A Brighter Side to Thalidomide: Its Potential Use in Immunological Disorders

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Abstract

Thalidomide and its derivatives are immunomodulatory drugs (IMiDs) known for their sedative, teratogenic, anti-angiogenic, and anti-inflammatory properties. Commonly used in the treatment of cancers such as **Multiple Myeloma** and **Myelodysplastic Syndrome**, IMiDs have also been used in the treatment of an inflammatory skin pathology associated with **Hansen's disease/Leprosy** and show promise in the treatment of autoimmune disorders including **Systemic Lupus Erythmatosus** and **Inflammatory Bowel Disease**. Recent structural and experimental observations have revolutionized our understanding of these properties by revealing the fundamental molecular events underpinning IMiD activity. Here, we review these findings, their relevance to IMiD therapy in immunological disorders, and discuss how further research might unlock the vast clinical potential of these compounds.

Evolving Interest in the Thalidomide Mechanism of Action

Thalidomide remains best known as the cause of congenital limb abnormalities in what is considered by many to be the greatest medical scandal of the last century. In 1957 and marketed by the German pharmaceutical company Chemie-Grunenthal, thalidomide, under the trade-name Contergan, was introduced to the world as a completely non-toxic sedative that could be used safely, even during pregnancy [1]. Prescribed to alleviate morning sickness, it was widely distributed with tragic consequences; until its discontinuation in 1961, up to 10,000 babies were born with characteristic limb defects, referred to as **phocomelia**, with estimations rising to up to 100,000 if premature births and miscarriages were included [1-3]. According to **The Thalidomide Trust**, in the UK alone up to 467 individuals continue to live with consequences of the inadequate testing and regulatory regime governing drug distribution at that time. Worldwide, the figure could be as many as 3000 [4].

Over 55 years since its discontinuation controversy surrounding compensation for those affected continues to be a subject of media interest [1, 2, 4]. Recent decades have seen a more positive side to thalidomide emerge. It was first introduced as an anti-inflammatory therapy with remarkable efficacy in the treatment of **Erythema Nodosum Leprosum (ENL),**[5] an inflammatory condition associated with leprosy, and later, in the treatment of Multiple Myeloma [6]. Recognition of thalidomide's therapeutic value led to renewed interest and the development of thalidomide derivatives with enhanced potency, most notably **lenalidomide**. These next generation thalidomide derivatives, developed by the pharmaceutical company Celgene and collectively referred to as IMiDs, have received approval or are in development for the treatment of cancers including **Mantle Cell Lymphoma**, **Acute Myeloid Leukemia**, and **hepatocellular carcinoma (HPC)**, in addition to autoimmune disorders such as **psoriasis**, Systemic Lupus Erythmatosus (SLE) and Inflammatory Bowel Disease (IBD) (Table 1).

Unusually, IMiDs have achieved widespread use in the absence of a clear mechanistic understanding of their properties. Indeed, the precise molecular target of thalidomide has until recently remained unclear. Pioneering biochemical work using thalidomide conjugated beads enabled the seminal discovery that Cereblon, a substrate adaptor in the **Cullin4A (CUL4A)** ubiquitin ligase complex,[7] is a receptor for these compounds and responsible thalidomide teratogenicity [8]. Interest following this discovery prompted a series of studies, mostly using *in vitro* cell lines, that have defined key molecular processes underlying IMiD therapy in Multiple Myeloma and a subset of Mylodysplastic Syndrome subjects haboring a deletion in the long arm of chromosome 5 (**MDS del (5q)**). To date, however, few studies have addressed the implications of these findings when considering the anti-inflammatory properties of IMiDs. Indeed, recent advances in our understanding of the IMiD mechanism of

action have seldom been considered from an immunological perspective. Here, by discussing potential mechanisms underlying IMiD therapy in immunological disorders, we aim to encourage discussion on this comparatively neglected aspect of IMiD research.

The Anti-inflammatory Properties of Thalidomide: Use in Hansen's Disease/Leprosy

The story of thalidomide's anti-inflammatory properties begins with a single case report published not long after thalidomide's market withdrawal; a patient suffering from Hansen's disease, commonly known as Leprosy, and taking thalidomide for its sedative properties, observed the complete resolution of inflammatory skin lesions associated with this condition [5].

The result of infection with ***Mycobacterium Leprae***, Leprosy is initially a disease of the **Peripheral Nervous System (PNS)**. Infection first occurs in **Schwann cells**, [9] a neuronal cell lineage responsible for synthesis of the **Myelin Sheath**. The resulting neuronal damage causes a loss of sensory function [9]. Schwann cell migration then facilitates the spread of infection to other tissues triggering a **T helper 1** (Th1) type immune response [9, 10]. In many cases the infection is effectively contained and in some cases resolved, resulting in a mild form of disease termed **Tuberculoid Leprosy** [11]. However, in susceptible individuals, **anergic** T-cell responses permit further bacterial infiltration and the development of **Lepramatous Leprosy**, an extensive infection frequently accompanied (Approximately 50% of patients) by the development of painful skin lesions covering the whole body [10-12]. This dermatological condition, termed Erythema Nodosum Leprosum (ENL), is the result of immune reactions to *M. Leprae* and constitutes a major cause of morbidity in Leprosy [11]. Treatment options for ENL involve administration of either **Clofazimine** or thalidomide, and although there is some disagreement over which is the more appropriate,[11, 13] the efficacy of thalidomide is typically described as exceptional, with an estimated 70-90% of patients responsive to therapy and many exhibiting a rapid and complete recovery in a matter of days following treatment onset [11, 14].

Studies addressing the mechanism underlying these properties have typically focused on thalidomide's effect on the cytokine milieu. Initial studies of *in vitro* stimulated human monocytes revealed a suppressive effect of thalidomide on the pro-inflammatory cytokine TNFα induced *via* the **Lipopolysaccharide** (LPS) receptor, **Toll-like receptor** 4 (TLR4) [15, 16]. The production of other cytokines, including IL12,[17] and in our hands, IL6 and IFN-β, [18] were also suppressed in thalidomide treated, LPS stimulated human monocytes and murine peritoneal macrophages, respectively.

