Lenalidomide and its related ‘analogues’ modulate the substrate specificity of the CRL4<sup>CRBN</sup> E3 ubiquitin ligase complex. Polyubiquitination and subsequent proteasomal degradation of IKZF1 and IKZF3 in multiple myeloma and CK1α in del(5q) MDS has recently been linked to therapeutic efficacy of this class of compounds. Harnessing ubiquitin ligase substrate specificity, may in time facilitate the degradation of other ‘undruggable’ proteins and allow for separation of detrimental side effects of IMiD compounds from those associated with therapeutic efficacy.

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**Introduction**

Lenalidomide is a member of a class of molecules that have been termed immunomodulatory drugs (or IMiDs). It exhibits significant therapeutic activity in diseases such as multiple myeloma [1,2], myelodysplastic syndrome with del(5q) [3–5] and mantle cell lymphoma [6,7*]. Despite its demonstrated efficacy however, the basis for lenalidomide’s mechanism of action has only recently been elucidated.

**History of lenalidomide and clinical use**

Following the association of ‘phocomelia’ (congenital deformity with arm/leg shortening) and ‘amelia’ (absence of arms and/or legs) with thalidomide use as an anti-emetic during pregnancy in the 1950s, it seemed unlikely that this agent, or its derivatives, might subsequently be repurposed for therapeutic use in human disease. Almost six decades later, lenalidomide, a thalidomide analogue, is FDA-approved for use in multiple myeloma, del(5q) myelodysplastic syndrome and more recently, mantle cell lymphoma (Figure 1 and Table 1). Further emerging evidence suggests, that lenalidomide may have therapeutic potential particularly as combination therapy in other B-cell malignancies (such as DLBCL) [8*].

Over the past decade, studies in multiple myeloma have demonstrated a therapeutic benefit of lenalidomide treatment (typically in combination with steroids or additional agents) in the disease relapse or refractory setting [1,9*,10*,11], in the context of a new diagnosis [2,12,13] and potentially following stem cell transplantation [14,15]. These studies build on previous findings associated with thalidomide use in myeloma and are pertinent for more recently derived analogues such as pomalidomide [16*,17].

In MDS, transfusion-dependent patients harboring a chromosome 5q31 deletion (comprising 15–20% of MDS subtypes [18]) derive significant therapeutic benefit from lenalidomide with over 65% achieving transfusion independence [3–5]. Whilst more than 50% of patients achieve cytogenetic remissions through the selective apoptosis of abnormal MDS clones, the presence of additional mutations such as those within p53 have been associated with lenalidomide resistance [19]. By contrast, although responses to lenalidomide are still evident in those patients who lack the chromosome 5q deletion, these usually occur at significantly lower frequencies [20].

**Lenalidomide dictates the substrate specificity of the E3 ubiquitin ligase**

In spite of the demonstrated efficacy of the IMiD compounds in various disease entities, the mechanistic basis of their pleiotropic effects has only recently become apparent.

For a long time, the IMiD compounds have been most notable for their effects on cytokine modulation (through TNF-α inhibition in activated monocytes [21]) and T-cell co-stimulation through IL-2 production [22*]. Additional suggested mechanisms of action particularly in myeloma have included induction of cell cycle arrest through increased expression of the cyclin-dependent kinase inhibitor p21 (Cdkn1a) [23], decreased expression of interferon regulatory factor 4 (IRF4) [24], induction of apoptosis and inhibition of angiogenesis [25,26].
More recent work has demonstrated that lenalidomide, and the other IMiDs, bind cereblon (CRBN) through their common glutarimide ring and modulate the substrate specificity of the CRL4<sub>CRBN</sub> E3 ubiquitin ligase complex [23,27**,28**,29**] (Table 1). Formed by the binding of RING finger protein regulator of cullins 1 (ROC1) and DNA damage binding protein-1 (DDB1) to the cullin ring 4 ligase (CUL4A), the CRL4<sub>CRBN</sub> E3 ubiquitin ligase complex uses CRBN as a substrate adaptor to polyubiquitinate specific substrates tagging these proteins for degradation (Figure 2). In this context, the IMiDs are the first clinically-approved drugs that target an E3 ubiquitin ligase and thus represent a novel mechanism of action.

The interaction with the E3 ligase complex is notable for a number of findings:

- The binding of CRBN to substrates in the presence of IMiDs varies between human and mouse CRBN [30**].

From a structural perspective, the binding site of the IMiD compounds’ glutarimide ring lies within CRBN’s putative substrate-binding pocket [33**]. By contrast, the variable phthaloyl ring that makes each IMiD compound unique, likely confers substrate specificity for proteins that are recruited to the CRBN ligase complex. This model may shed light on why lenalidomide is more efficacious in del(5q) MDS in comparison with thalidomide. Of significance, as the chemical structure of this variable ring is amenable to alteration, this suggests it may be possible to direct the specificity of substrates recruited to the E3 ligase complex for subsequent degradation.

