in human cardiac and limb development (Capdevila, Tsukui, Rodriquez Esteban, Zappavigna, & Izpisua Belmonte, 1999; Paige et al., 2012).

Glutamine synthetase (GS) was also identified as a candidate CRBN substrate based on SILAC analysis under thalidomide treatment for 4 h (Nguyen et al., 2016). In this study, GS was ubiquitinated in a CRBN-dependent manner but without involvement of the effect of IMiDs; when the glutamine concentration was raised after 36 to 48 h of glutamine starvation, GS binding to CRBN was stimulated, leading to ubiquitination and degradation of GS by $CRL4^{CRBN}$. Additionally, the authors found that the conserved KxxK motif (K = lysine) in the N-terminus of GS was an acetylation target of CBP/p300, and acetylation is crucial for CRBN binding and degradation.

6. Concluding remarks

Since the work of Ito et al. (2010), CRBN and its binding compounds have mostly been studied from the perspective of its main function, and while the identified neosubstrates explain anti-cancer activity, the molecular mechanisms of the side effects remained unclear. However, two groups independently reported a neosubstrate that could explain the teratogenic effects of thalidomide. Firstly, Fischer and colleagues identified SALL4 as a thalidomide-dependent neosubstrate of $CRL4^{CRBN}$ by mass spectrometry-based proteomics using human embryonic stem cells (Donovan et al., 2018). SALL4 is a *spalt*-like developmental transcription factor important for limb development, and genetic mutations have been linked to clinical syndromes such as Duane Radial Ray syndrome (DRRS) and Holt-Oran syndrome (HOS) (Knobloch & Ruther, 2008; Kohlhase et al., 2003). As phenotypes of DRRS and HOS overlap with thalidomide embryopathy, the association of SALL4 and $CRL4^{CRBN}$ was further investigated, revealing thalidomide (IMiDs)- and CRBN-dependent degradation of SALL4 in cell lines, and thalidomide-dependent CRBN binding and ubiquitination of SALL4. Furthermore, in mice and zebrafish, thalidomide-dependent SALL4 degradation does not occur due to species-specific differences in amino acid sequences. Celgene have also focused on SALL4, as it is an embryonic C2H2 zinc-finger transcription factor involved in fetal limb development possessing the degron motif found in other C2H2 $CRL4^{CRBN}$ substrates, and is associated with human congenital birth defects. In addition to the biochemical analysis of thalidomide-dependent SALL4 degradation, this group also showed that thalidomide treatment decreased SALL4 protein expression in rabbit embryos (Matyskiela et al., 2018). These two new reports help to clarify the molecular mechanism of the side effects of thalidomide. However, further investigations are needed to fully address the teratogenicity problem; firstly, the role of SALL4 in animal teratogenicity is still based on circumstantial evidence. Confirmation using animals expressing non-degradable SALL4 mutants could provide insight. Additionally, SALL4 is not degraded in zebrafish, even though fin and ear development are affected by thalidomide in this species. Other proteins including MEIS2 or CD147 may be important for thalidomide teratogenicity (Eichner et al., 2016; Fischer et al., 2014).

Accumulating evidence is being used to develop new drugs to degrade target proteins via $CRL4^{CRBN}$. In addition, the molecular regulatory

