

The novel mechanism of lenalidomide activity

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Lenalidomide acts by a novel drug mechanism—modulation of the substrate specificity of the CRL4^{CRBN} E3 ubiquitin ligase. In multiple myeloma, lenalidomide induces the ubiquitination of IKZF1 and IKZF3 by CRL4^{CRBN}. Subsequent proteasomal

degradation of these transcription factors kills multiple myeloma cells. In del(5q) myelodysplastic syndrome, lenalidomide induces the degradation of CK1 α , which preferentially affects del(5q) cells because they express this gene at haploinsufficient

levels. In the future, modulation of ubiquitin ligase function may enable us to target previously “undruggable” proteins. (*Blood*. 2015;126(21):2366-2369)

Introduction

Lenalidomide is a highly effective treatment for multiple myeloma,¹⁻⁵ other B-cell neoplasms including mantle cell lymphoma⁶ and chronic lymphocytic leukemia,^{7,8} and myelodysplastic syndrome (MDS) with del(5q).⁹⁻¹¹ Lenalidomide is a derivative of thalidomide, infamous for its teratogenicity following use as an anti-emetic in pregnant women. Thalidomide, lenalidomide, and another derivative, pomalidomide (Figure 1), increase IL-2 production in T lymphocytes¹²⁻¹⁴ and decrease pro-inflammatory cytokines,¹⁵ leading to their designation as immunomodulatory drugs (IMiDs). Despite their widespread clinical use, these drugs were developed before elucidation of the molecular basis of their activity.

Recent studies have revealed that thalidomide, lenalidomide, and pomalidomide share a novel pharmacologic mechanism of action (Figure 2). The drugs bind to an E3 ubiquitin ligase complex and modulate its substrate specificity, resulting in the proteasomal degradation of specific disease-related proteins. These are the first approved drugs to target an E3 ubiquitin ligase, demonstrating the potential for the development of new therapies that modulate ubiquitination. In lymphocytes, these drugs induce rapid and effective degradation of Ikaros (IKZF1) and Aiolos (IKZF3), 2 zinc finger transcription factors that would generally be considered “undruggable.”

Thalidomide, lenalidomide, and pomalidomide may represent the first in a new class of pharmacologic agents that alter the abundance of specific proteins by targeting ubiquitin ligase activity. Here, we review recent advances in the understanding of this mechanism as well as questions left unanswered.

Lenalidomide modulates the CRL4^{CRBN} E3 ubiquitin ligase

In 2010, Ito et al¹⁶ identified cereblon (CRBN) as the primary molecular target of thalidomide. CRBN is the substrate adaptor of the CRL4^{CRBN} E3 ubiquitin ligase, a cullin-ring ligase composed of damaged DNA-binding protein 1 (DDB1), cullin 4a (CUL4A), and regulator of cullins 1 (ROC1).

Proteome-wide surveys revealed that IKZF1 and IKZF3 are selectively ubiquitinated and degraded in the presence of lenalidomide in multiple myeloma cells.^{14,17} Additional proteomic studies identified casein kinase 1A1 (gene: *CSNK1A1*, protein: CK1 α) as a lenalidomide

target in myeloid cells, and CK1 α is ubiquitinated and degraded in a CRBN-dependent manner.¹⁸

Further biochemical investigation revealed that lenalidomide induces the recruitment of IKZF1, IKZF3, and CK1 α to CRL4^{CRBN} and promotes their ubiquitination by this complex. Lenalidomide-induced ubiquitination of these substrates can be recapitulated in vitro, confirming direct interaction between these targets and CRBN. Lenalidomide-induced substrate recognition is exquisitely specific, because other highly homologous members of the Ikaros and casein kinase families are not affected. A 60–amino acid region within the IKZF3 protein constitutes a lenalidomide-inducible degron. Fusion of this sequence to a heterologous protein is sufficient for lenalidomide-induced degradation, but single-point mutations in this region can abrogate the lenalidomide response.¹⁴ Once ubiquitinated, IKZF1, IKZF3, and CK1 α are degraded by the proteasome, ultimately leading to decreased protein levels. Taken together, these findings demonstrate that lenalidomide and other IMiDs act by a novel drug mechanism—modulation of the substrate specificity of the CRL4^{CRBN} E3 ubiquitin ligase.

The crystal structure of CRBN-DDB1 bound to lenalidomide provides further mechanistic insight into how IMiDs act on CRL4^{CRBN}. The IMiD compounds bind CRBN through their shared glutarimide ring, leaving portions of their variable phthaloyl ring solvent-exposed (Figure 1). Although the structure of the CRBN-lenalidomide-substrate complex is not yet available, the IMiD binding site sits in CRBN's proposed substrate binding pocket.^{19,20} This suggests that lenalidomide may act as “molecular glue,” bridging the interface between CRBN and recruited substrates. If this is the case, alterations in the chemical structure of IMiDs could potentially affect the substrates that are recruited to CRL4^{CRBN}. Indeed, a novel thalidomide analog, CC-122 (Figure 1B) has a strong effect on IKZF1/IKZF3 without any significant effect on CK1 α protein levels.¹⁸ This provides a proof of concept that subtle changes in the chemical structure of IMiDs can alter their substrate specificity.