While there is no direct evidence linking this pathway to ENL pathogenesis, TLR induced immune responses form an essential part of the host response to intracellular bacteria and are likely to be active in patients with Leprosy. For example, a study of ***Mycobacterium Tuberculosis***, which is highly similar to *M. Leprae*, revealed that the induction of pro-inflammatory cytokines IL6, TNFα and IL12p40 in response to infection was dependent on both Toll-like receptor 2 and 9 (TLR2/TLR9) *in vitro* [19]. This type of pro-inflammatory response can in turn drive theTh1 responses that are characteristic of infection with **Mycobacteria** [19]. Indeed, $Tlr2^{-/-}/Tlr9^{-/-}$ double knockout mice fail to exhibit hallmarks of a Th1-type immune response, such as increased serum IFN-γ, in response to infection with *M. Tuberculosis* [19]. In a separate study, the activation of Toll-like receptor 7 (TLR7) by phagocytised bacteria was seen to provoke type-1 interferon production by conventional dentritic cells stimulated *in vitro* [20]. This likely provides a strong protective affect as $Tlr7^{-/-}$ mice exhibit decreased survival during bacterial infection, in this case with ***B Streptococcus*** [20]. Thus, we consider that cytokine and type-1 interferon production induced *via* TLRs 2, 7, and 9 may potentially mediate host responses to *M. Leprae* and be implicated in the pathogenesis of ENL.

Circumstantial evidence is supportive of this hypothesis. For instance, peripheral blood mononuclear cells (PBMC) isolated from ENL patients are hyper-responsive to the TLR9 agonist **CpGB DNA**, producing increased levels of TNF, IL6 and IL1β following stimulation *in vitro* [21]. Furthermore, ENL derived skin biopsies exhibit abnormally high levels of TLR9 expression on various cell types including B-lymphocytes, monocytes and plasmacytoid dentritic cells (pDCs) [21]. Importantly, TLR7/TLR9 induced type-1 interferon production can drive systemic autoimmune disease with symptoms similar to those found in ENL, such as in skin lesions seen in SLE [22]. Thus, it is reasonable to suppose that ENL symptoms may arise from excessive TLR signalling in response to *M. Leprae* infection, and therefore, that the inhibitory effect of IMiDs on TLR induced cytokine and type-1 interferon production might potentially contribute to the efficacy of these compounds in the treatment of ENL. However, additional studies are warranted to validate this hypothesis. Furthermore, the effect of IMiDs on these same signalling pathways varies widely according to the cell type under investigation. For instance, lenalidomide treatment augmented type-1 interferon production in *in vitro* cell lines derived from a subset of B cell lymphoma; activated B-cell like (ABC) diffuse large B cell lymphoma (DLBCL)[23].

Thalidomide and in particular, its derivatives lenalidomide and pomalidomide, are known for their co-stimulatory effect on T-cell activation [6, 24]. Specifically, the stimulation of CD3 or PMA/Ionomycin activated human T-cells (both $CD4^+$ and $CD8^+$) with IMiDs has been reported to up-regulate TCR induced TNFα, IL2 and IFNγ production *in vitro* [24-27]. Another study, the production of IL2, which has essential functions in promoting regulatory T-cell (Treg) differentiation, survival and function,[28] was suggested to underlie thalidomide efficacy in ENL following observations of enhanced Il2 production by patient derived PBMC, stimulated *ex vivo* with PMA and Ionomycine [29]. Indeed, IL2 has an

essential role in maintaining immune homeostasis as illustrated by the phenotype of *Il2*$^{-/-}$ mice, which spontaneously develop Colitis accompanied by unrestrained T-cell proliferation [30, 31]. The possibility; that thalidomide promotes Treg expansion *via* the up-regulation of IL2, remains to be clearly demonstrated. However, at least one study has noted an increase in gene expression of the Treg marker *FOXP3* in PBMC isolated from ENL patients undergoing thalidomide treatment [29]. Moreover, analyses of PBMC derived from multiple myeloma and MDS del(5q) patients undergoing treatment with pomalidomide and lenalidomide, respectively, revealed an increase in Treg frequency as a proportion of total *CD4*$^{+}$ T-cells following treatment onset [32, 33].

The *in vitro* observations of the immune-suppressive and co-stimulatory effects of thalidomide in monocytes and T-cells, respectively, are in principle, capable of explaining the efficacy of thalidomide in ENL. However, at present, both hypotheses lack sufficient experimental support. In particular, observations of serum cytokine levels in patients undergoing thalidomide treatment for ENL are inconsistent. A study of 20 Nepalese ENL patients concluded that disease resolution was accompanied by an increase in serum levels of TNFα and IL12 [29]. Furthermore, T-cells isolated from these patients and stimulated *ex vivo* (PMA/ Ionomycin) exhibited increased production of IL2, IL4 and IFN-γ [29]. Based on these data we might conclude that the co-stimulatory property of thalidomide might be critical to resolving ENL symptoms. However, a separate study of 9 ENL patients from Mexico reported a decrease in serum TNFα and IFNγ in response to thalidomide treatment that also correlated with reduced severity of disease symptoms [34]. Further clinical studies are required to understand whether the *in vitro* observations of thalidomide's immune-suppressive and co-stimulatory activity are relevant to its therapeutic effect in ENL.

Thalidomide in the Treatment of Autoimmune Diseases

While thalidomide's anti-inflammatory properties are best characterised in the context of ENL, numerous case reports and small-scale studies suggest that thalidomide and its derivatives may be effective in the treatment of diseases such as **Rhuematoid Arthritis**, **Ulcerative Colitis**, **Crohn's disease**, (see Box 1) and dermatological complications associated with **Behcet's disease** and SLE [14, 35, 36]. For the most part, however, these studies are not supported by large-scale controlled clinical trials. Consequently, while thalidomide exhibits potential in the treatment of autoimmune disease, it has so far failed to gain widespread acceptance as a treatment for immunological disorders other than ENL (Box 2).

Box 1. Clinicians corner

- The use of thalidomide requires strict monitoring and patient compliance. Although reports indicate that lenalidomide and pomalidomide fail to induce teratogenicity in zebrafish and chick models of development,[37, 38] these compounds bind Cereblon and should therefore be considered teratogenic in humans [27, 39]. Thalidomide derivatives should therefore be used cautiously.

- Thalidomide is likely to prove effective in IBD (both Ulcerative Colitis and Crohn's disease)[35, 36], however, opinions differ over whether the side effect profile can be considered tolerable. Adverse events requiring suspension of treatment are experienced in approximately 20% of patients undergoing long term treatment [35]. Specifically, peripheral neuropathy (20-26% of patients), a neurological condition described as a 'pins and needles' sensation (tingling in hands and feet) can result in permanent neurological damage if treatment is not suspended. Furthermore, the

sedative effects of thalidomide (32-60% of patients) are sufficiently problematic such that existing first line treatments-such as 5-aminosalicyclic acid compounds- may be preferable [35, 36]. Nevertheless, thalidomide might be considered in patients refractory to existing treatments. Further studies are warranted to study the applicability of thalidomide derivatives for the treatment of IBD.