In myeloma, the B-cell lymphoid transcription factors, IKZF1 and IKZF3 are targeted for degradation in the presence of lenalidomide

Using two distinct approaches (SILAC-based quantitative mass spectrometry and a luciferase-based ORF screen), we and others have recently shown that lenalidomide induces the specific ubiquitination and proteosomal degradation of two B-cell lymphoid transcription factors, IKZF1 (Ikaros) and IKZF3 (Aiolos) in multiple myeloma cells [23,28**,29**]. Homologous members of
Table 1

A table outlining the chemical structures of current IMID compounds in use together with their most common dosing schedules and half-lives. The commonly shared glutarimide ring structure is denoted in red. Clinical indications are also shown together with common side effects as has been described for each compound.

<table>
<thead>
<tr>
<th>IMID compound</th>
<th>Structure</th>
<th>Common dosing schedule</th>
<th>Half-life</th>
<th>Potency (cf. thalidomide)</th>
<th>Clinical indication/uses</th>
<th>Common side effects</th>
</tr>
</thead>
</table>
| Thalidomide   | ![Thalidomide structure](image) | 200–400 mg orally daily | 5–7 hours | N/A | | *Multiple myeloma — new diagnosis and relapse setting [1,2]*  
*Erythema nodosum leprosum [44,45]* | *Teratogenicity (phocomelia)*  
*Peripheral neuropathy*  
*Sedation/anti-emesis*  
*Constipation*  
*Thrombosis*  
*Cytopenias* |
| Lenalidomide  | ![Lenalidomide structure](image) | 5–10 mg orally daily;  
21 days in a 28 day cycle or continuous dosing | 3–5 hours | ++ | | *Multiple myeloma — new diagnosis and relapse setting*  
*MDS — del(5q) abnormality [4,5]*  
*Mantle cell lymphoma [7]*  
*Other B-cell malignancies (DLBCL)* | *aGvHD*  
*Fatigue, diarrhea, constipation*  
*Increased incidence of secondary malignancies [15,46]*  
*Thrombosis*  
*Cytopenias* |
| Pomalidomide  | ![Pomalidomide structure](image) | 4 mg orally daily;  
21 days in a 28 day cycle | 7.5–9.5 hours | ++++ | | *Multiple myeloma — particularly in cases of thalidomide/lenalidomide resistance* | *Cytopenias*  
*Thrombosis*  
*Peripheral neuropathy — mild* |
CRBN is a member of the CRL4 E3 ubiquitin ligase complex—comprised of cullin ring 4 ligase (CUL4A), DNA damage binding protein-1 (DDB1) and RING finger protein regulator of cullins 1 (ROC1). Together these form a scaffold for the binding of E2. In the presence of IMiD compound, CRBN’s substrate affinity significantly increases and substrate binding occurs. Polyubiquitination of the tethered substrate marks the protein for subsequent proteosomal degradation.

the IKZF family (IKZF2, IKZF4 and IKZF5) are not degraded, highlighting the specificity of lenalidomide’s activity. Within IKZF3, a 60 amino-acid region constitutes a lenalidomide-inducible ‘degron’ which is necessary and sufficient for lenalidomide’s efficacy. This degron sequence is capable of conferring lenalidomide-dependent degradation of a heterologous protein. Single point mutations within this region can abrogate downstream effects. Importantly, this interaction demonstrates an ability to degrade two zinc-finger transcription factors that would otherwise be considered ‘undruggable’.

This recently deciphered mechanism of action is consistent with observed correlation between cereblon expression levels and sensitivity to IMiD therapy in patients with multiple myeloma [23]. Typically, reduced CRBN associates with IMiD resistance, while the opposite is also true.

Downstream, degradation of IKZF1/IKZF3, contributes to T-cell co-stimulation by derepressing IL-2 transcription [22*]. This observation may explain some of lenalidomide’s immunomodulatory effects in myeloma. IRF4, a transcription factor essential for development of multiple myeloma is also regulated by IKZF3, and the expression of the IRF4 gene is modulated by IMiD compounds [24,34].

**IKZF1 and IKZF3 as transcriptional regulators of lymphopoiesis**

Belonging to the family of Ikaros zinc-finger transcription factors, IKZF1 (Ikaros) and IKZF3 (Aiolos) are two of the family’s most well characterized members. Existing in both homodimeric and heterodimeric forms, these factors play an important role in both B and T lymphoid cell differentiation and development. Unlike IKZF1 which is expressed from the pluripotent stem cell phase through to the mature lymphocyte, IKZF3 first becomes detectable at the common lymphoid progenitor (CLP) phase and is expressed at higher levels in more mature B and T-cells [35].