Therapeutic activity of lenalidomide in multiple myeloma and other B-cell neoplasms

Lenalidomide is remarkably effective in multiple myeloma, with response rates for combined lenalidomide and dexamethasone exceeding 70% as a first-line therapy.¹⁻⁵ Degradation of IKZF1 and IKZF3

Submitted July 1, 2015; accepted September 23, 2015. Prepublished online as *Blood* First Edition paper, October 5, 2015; DOI 10.1182/blood-2015-07-567958.

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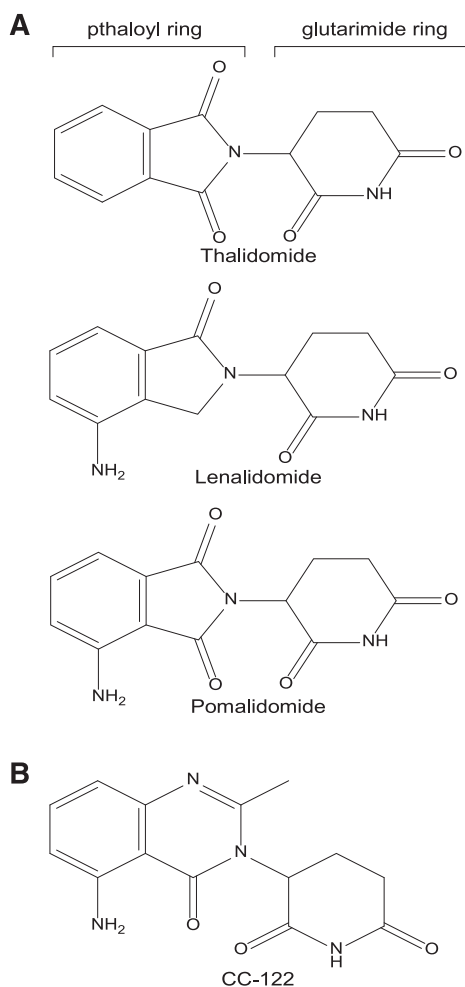


Figure 1. Chemical structures of IMiD compounds. (A) Chemical structures of thalidomide, lenalidomide, and pomalidomide, with pthaloyl and glutarimide rings indicated. (B) Chemical structure of CC-122.

provides a mechanism for lenalidomide's effects in multiple myeloma. IKZF1 and IKZF3 are transcription factors that are essential for B-cell differentiation, and multiple myeloma cells require ongoing expression of IKZF1 and IKZF3 for survival. In cell lines, lenalidomide response is associated with sensitivity to IKZF1/3 knockdown. Addition to

IKZF1 and IKZF3 expression may explain the efficacy of lenalidomide in other B-cell malignancies, including mantle cell lymphoma and chronic lymphocytic leukemia (CLL).

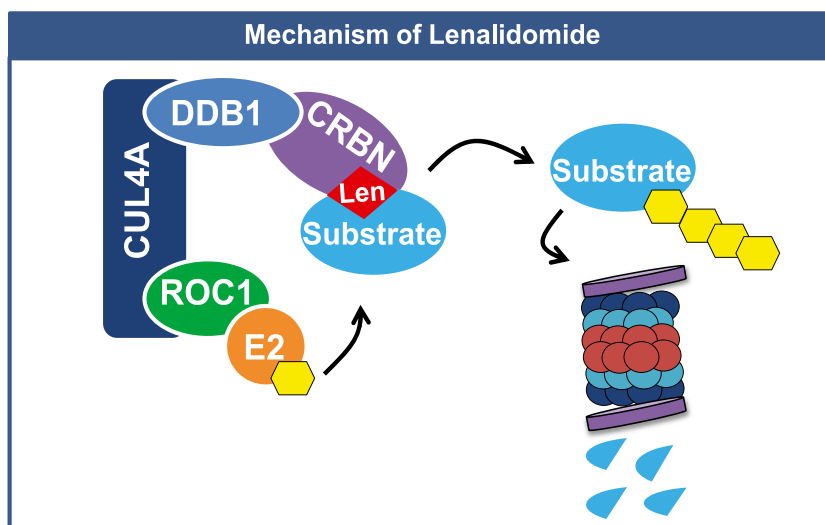
IKZF1 and IKZF3 regulate a transcriptional network that is essential for multiple myeloma cells. In particular, IKZF3 regulates expression of interferon regulatory factor 4 (IRF4), a transcription factor that has been previously demonstrated to be an essential gene in multiple myeloma²¹ and is linked with lenalidomide's activity in disease.²² IRF4 is a master regulator of an aberrant myeloma-specific gene program, which encompasses a positive feedback loop with the oncogene *MYC* as well as many other genes essential to myeloma survival. Regardless of their underlying genetic alterations, myeloma cells are addicted to this IRF4-controlled expression profile.²¹ Through targeted degradation of IKZF3 and the subsequent decrease in IRF4 expression, lenalidomide exploits the IRF4 addiction of multiple myeloma cells to contribute to its overall therapeutic window.