- Thalidomide derivatives may also be considered suitable for dermatological complications associated with Behcet's disease and SLE. For instance, lenalidomide is reported to be well tolerated and effective in cutaneous lupus erythmatosus [40, 41].

Box 2. Expanding the clinical potential of IMiDs

IMiDs are the only class of drugs to initiate the proteosomal degradation of therapeutic targets by binding to an E3 ubiquitin ligase complex. Ligand activated ubiquitin ligases have been previously described; the aryl hydrocarbon receptor (Ahr) performs an analogous role to Cereblon in the CUL4B ubiquitin ligase complex. In this case, the binding of Ahr Ligands initiates recruitment and degradation of the estrogen receptor (ERα)[42]. Another example is the binding of Auxin, a plant growth hormone, to the TIR1 ubiquitin ligase, resulting in the degradation of the Aux/IAA family of transcriptional repressors [43]. The authors of this study noted the implications of their findings; that small molecules might be designed to promote protein-protein interactions with ubiquitin ligases [43]. The recent development of molecules that can effectively re-program $CUL4A^{CRBN}$ to degrade specific proteins promises to greatly expand the therapeutic potential of IMiDs.

Proteolysis Targeting Chimera (PROTAC)

The targeting of a desired protein is made possible by the non-specific, promiscuous nature of $CUL4A^{CRBN}$. Proteins that come within a given range, as defined by the rotation of CUL4A around the DDB1/Cereblon substrate adaptor, are targeted for ubiquitination [44]. This has enormous implications for the design of IMiD based therapeutics; IMiDs based molecules can be designed to bring disease causing proteins into the effective range of $CUL4A^{CRBN}$. Two groups have independently demonstrated the potential for this approach. Both studies combined IMiDs with chemical inhibitors of BRD4, a transcriptional regulator and therapeutic target in various types of cancer. The resultant compound effectively induced the Cereblon mediated degradation of BRD4 in Burkitts lymphoma cell lines while overcoming the problem of acquired resistance seen with standard BRD4 inhibitors [45]. A similar compound proved effective in the treatment of murine leukemia *in vivo* [46]. This sort of IMiD based hybrid molecule, referred to as Proteolysis targeting chimera (PROTACs), have not yet been applied to the treatment of inflammatory and autoimmune diseases, but hold great potential.

Molecular Mechanisms in IMiD Therapy

Past studies have therefore described putative mechanisms that may potentially explain the properties of IMiDs in the treatment of ENL and potentially, in autoimmune diseases such as IBD and SLE. Now, the identification of Cereblon as a receptor for thalidomide has provided an opportunity to consider the molecular aspects of putative IMiD therapy in these diseases.

Cereblon: The Thalidomide Receptor

Using innovative IMiD coated beads, Cereblon was identified as a direct target of thalidomide, pomalidomide and lenalidomide [8, 27]. Cereblon, which shares homology to

the yeast protein Lon (N-terminal domain of the ATP –dependent protease La),[47] was first reported as the causative factor of a rare form of inherited cognitive disorder (Autosomal Recessive Nonsyndromic Mental Retardation (**ARNSMR**)) based on genetic linkage analysis of an affected family [48]. Shortly thereafter, a mass spectroscopy based proteomic screen identified Cereblon as a putative component of the CUL4A//DDB1/RBX1 E3 ubiquitin ligase complex [7]. Cereblon is now also known to co-immunoprecipitate with the related ubiquitin ligase CUL4B [38, 49]. Added to this, proteomic screens have identified a large number of additional putative substrate adaptors, termed CUL4A associated factors (DCAFs),that are thought to function interchangeably with Cereblon in these complexes [7, 50]. Thus, there is potential for significant complexity, the importance of which remains poorly understood in relation to IMiD properties.

The ubiquitin ligase function of Cereblon has since been experimentally verified with studies indicating that under physiological conditions **CUL4A^{CRBN}** has important functions in maintaining metabolic homeostasis. For instance, degradation of metabolic enzyme Glutamate Synthetase (GS), which occurs in hepatic cell lines when glutamine concentrations are high,[51] was shown to be dependent on Cereblon in the Hep3B cell line [52]. Pull-down experiments in HEK293T cells further revealed that following treatment with glutamine, acetylation of two tandem lysine residues (K11, K14) in GS resulted in its recruitment to CUL4A^{CRBN} and subsequent degradation *via* the ubiquitin proteasome pathway [52]. Although the *in vivo* relevance of these observations was not addressed, *Crbn*$^{-/-}$ mice are reported to be highly susceptible to weight gain when fed a high fat diet [53]. Evidence therefore suggests that CUL4A^{CRBN} may be a physiologically relevant regulator of glutamine homeostasis (Box 3). These findings suggest that diet may be an important consideration for patients underoing IMiD therapy, although this remains to be investigated.

Box 3. Cereblon and the immune system.

Only a handful of studies have investigated Cereblon function in the immune system. Using the CRISPR-Cas9 system to generate Cereblon deficient mice for the study of TLR responses, one study documented a small, albeit significant increase in TLR-induced TNFα, IFN-β, IL12p40 and IL6 production in Cereblon deficient macrophages [18]. These observations are consistent with other findings showing that *Crbn*$^{-/-}$ mice exhibit a slightly increased susceptibility to endotoxic shock [54]. In this study, Cereblon knockdown (siRNA) in the THP-1 cell line was seen to augment LPS induced NFkB activation [54]. Furthermore, over-expression assays in HEK293T cells suggest a putative role for Cereblon in the regulation of TRAF6 ubiquitination, although further experiments using physiological systems are required to validate this point [54].

In our view, the contribution of Cereblon to regulating innate immune signaling pathways, and macrophage function, is quite small. A recent study suggests that Cereblon may have a more important role in shaping adaptive immunity. T-cells isolated from Cereblon deficient mice exhibit increased cytokine production in response to TCR activation [55]. Furthermore, T-cell specific deletion of Cereblon increases disease severity in Experimental Autoimmune Encephalomyelitis (EAE), a Th17 mediated murine model of Multiple Sclerosis involving central nervous system (CNS) inflammation [55]. While these findings may suggest a role for Cereblon in regulating T-cell activation, similar observations have been reported for CD147$^{-/-}$ mice, which exhibit increased susceptibility to EAE and enhanced Th17 cell differentiation *in vitro* [56]. The phenotype of *Crbn*$^{-/-}$ mice may therefore reflect a loss of Cereblon chaperone function in CD147 maturation [38]. Further investigation is required to determine whether Cereblon has a meaningful role in immune regulation.