mechanism of $CRL4^{CRBN}$ is gradually becoming clear. Using genome-wide single-guide RNA (sgRNA) screening, knockout of constitutive photomorphogenesis 9 (COP9) signalosome (CSN) subunits in multiple myeloma (MM1.S) cells weakened the pomalidomide sensitivity of this cell line, and inhibited pomalidomide-dependent IKZF3 degradation (Liu et al., 2019). When CSN subunits were knocked out, CRBN protein levels decreased. CSN is known to negatively regulate SCF ubiquitin ligase activity. Knockdown of CSN subunits led to increased neddylation of CUL1, and increased Fbxo7 protein levels. The authors concluded that CSN inhibits CRBN-mediated ubiquitination and degradation by SCF^{Fbxo7}. Two different groups independently reported an E2 enzyme that regulates drug-dependent substrate ubiquitination by $CRL4^{CRBN}$. A group from the Broad Institute used genome-scale CRISPR-Cas9 screening in the lenalidomide-sensitive MM1.S cell line (Sievers, Gasser, Cowley, Fischer, & Ebert, 2018). They treated MM1.S cells infected with human lentivirus coding gRNA library with lenalidomide (or dimethylsulfoxide for controls). By harvesting genomic DNA from the cells and identifying the amplified gRNA in lenalidomide-treated cells using next-generation sequencing, they identified several genes required for lenalidomide activity in addition to CRBN, such as DDB1 and cullin-RING regulators (CSN subunits and UBE2M). Among the identified genes were E2 ubiquitin conjugating enzymes UBE2G1 and UBE2D3, and these underwent further investigation. Importantly, they found that (i) the COP9 signalosome and UBE2M alter Cul4A neddylation and CRBN protein levels, and (ii) UBE2G1 and UBE2D3 cooperate with lenarlidomide-induced ubiquitination of IKZF1 and IKZF3. Celgene performed CRISPR-Cas9 screening of an E2 enzyme library and identified UBE2G1 as the most critical E2 enzyme mediating pomalidomide-dependent IKZF1 degradation (Lu et al., 2018). Further investigation revealed that although knockout of UBE2D3 had little effect, double knockout of UBE2G1 and UBE2D3 completely blocked pomalidomide-dependent degradation of IKZF1. Subsequent reconstruction of in vitro ubiquitination assays illuminated the role of these E2 enzymes in CRBN-neosubstrate ubiquitination; UBE2D3 triggers the first ubiquitination of neosubstrates, and UBE2G1 catalyzes the following poly-ubiquitination. These findings proved essential in addressing the problem of myeloma patients acquiring resistance to treatment with IMiDs; both CRBN levels and other $CRL4^{CRBN}$-related factors should be taken into consideration.

Since the molecular basis of the action of CRBN-binding drugs such as thalidomide has been clarified, the 'targeted protein degradation (or induced protein degaradation)' approach has been developed. This approach attempts to actively degrade the proteins responsible for cancer and other diseases by artificially manipulating the ubiquitin-proteasome system using the compounds described below as tools.

This concept, originally proposed as proteolysis-targeting chimera (PROTAC) by Crews and Deshaies in 2001, aimed to achieve targeted protein degradation by synthesizing a fused drug in which two ligands that bind either the target substrate to be degraded or ubiquitin ligase are fused (Sakamoto et al., 2001). In 2015, Bradner and colleagues developed the JQ1 and thalidomide fusion compound dBET1 (Fig. 1g), and this had an inhibitory effect on AML and similar cells (Winter et al., 2015). JQ1 targets Bromodomain and Extra-Terminal motif (BET) transcription factor proteins including BRD4, and dBET1 treatment of AML cells resulted in degradation of BRD4 via CRBN. Similar results were presented soon after, and compound DAS-6-2-2-6-CRBN (Fig. 1h) was developed, in which Dasatinib (an inhibitor of BCR-ABL) and pomalidomide were fused to achieve protein degradation of BCR-ABL, a oncogenic driver for chronic myeloid leukemia (CML) (Lai et al., 2016). Development of various CRBN-based degraders that induce the breakdown of protein of interest (POI) such as CDK9, ALK, Sirt2 and Tau has since been reported (Bondeson et al., 2018; Lai & Crews, 2017; Powell et al., 2018; Schiedel et al., 2018; Silva et al., 2019), and clinical trials are planned (Fig. 3).

There remain many questions about the molecular functions of CRBN, not least regarding the origin of the sedative effects of thalidomide, and the original function of CRBN in the absence of IMiDs. There is a report that uridine can bind to the thalidomide-binding pocket of CRBN (Hartmann et al., 2014) suggesting the existence of internal CRBN ligands, but the physiological significance of binding of uridine to CRBN is still largely unknown. Degradation of multiple proteins by CRBN is rapidly being achieved using novel compounds such as degraders described in this review. Manipulating $CRL4^{CRBN}$ activity to degrade various proteins specifically by linking a CRBN-binding drug to another compound is attracting much attention as a potential next-generation therapeutic method as former RNAi and the genome editing (Fire et al., 1998; Gaj, Gersbach, & Barbas 3rd., 2013).

Declartion of compteting statement

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