Underscoring their role in lymphoid development, mutations in IKZF1 have been associated with a differentiation block, manifested as a lack of CLPs, mature B cells and natural killer (NK) cells and absent or aberrant T-cell differentiation [36,37]. By contrast, IKZF3 knockout mice develop a precursor B-cell hyperplasia and have an increased propensity for the development of autoantibodies and lymphoma.

As further evidence of their importance in B and T-cell development, IKZF1 and IKZF3 are associated with loss-of-function and dominant-negative mutations in B-cell and T-cell ALL [38]. The resultant differentiation block causes a failure to generate mature lymphocytes and drives development of leukemia.

**Casein kinase 1α is targeted for degradation by lenalidomide in the del(5q) subtype of myelodysplastic syndrome**

An alternative CRBN substrate has recently been identified in the lenalidomide-responsive del(5q) subtype of MDS in which variable parts of the long arm of chromosome
5 are deleted [30**]. Phenotypically, del(5q) MDS is associated with a macrocytic anemia, thrombocytosis with small monolobated megakaryocytes in the bone marrow and a female preponderance. It is a disease whose hallmark is the haploinsufficient expression of a number of genes including \textit{RPS11} [39,40].

Casein kinase 1\(\alpha\) (CK1\(\alpha\)) is a serine/threonine kinase encoded by the casein kinase 1A1 gene (\textit{CSNK1A1}) on the long arm of chromosome 5 in the distal common deleted region (CDR) (5q32) for del(5q) MDS. CK1\(\alpha\) functions as a negative regulator of p53 and \(\beta\)-catenin protein levels. Although heterogeneous inactivation of this gene increases \(\beta\)-catenin levels and provides hematopoietic stem cells with a growth advantage, further reductions of CK1\(\alpha\) are associated with hematopoietic stem cell failure and p53 activation [41\textsuperscript*]. We recently demonstrated that lenalidomide targets CK1\(\alpha\) for ubiquitination by the same E3 ubiquitin ligase complex, ultimately driving its degradation [30\textsuperscript**]. Although degradation of CK1\(\alpha\) may be tolerated by normal cells that express two copies of \textit{CSNK1A1}, haploinsufficient or reduced expression of this gene (as occurs in del(5q) cells) renders these cells particularly sensitive to lenalidomide therapy [41\textsuperscript*]. By contrast, the overexpression of \textit{CSNK1A1} is associated with reduced lenalidomide sensitivity in patients with del(5q) MDS. To date, CK1\(\alpha\)’s specific binding domain to CRBN remains unidentified.

Downstream, inhibition of casein kinase activates p53 driving apoptosis of del(5q) bearing cells. This finding may explain why MDS patients harboring a p53 mutation in addition to the 5q abnormality often exhibit inferior responses to lenalidomide therapy [19,42].

\textbf{Testing of IMiD compounds in murine models}

The basis of undetected teratogenicity in rodent models in which thalidomide was tested, attracted significant scrutiny in the years that followed its devastating effects in humans. This tragedy highlighted a significant deficiency in pre-clinical testing at the time [43] and has also limited our ability to study the biological effects of the IMiD compounds as a class of drugs.

Recent work has demonstrated that overexpression of human CRBN in murine cells enables murine cells to degrade substrates in response to lenalidomide. In the murine Crbn cDNA, a single point mutation (I391V) confers sensitivity to IMiD compounds in murine cells and explains the species-specific variation in response [30\textsuperscript**]. Based on these findings, we envisage the development of better \textit{in vivo} models, that will assist in mechanistic understanding of this group of compounds and their pleiotropic activity and in testing novel molecules \textit{in vivo}. As the basis of side effects such as increased thrombotic risk, neurotoxicity and teratogenicity associated with this class of drugs remains somewhat unexplained, the findings to date suggest that additional substrates are as yet unidentified.

\textbf{Conclusion}

In spite of their turbulent history, the IMiD compounds have been repurposed in the last decade and demonstrate efficacy in a number of hematological malignancies. Acting to modulate the substrate specificity of the CRL4\textsuperscript{CRBN} E3 ubiquitin ligase complex, these compounds draw attention to a novel mechanism of action in which ‘undruggable’ targets could one day be degraded. The biological effects of these compounds suggest that a number of additional substrates are as yet unidentified. Whether these will share a common means of interacting with CRBN remains to be determined. Harnessing the substrate specificity of these compounds by identifying additional analogues may deliver a potent tool for targeting proteins of interest for degradation and may also enable us to separate the teratogenic effects of these compounds from those associated with therapeutic benefit.

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New promising use of lenalidomide overcomes the negative impact of ABC subtype of DLBCL.


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