Interestingly, the GS conjugated acetylation motif binds to Cereblon at the same location as IMiDs; an N-terminal hydrophobic pocket (Figure 2) [52, 57]. Thus, mutation of Tyrosine and Tryptophan residues at position 384 and 386 (human Cereblon), respectively, to Alanine (**CRBN$^{YW/AA}$**), which is reported to abolish IMiD binding,[8] also prevented GS-Cereblon protein interactions [52]. We might therefore expect that thalidomide should compete with GS for Cereblon binding, however, for reasons that remain unclear, this appears not to be the case [52]. Further studies are thus required to understand how IMiD binding might affect the physiological function of Cereblon and whether this influences disease outcomes in conditions where IMiDs are used frequently, such as Multiple Myeloma and MDS del(5q).

CUL4A^{CRBN} ***Mediated Degradation of Ikaros and Aiolos Relieves Transcriptional Repression of the Il2 Promoter***

Proteomic and biochemical analyses have determined that, following IMiD binding, the ubiquitin ligase function of Cereblon is redirected to target proteins that would not ordinarily be subject to CUL4A^{CRBN} regulation. IMiD binding to Cereblon results in the recruitment of target proteins, termed neo-substrates (to distinguish them from physiological substrates like GS), resulting in their ubiquitin mediated proteasomal degradation [58-62]. Mass-spectrometry based proteomic studies have identified neo-substrates including the transcription factors Ikaros Family Zinc Finger Protein 1 and 3 (IKZF1 and IKZF3), also referred to as Ikaros and Aiolos, and the Casein kinase 1α (CK1α)[59-62]. The IMiD induced degradation of neo-substrates is at least one contributing factor to IMiD efficacy in the treatment of Multiple Myeloma and MDS del5(q), with knockdown (shRNA) of IKZF1, IKZF3 and CK1α proving cytotoxic in *in vitro* cell lines derived from these malignancies (see box 4, box 5)[59-61, 63, 64], and reviewed elsewhere[63, 64].

Box 4. IMiD therapy in cancer

Multiple Myeloma

Shortly after the identification of Cereblon as an IMiD receptor, the efficacy of IMiDs in Multiple Myeloma was investigated. Cereblon knockdown in Multiple Myeloma cell lines proved cytotoxic, however, stably transfected clones were resistant to IMiD treatment [65]. Therefafter, two groups simultaneously reported the transcription factors Ikaros (IKZF1) and Alios (IKZF3) as targets of $CUL4A^{CRBN}$. IMiD binding initiated the degradation of IKZF1 and IKZF3 resulting in cytotoxicity in multiple myeloma cell lines *in vitro* [60, 61].

MDS del(5q)

Similar proteomic studies identified CK1α as a target in MDS del(5q)[59]. Lenalidomide stimulated the degradation of CK1α resulting in cytotoxicity in MDS del(5q) cell lines[59]. A recent study revealed that IKZF1 degradation is also important to IMiD therapy in this condition. IKZF1 is a transcriptional suppressor of GRP68[66]. Following IKZF1 degradation, GRP68 is up-regulated triggering a calcium-inducing signalling cascade and the activation of CAPN1, a calcium activated mediator of apoptosis [66]. MDS del(5q) cells are uniquely sensitive to this pathway owing to haplo-insufficiency for the CAPN1 inhibitor Caplastatin (CAST), which is encoded by a gene in the deleted 5q region [66].

Multiple Mechanisms to Explain the Effects of IMiD Therapy in Cancer?

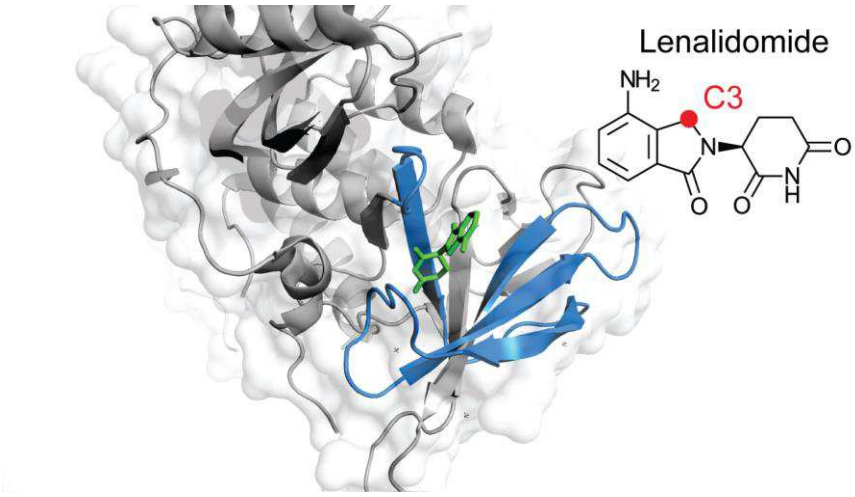
Neo-substrate (IKZF1, IKZF3, CK1α) degradation alone failed to explain why, for instance, proteasome inhibitors are effective in combination with IMiDs in Multiple Myeloma, despite the degradation of IKZF1 and IKZF3 occurring *via* the ubiquitin proteasome pathway [64]. These problems were solved with the identification of CD147 as a target of IMiDs. CD147 knockdown is cytotoxic in both MDS del(5q) and multiple myeloma cell lines *in vitro* [38]. Together with the degradation of $CUL4A^{CRBN}$ neo-substrates, these mechanisms are suggested to combine to result in the observed IMiD effects during cancer treatment[64].

Box 5. Neo-substrate degradation influences functional differences in IMiD compounds.

Target specificity varies among IMiD compounds. So for example, thalidomide, lenalidomide and pomalidomide can all effectively induce the degradation of IKZF1 and IKZF3, while

only lenalidomide is effective in stimulating the degradation of CK1α in MDS del(5q) cell lines [59]. Protein interaction assays using Flurescence Resonance Energy Transfer (FRET) indicate that although thalidomide and pomalidomide are able to facilitate Cereblon-CK1α complex formation, this occurs at very low efficiency compared with lenalidomide [58]. Structural studies indicate that in the case of thalidomide and pomalidomide, the addition of a carbonyl group at position C3 (Highlighted on the chemical structure of lenalidomide below, Figure 1) clashes with the resolved crystal structure of Cereblon in complex with CK1α [58].