IKZF3 is also a transcriptional repressor of the interleukin 2 gene (*IL2*). Lenalidomide-induced degradation of IKZF3 is associated with increased *IL2* transcription and production, providing an explanation for at least some of lenalidomide's immunomodulatory effects. Increased *IL-2* production has been shown to increase the proliferation of natural killer (NK), NKT cells, and CD4⁺ T cells in CLL.²³ This lenalidomide-induced activation of an antileukemic immune response may contribute to lenalidomide's therapeutic efficacy in CLL.

Given lenalidomide's mechanism of action, it is intriguing that IMiDs and proteasome inhibitors are synergistic in the treatment of multiple myeloma.²⁴ Recent evidence suggests that this synergy may result from the pharmacokinetic properties and dosing schedules of these drugs. Although treatment with proteasome inhibitors can block lenalidomide-induced degradation of IKZF1 and IKZF3 in vitro,¹⁴ this effect depends on both the order of administration and the dose. When the drugs are administered at the same time, lenalidomide-induced degradation occurs before the onset of proteasomal blockade.²⁵ Similarly, low doses of proteasome inhibitor cause death of myeloma cells while still allowing proteasomal degradation of lenalidomide's substrates.²⁵ Such dose effects may be particularly relevant in the clinic because lenalidomide is given daily, whereas proteasome inhibitors are administered as pulse infusions. These findings remain to be confirmed clinically, but provide a biologically plausible explanation for these synergistic effects.

Taken together, these findings demonstrate how multiple drug effects may result from perturbations of a few key proteins. In the future, the identification of additional conditions whose pathogenesis relies on IKZF1 or IKZF3 may reveal additional clinical indications for IMiDs.

Figure 2. Mechanism of lenalidomide in multiple myeloma and del(5q) MDS. Lenalidomide binds to CRBN, the substrate adaptor of the CRL4^{CRBN} E3 ubiquitin ligase. Lenalidomide induces the recruitment of specific substrates to CRL4^{CRBN} and their ubiquitination by this ligase. In multiple myeloma, the ubiquitination and subsequent proteasomal degradation of two B-cell transcription factors, IKZF1 and IKZF3, kills multiple myeloma cells. In del(5q) MDS, the ubiquitination and degradation of casein kinase 1A1 causes the death of del(5q) cells, because they express this protein at haploinsufficient levels. Yellow hexagons represent ubiquitin molecules.



The mechanism of lenalidomide in del(5q) MDS

Lenalidomide is highly effective in MDS with del(5q), leading to a complete cytogenetic remission in ~50% of patients and transfusion independence in >70% of del(5q) MDS patients.⁹⁻¹¹ In del(5q) MDS, heterozygous deletion of the long arm of chromosome 5 causes haploinsufficient expression of several genes that have been shown to cause the symptoms of the disease, such as macrocytic anemia,²⁶ dysplastic megakaryopoiesis and thrombocytosis,^{27,28} and a risk of progression to acute myeloid leukemia.

In proteomic studies, we recently identified a lenalidomide-dependent CRBN substrate in myeloid cells, casein kinase 1A1, that is critical for the activity of lenalidomide in del(5q) MDS.¹⁸ Casein kinase 1A1 is one of only about 40 genes encoded within the del(5q) common deleted region and is expressed at ~50% of normal levels in del(5q) cells. Through its interactions with MDM2 and the β -catenin destruction complex, CK1 α negatively regulates both p53 and β -catenin protein levels. Heterozygous loss of *CSNK1A1* in the hematopoietic system causes increased levels of β -catenin and clonal dominance that contributes to the pathogenesis of del(5q) MDS. In contrast, homozygous loss of *CSNK1A1* leads to p53 induction and the death of hematopoietic cells. In addition, recurrent somatic mutations have been identified in the nondeleted allele of *CSNK1A1*, which increase β -catenin expression without altering p53 levels.²⁹ Thus, haploinsufficient expression of CK1 α plays an important role in the pathogenesis of del(5q) MDS.