Binding of the substrates themselves is remarkably similar. While IKZF1 and CK1α share no sequence homology, each shares a similar tertiary structure (β-hairpin loop) that mediates binding to an identical region of Cereblon (aa351-400) (highlighted blue on the crystal structure of human Cereblon in complex with lenalidomide,[57] below)[58]. These studies provide a mechanistic explanation for the functional differences existing between IMiD compounds and why, for example, all three compounds are effective in Multiple Myeloma but only lenalidomide is effective in MDS del(5q)[59]. In contrast to selectivity in neo-substrate degradation, thalidomide, lenalidomide and pomalidomide are reported to be equally effective at inducing the destabilisation of CD147/MCT-1[38]. Thus, neo-substrate specificity appears to be a primary source of diversity among IMiD compounds.



A handful of studies have investigated the relevance of this process in contributing to the anti-inflammatory properties of IMiDs. In particular, the degradation of IKZF1 and IKZF3 has been suggested to influence IMiD-mediated costimulation and IL2 production in CD3 activated T-cells by relieving transcriptional repression of the *Il2* promoter [27, 62]. Indeed, treatment with pomalidomide or lenalidomide resulted in diminished protein levels of IKZF1/IKZF3 in primary human T-cells *in vitro*, [60, 62] which was rescued by Cereblon knockdown (siRNA) [62]. In support of this, the function of IKZF1 and IKZF3 as transcriptional repressors of *Il2* has been well documented and **ChIP-seq** experiments have confirmed that both IKZF1 and IKZF3 bind up-stream of the *Il2* promoter in mice [67, 68]. Specifically, $CD4^+$ T-cells isolated from *Ikzf1*$^{-/-}$ mice, or from transgenic mice expressing a dominant negative mutant of IKZF1 (IK7DN) have been shown to produce increased amounts of IL2 following stimulation with anti-CD3 and anti-CD28 *in vitro*[68]. Meanwhile, IKZF3 can promote **T-helper 17** (Th17) cell differentiation *via* transcriptional repression of the *Il2* promoter. *Ikzf3* mRNA was markedly elevated in murine splenic *CD4*$^+$ T-cells activated with CD3 and cultured under Th17 (but not Th1 or Th2) polarizing conditions (medium supplemented with cytokines that promote T-cell differentiation into an IL17 producing subtype, for example IL6 with TGFβ) *in vitro* [67]. In this same study, *CD4*$^+$ T-cells derived from *Ikzf3*$^{-/-}$ mice produced increased amounts of IL2 and consequently, exhibited impaired Th17 differentiation relative to wild type controls [67]. Taken together, these studies suggest that IMiD-induced degradation of IKZF1 and IKZF3 might be responsible, at least in part, for IMiD-induced up-regulation of IL2 in activated T-cells. IMiD-induced augmentation of IL2 production, *via* the degradation of IKZF1 and IKZF3, may promote Treg survival,[69] suppress pathogenic Th17 cell expansion,[70] and thereby potentially contribute to the efficacy of IMiDs in the treatment of ENL. Further *in vivo*

studies are required to understand and validate the importance of this pathway in relation to the immune-suppressive effects of IMiDs.

Cereblon: A Chaperone in CD147 Membrane Trafficking?

The ubiquitin ligase function of $CUL4A^{CRBN}$ is by far the best studied aspect of IMiD signalling; however, other consequences to IMiD binding to Cereblon have been suggested. For example, sucrose gradient fractionation has revealed that Cereblon functions in at least two distinct protein complexes; IMiD complexed with CUL4A (~300KDa), and a lower molecular weight complex of Cereblon with the cell surface receptor CD147 and its co-receptor Monocarboxylate Transporter-1 (MCT-1) (~120KDa) [38]. CD147 (Basigin, or Extracellular matrix metalloproteinase inducer (EMMPRIN)) is a trans-membrane glycoprotein with diverse functions and implicated the regulation of cell metabolism, invasion of erythrocytes by ***Plasmodium Falciparum***,[71] metastasis, as well as T-cell maturation [72]. IMiD binding was shown to have an inhibitory effect on the Cereblon-CD147 protein interaction resulting in the degradation of CD147 and MCT-1 in lenalidomide sensistive (MMS1, U266, AMO1, L363) but not lenalidomide resistant (KMS12BM, RPMI8226, JJN3, INA-6) Multiple Myeloma *in vitro* cell lines [38]. The degradation of CD147/MCT-1, and indeed also of IKZF1 and IKZF3, was observed to occur even in a cell line (MMS1) deficient for both CUL4A and CUL4B (shRNA). This unexpected observation, taken together with an accumulation of CD147/MCT-1 in the endoplasmic reticulum of IMiD treated cells, led the authors to suggest an alternative hypothesis; that the degradation of CD147/MCT-1 may reflect a distinct, chaperone-like function of Cereblon in the regulation of CD147/MCT1 maturation, trafficking and expression at the cell membrane [38]. This remains speculative however, and further studies are required to understand the precise mechanism whereby CD147/MCT-1 are degraded. Moreover, that IKZF1 and IKZF3 are

degraded in MMS1 cells deficient for both CUL4A and CUL4B is curious, and requires explanation.

The authors of this study present evidence to support the role of this process in effecting thalidomide teratogenicity. For instance, in experiments using the zebrafish model of development, morpholino mediated knockdown of CD147 phenocopied thalidomide teratogencicity, manifesting in reduced head, fin and eye sizes, [38] which is consistent with observations of thalidomide treatment or Cereblon knockdown in this same model [8]. However, further investigation is required to understand whether this process is mechanistically and functionally relevant to IMiD therapy in ENL. It might be reasonable to suppose that, at least in some disorders, loss of CD147 expression could result in a T-cell mediated anti-inflammatory response. Transient blockade of CD147 using blocking monoclonal antibodies has been shown to be effective in murine EAE, primarily a Th17 mediated disorder [73]. Moreover, human clinical trials have shown anti-CD147 blocking antibodies to be effective in the treatment of corticosteroid refractory Graft vs. Host Disease (GVHD) [74]. Further data may help elucidate whether Cereblon's putative chaperone function in CD147 membrane trafficking is relevant in ENL (or other) diseases.

IMiDs Disrupt a Cereblon-Rabex-5 Complex

A recent study from our laboratory used *in vitro* over-expression/pull-down experiments in HEK293T cells to show that IMiD treatment could disrupt protein-protein interactions between Cereblon and an important regulator of immune homeostasis, the **guanosine nucleotide exchange factor (GEF),** Rabex-5[75, 76]. Mutation of the IMiD binding site ($CRBN^{YWAA}$) rescued these protein interactions confirming that this is a direct result of IMiD binding to Cereblon [75]. Notably, protein levels of Rabex-5 were unaffected by IMiD treatment or Cereblon overexpression, suggesting that Rabex-5 was not a substrate of

$CUL4A^{CRBN}$ [75]. Studies of Rabex-5 deficient mice revealed critical functions in the regulation of immune homeostasis in the skin; *Rabex-5*$^{-/-}$ mice rapidly succumb to a MYD88 dependent inflammatory skin condition of unclear aetiology [76, 77]. Thus, we considered that Rabex-5 might be a putative effector of IMiD properties in inflammatory skin conditions such as ENL. Further investigation will be required to understand whether Rabex-5 function is influenced by the loss of interaction with Cereblon, and if so, whether this observation might help explain the acute activity of IMiDs in the treatment of inflammatory skin conditions (ENL, psoriasis), as opposed to other organs.

Multiple Target Molecules Imply a Complex Mechanism of Action

With many IMiD target molecules now identified (in addition to those discussed above; MEIS2, GSPT1[44, 78]), understanding which target molecules are of genuine relevance to IMiD therapy, particularly in complex immunological disorders such as ENL, is a considerable challenge. Many of the targets identified have important functions; either as transcription factors (IKZF1, IKZF3),[60, 61] kinases (CK1α),[59] cell surface receptors (CD147/MCT-1),[38] or ubiquitin ligases (Rabex-5),[75] raising the prospect of downstream/secondary targets, the identity and significance of which is poorly understood. Indeed, this list is not exhaustive and we expect that many other such proteins are likely to be reported in future studies.

Added to this, much of the work has been performed in *in vitro* cancer cell lines and therefore, at least at present, the relevance of these processes to IMiD efficacy in ENL is at present largely conjecture. How the myriad primary targets, in addition to putative secondary targets, synthesise to result in the therapeutic effects of IMiDs in the treatment of autoimmune and inflammatory disorders, will require the use of more sophisticated approaches, including global gene expression and bioinformatics platforms, and importantly, the development of

appropriate *in vivo* systems for IMiD research. It will be necessary for studies to move beyond a simplified, single target molecule approach, that has characterised the field to date.

Future Directions in IMiD Research: Humanized Mice

The studies outlined above describe Cereblon mediated processes that may potentially explain some of the positive effects of IMiD treatments in immunological disorders such as ENL, IBD and SLE. The challenge now is to understand the contribution of Cereblon to mediating the anti-inflammatory properties of IMiDs *in vivo*. This is currently hampered by the inability to use murine models of disease.

Indeed, murine resistance to IMiD properties has been widely documented. Moreover, thalidomide administration induces birth defects, which are evident in chick and rabbit models of development, but fail to manifest in rodents [79]. Similarly, pomalidomide induced up-regulation of IL2 production is observed in human, but not murine PBMC (CD3 activated) *in vitro* [57]. Murine resistance to IMiDs used in anti-cancer treatments has also been noted [59]. Remarkably, these observations are explained by a single amino acid difference between human and mouse Cereblon. The substitution of Valine for Isoleucine at a position adjacent to the IMiD binding site (391, 387 in murine and human Cereblon respectively) prevents the degradation of IKZF1, IKZF3 and CK1α in murine cell lines [59]. For instance, over-expression of murine Cereblon^{I391V} enables lenalidomide induced degradation of CK1α in the murine leukemia cell line Ba/F3 [59]. Therefore, at least *in vitro*, IMiD responses can be ‘humanised’to a certain degree, by the smallest of changes. We expect that in coming years, the development and *in vivo* analysis of humanized CRBN^{I391V} mice will be the subject of intense effort.

Concluding Remarks

For many, thalidomide remains and will always be synonymous with tragedy. Images of thalidomide induced birth defects remain burned into the collective memory and it is with this in mind, and with great sensitivity, that we approach the subject of thalidomide's more positive attributes. Perhaps, it may be said that recent decades have seen thalidomide undergo some degree of atonement. Research inspired, at least in part, by the observation of thalidomide's anti-inflammatory properties and in particular the seminal report of thalidomide's inhibitory effect on Toll-like receptor induced TNFα production,[15] has resulted in the development of thalidomide derivatives that have borne great impact on the treatment of a few cancers including Multiple Myeloma and MDS del(5q) [6, 63]. Today, lenalidomide (Revlimid®) is widely used to treat many of these malignancies.

The use of IMiDs in the treatment of immunological disorders is clearly a much smaller market. However, in our view, it holds great potential. In particular, the use of IMiDs in autoimmune diseases such as IBD and SLE is, at least in part, hampered by the absence of data on IMiD efficacy in these conditions. Concerns over teratogenicity will no doubt continue to weigh heavily in the minds of medical practitioners, particularly given the prevalence of such diseases in individuals of reproductive age. However, thalidomide might be considered as an alternative in patients who prove refractory to existing treatments, and in our view, this makes understanding the anti-inflammatory properties of IMiDs an important topic for future research. The identification of Cereblon as an IMiD receptor has markedly enhanced our understanding; going forward, the anticipated development of humanised Cereblon mice might enable the *in vivo* characterisation of IMiD properties in murine models of autoimmune disease. Are the co-stimulatory or immune suppressive properties of thalidomide critical to its efficacy in these diseases? Just how important is Cereblon to these properties? (see outstanding questions box) Answers to these questions might, in turn, inform

clinical studies and ultimately, help expand the use of IMiDs in the treatment of immunological disorders.

Glossary

Acute Myloid Leukemia
Aggressive cancer affecting the myeloid lineage of hematopoietic cells.

Anergic
A t-cell is said to be anergic when it fails to respond to its cognate antigen.

Apremilast
Next generation IMiD with potent anti-inflammatory activity.

ARNSMR
Rare and relatively mild inherited cognitive disorder.

B Streptococcus
Gram negative strain of extracellular bacteria.

Behcet's disease
Complex inflammatory disorder affecting mucosal surfaces.

Chaperone
Assists with correct folding/maturation of another protein.

ChIP-seq
Technique to demonstrate transcription factor binding. Immuno-precipitation of TF bound chromatin followed by sequence analysis.

Clofazimine
Antibiotic effective against mycobacteria.

CpGB DNA
DNA motif abundant in bacterial DNA.

Cullin4A (CUL4A)
Central scaffold protein in modular E3 ubiquitin ligase complex.

$CUL4A^{CRBN}$
Modular E3 ubiqutin ligase. $CUL4A^{CRBN}$ denotes the Cereblon containing CUL4A complex.

$CRBN^{YWAA}$
Mutation that eliminates IMiD binding. Y384/W386 to Alanine.