However, haploinsufficient CK1 α expression may also make del(5q) cells more vulnerable to further decreases in casein kinase expression. Such cancer vulnerabilities resulting from heterozygous deletions have been termed CYCLOPS³⁰ genes and recent efforts have attempted to drug known vulnerabilities in the spliceosome, proteasome, and ribosome. According to this hypothesis, lenalidomide induces the degradation of CK1 α , which can be tolerated by normal cells with 2 copies of *CSNK1A1*, but kills del(5q) cells because they express this gene at 50% of normal levels.

Using shRNA knockdown of *CSNK1A1* and a genetically defined *CSNK1A1* knockout mouse model, we demonstrated that hematopoietic cells heterozygous for *CSNK1A1* are more sensitive to lenalidomide than wild-type cells with 2 copies of this gene. Lenalidomide induces p53-mediated cell-cycle arrest and cell death in cells heterozygous for *CSNK1A1*. Overexpression of *CSNK1A1* reduces the lenalidomide sensitivity of bone marrow cells from patients with del(5q) MDS but not cells from patients with normal karyotype MDS or normal donors. Similar results are not observed with overexpression of *IKZF1*, suggesting that degradation of *CSNK1A1* is the major contributor to lenalidomide's therapeutic window.¹⁸

Remarkably, the increased lenalidomide sensitivity of cells heterozygous for *CSNK1A1* can be rescued by heterozygous deletion of *TP53*. This fits well with the known mechanism, because genetic loss of *TP53* can rescue p53 activation resulting from homozygous loss of *CSNK1A1*. More importantly, it fits with known clinical mechanism of resistance, because lenalidomide resistance in patients with del(5q) MDS is associated with the selection of p53 mutant clones.³¹

Our findings demonstrate that lenalidomide induces the ubiquitination of CK1 α by CRL4^{CRBN}, resulting in the degradation of CK1 α by the proteasome. Del(5q) cells express CK1 α at lower baseline levels, so they are more sensitive to its degradation than wild-type cells with 2 copies of the gene. To our knowledge, this represents the first example of an Food and Drug Administration–approved drug that derives its therapeutic window from specifically targeting a haploinsufficient protein.

IMiDs in rodent models

Interrogation of the biological and clinical effects of lenalidomide has been impaired by the inactivity of IMiDs in murine models. Thalidomide has no teratogenicity in rodent models,³² and lenalidomide is not effective in murine multiple myeloma.³³ However, expression of human *CRBN* in murine cells restores lenalidomide activity. We identified a single, nonconserved amino acid residue that is responsible for the species-specific effects of lenalidomide; alteration of this one amino acid in murine Crbn is sufficient to enable lenalidomide-dependent degradation of IKZF1, IKZF3, and CK1 α .¹⁸ These insights will facilitate the development of improved models in which to dissect lenalidomide's pleiotropic effects in vivo.

Conclusion and future directions

Lenalidomide, thalidomide, and pomalidomide are the first approved drugs to target an E3 ubiquitin ligase, leading to the modulation of substrate specificity. Elucidation of this mechanism highlights many new opportunities for future research. IMiDs have pleiotropic effects that derive, at least in part, from the targeted degradation of key cellular regulators, and it is likely that new substrates remain to be identified. For example, a thalidomide-dependent CRBN substrate that mediates teratogenicity has not been conclusively determined, nor has a substrate been identified that mediates the IMiD-dependent repression of tumor necrosis factor- α production. Lenalidomide treatment is associated with an increased risk of secondary malignancies in patients who received alkylating agents,³⁴ but the molecular basis of this is unknown. The potential role of posttranslational modifications of CRBN or its substrates in lenalidomide activity remains to be determined.

The term “immunomodulatory drugs” reflects specific activities of these drugs, but modulation of ubiquitin ligase activity has the potential for many novel effects that extend beyond their immunomodulatory properties. Subtle alterations in the chemical structure of IMiDs can alter their substrate specificity.¹⁸ In addition, it was recently shown that small-molecule conjugates of thalidomide and protein-specific binders can target novel proteins to CRL4^{CRBN} for ubiquitination.³⁵ Taken together, this raises the possibility that we may one day be able to target a wide variety of previously “undruggable” targets for ubiquitination and degradation.

Acknowledgments

This work was supported by the National Institutes of Health, National Heart, Lung, and Blood Institute (R01HL082945) and National Cancer Institute (P01CA108631), the Edward P. Evans Foundation, the Gabrielle's Angel Foundation, and a Leukemia and Lymphoma Society Scholar Award (B.L.E.); and grants T32GM007753 and T32GM722639 from the National Institute of General Medical Sciences and F30CA199988-01 from the National Cancer Institute (E.C.F.).

Authorship

Contribution: E.C.F. and B.L.E. wrote and edited the manuscript.

Conflict-of-interest disclosure: B.L.E. has consulted for Celgene. The remaining author declares no competing financial interests.

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