Crohn's disease

Inflammation affecting the upper digestive tract and terminal ileum.

Enantiomer
Mirror image chemical isoforms.

Erythema Nodosum Leprosum
Inflammatory reaction associated with Leprosy. Also referred to as a type-2 lepra reaction.

Flourescence Resonance Energy Transfer (FRET)
Flourescence based *in vitro* protein interaction assay. Proximity is measured by excitation at a particular wavelength.

Glutarimide
Derivative of Glutamic acid.

Guanosine Nucleotide Exchange Factor (GEF)
An activating enzyme that catalyses the transfer of GTP to GTPases.

Graft vs. Host Disease
Complication of organ transplantation, whereby the (host) immune system attacks the transplanted organ (graft).

Hansen's Disease
Also known as Leprosy, an infectious disease resulting from infection with a species of Mycobacteria.

Hepatocellular Carcinoma
Cancer of the Liver often caused by viral infection.

Inflammatory Bowel Disease (IBD)
Inflammation of the digestive tract. There are two principal catagories; Crohn's disease and Ulcerative Colitis.

Lenalidomide
Chemical derivative of thalidomide known for its use in the treatment of Multiple Myeloma and MDS del(5q).

Lepramatous Leprosy
Characterised by robust infection and weak antigen specific immune responses.

Lipopolysaccharide
Membrane component of gram negative bacteria.

Mantle Cell Lymphoma
Subset of Non-Hodgkin Lymphoma (cancer affecting the lymphoid system).

MDS del(5q)
Subset of Myelodysplastic syndrome. A deletion in chromosome 5 results in haploinsufficiency.

Morpholino
An RNA binding oligo that blocks protein synthesis by binding to cognate mRNA.

Multiple Myeloma
B-cell malignancy characterised by excessive immunoglobulin production.

Mycobacteria
Gram positive strain of intracellular bacteria.

Mycobacterium Leprae
Gram positive intracellular bacterium .

Mycobacterium Tuberculosis
Gram positive intracellular bacterium. Infection mostly occurs in the respiratory system.

Myelin Sheath
Protective layer that enables neuron conductivity.

Myelodysplastic Syndrome (MDS)
Heterogenous group of hematopoietic cancers.

Peripheral Nervous System (PNS)
The nervous system excluding the brain and spinal cord.

Phocomelia
Abnormally short limb outgrowth.

Plasmodium Falciparum
Mosquito born parasite responsible for malaria.

Pomalidomide
Differs from lenalidomide with the addition of a carbonyl group on the pthalimide ring.

Psoriasis
Autoimmune disease affecting the skin.

Pthalimide

Derivative of pthalic anhydride.

Rab5
GTPase. Considered the engine of early to late endosomal maturation.

Rabex-5
GEF thought to regulate Rab5 activation. Functions in intracellular transport and negatively regulates numerous signalling pathways.

Racemic
Composite mixture of enantiomers/isoforms.

Rheumatoid Arthritis
Autoimmune disease resulting in joint inflammation and damage to the surrounding bone and cartilage.

Schwann cell
Responsible for the synthesis of Myelin, essential for neuron function.

Systemic Lupus Erythmatosus (SLE)
Type-1 interferon driven multi-organ autoimmune disease characterised by autoantibody production.

T-Helper 1
Characterised by IFNγ production. Promote macrophage function and the clearance of intracellular bacteria.

T-Helper 17 (Th17)
Pro-inflammatory T-cell subset characterised by high levels of IL17 production.

The Thalidomide Trust
UK based charity.

Toll-like receptor
A family of receptors that recognises pathogen derived proteins or nucleic acids and initiates a pro-inflammatory cytokine response.

Tubercloid Leprosy
Characterised by limited infection and strong antigen specific immune responses.

Ulcerative Colitis
Inflammation mostly affecting the large intestine.

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Figure Legends

Figure 1. Chemical Characteristics of Thalidomide Derivatives. Thalidomide is a fusion of **pthalimide** and **glutarimide** (bottom left) ring moieties and is produced as a mixture of two **enantiomers** (mirror image chemical isoforms: (S) and (R)). Structural and experimental observations show that only the S enantiomer is Cereblon binding [27, 44]. The R enantiomer is suggested to mediate the sedative properties of thalidomide [80] . However, purification is meaningless as both isoforms are rapidly and spontaneously inter-converted in fluids[14]. Inter-conversion of enantiomers means that thalidomide is produced and administered as a **racemic** mixture. The glutarimide ring is responsible for Cereblon binding [27, 44, 57], while the pthalimide ring is implicated in the suppressive effect of thalidomide on lipopolysaccharide induced TNFα production [81]. Chemical modifications to the pthalimide ring result in the derivatives lenalidomide and **pomalidomide**, which are more effective inhibitors of this pathway [6]. **Apremilast** may also be regarded as a thalidomide related compound as it shares the pthalimide ring; however, this compound does not bind Cereblon [82].

Figure 2. The Crystal Structure of Lenalidomide in Complex with Human Cereblon. Lenalidomide (green) is embedded in a pocket composed of three Tryptophan residues [57]; W380, W386 and W400 (red). The glutarimide portion of the molecule is embedded in Cereblon while the pthalimide moiety is exposed. Lenalidomide is secured by three hydrogen bonds (dotted yellow lines). Each IMiD binds with essentially the same affinity and conformation, however, in the case of thalidomide and pomalidomide, two hydrogen bonds extending from the glutarimide ring are sufficient to anchor the molecule (the hydrogen bond extending from the pthalimide ring is not present) [27, 57]. Similar results have been obtained for the crystal structure of IMiDs in complex with *Gallus Gallus* Cereblon [44]. Mutation of

Y384 (yellow) and W386 (red, labelled) to Alanine (*CRBN*^{YWAA}) mostly eliminates IMiD binding [8, 27]. Endogenous molecules are also able to bind this pocket. For instance, the tandem acetylation motif on Glutamate Synthetase [52]. The ribonucleoside Uridine is also suggested to be a putative endogenous ligand [83]. Image generated using Pymol (Scrhodinger) with pre-existing pdb file (4tz4, reported [57]).

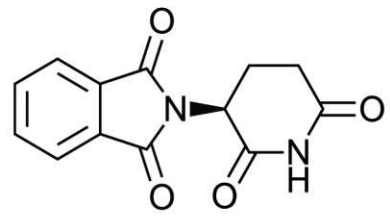
Key Figure, Figure 3. Molecular Processes Underlying IMiD Properties. (1) IMiD binding inhibits a chaperone-like function of Cereblon in promoting the trafficking and maturation of CD147. In the absence of Cereblon or following IMiD treatment, CD147 (blue) and its co-receptor MCT-1 (purple) fail to translocate into the Golgi apparatus and instead accumulate at the endoplasmic reticulum (red cross). CD147 therefore fails to mature into its high glycosylated form and does not express at the cell surface [38]. This process may explain why Cereblon fails to interact with Rabex-5, an important regulator of immune homeostasis that localises to a variety of intracellular vesicles [75, 76, 84]. (2) Cereblon also functions in a completely separate complex with the CUL4A E3 ubiquitin ligase (CUL4A serves as a scaffold. Complex also includes DDB1; a flexible linker connecting Cereblon, and RBX1; possesses E3ubiquitin ligase activity)[7]. Here, Cereblon functions as a substrate adaptor; binding to Cereblon brings substrates into proximity with CUL4A enabling their K48 linked ubiqutination (dashed black arrow) and subsequent degradation *via* the ubiquitin proteasome pathway (not shown) [44, 57, 58, 63]. IMiD binding changes the surface of Cereblon to facilitate protein-protein interactions with IKZF1, IKZF3, CK1α and perhaps other, yet to be identified proteins [59-61]. This process may occur in the nucleus,[64] however, CUL4A^{CRBN} cannot bind active IKZF1/IKZF3; the structure of IKZF1/IKZF3 when bound to cognate DNA sequences interferes with Cereblon binding [58]. Lenalidomide is

shown as a representative example. Lenalidomide, and not thalidomide or pomalidomide is able to induce the degradation of CK1α.

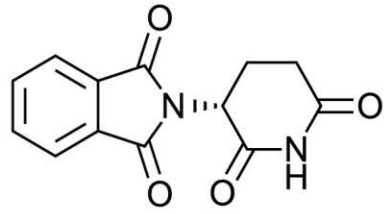
Figure 1 (Box 5) The Crystal structure of human Cereblon in complex with lenalidomide. Image generated using Pymol (Scrhodinger) with pre-existing pdb file (4tz4, reported [57]). Region in blue shows the substrate binding region of Cereblon. The critical C3 is marked on the chemical structure of lenalidomide.

Thalidomide

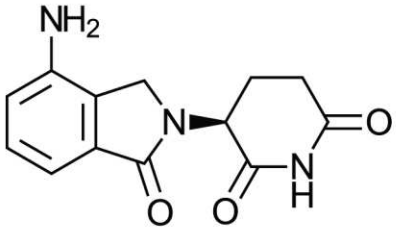
(S)



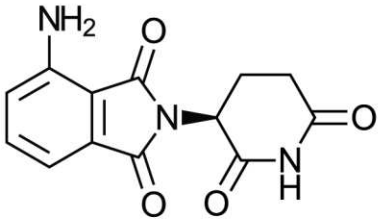
(R)



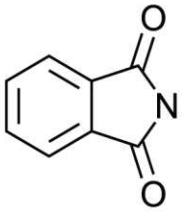
Lenalidomide



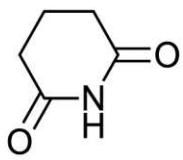
Pomalidomide



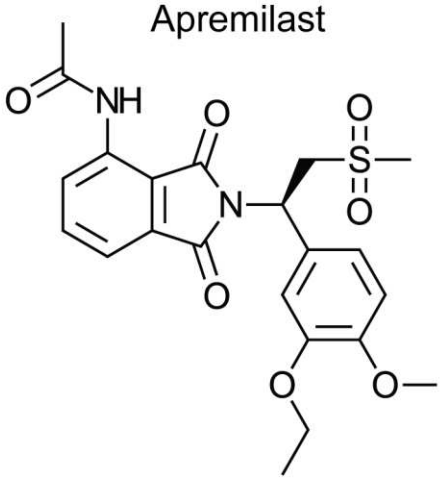
Pthalimide

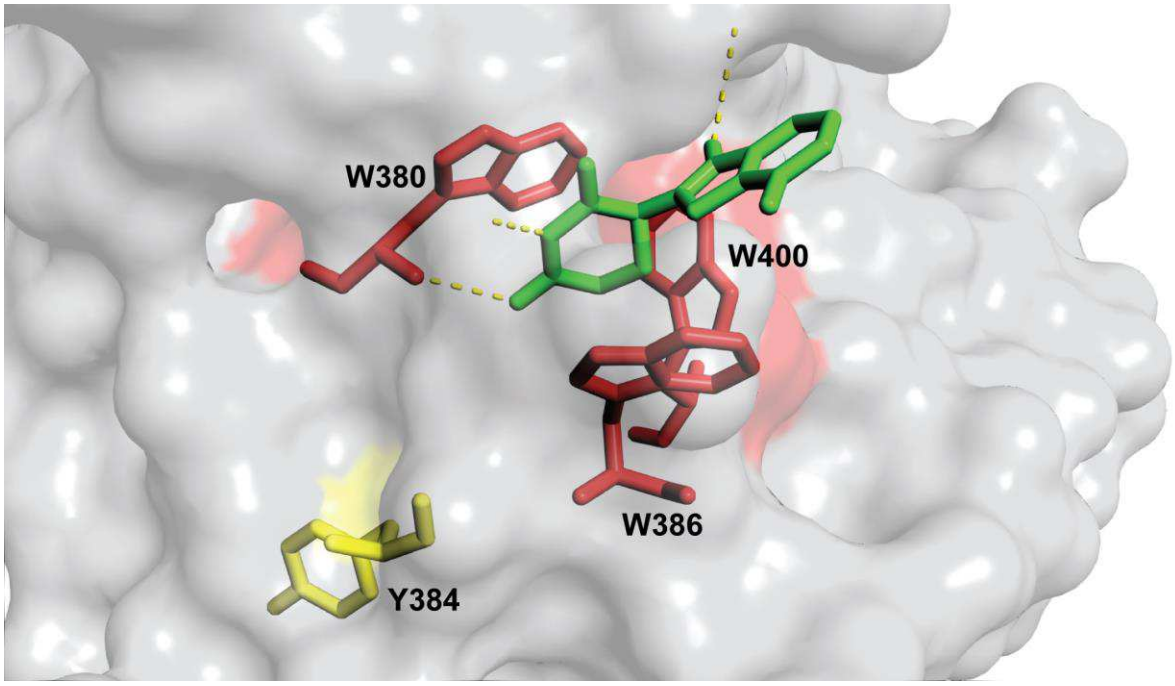


Glutarimide



Apremilast

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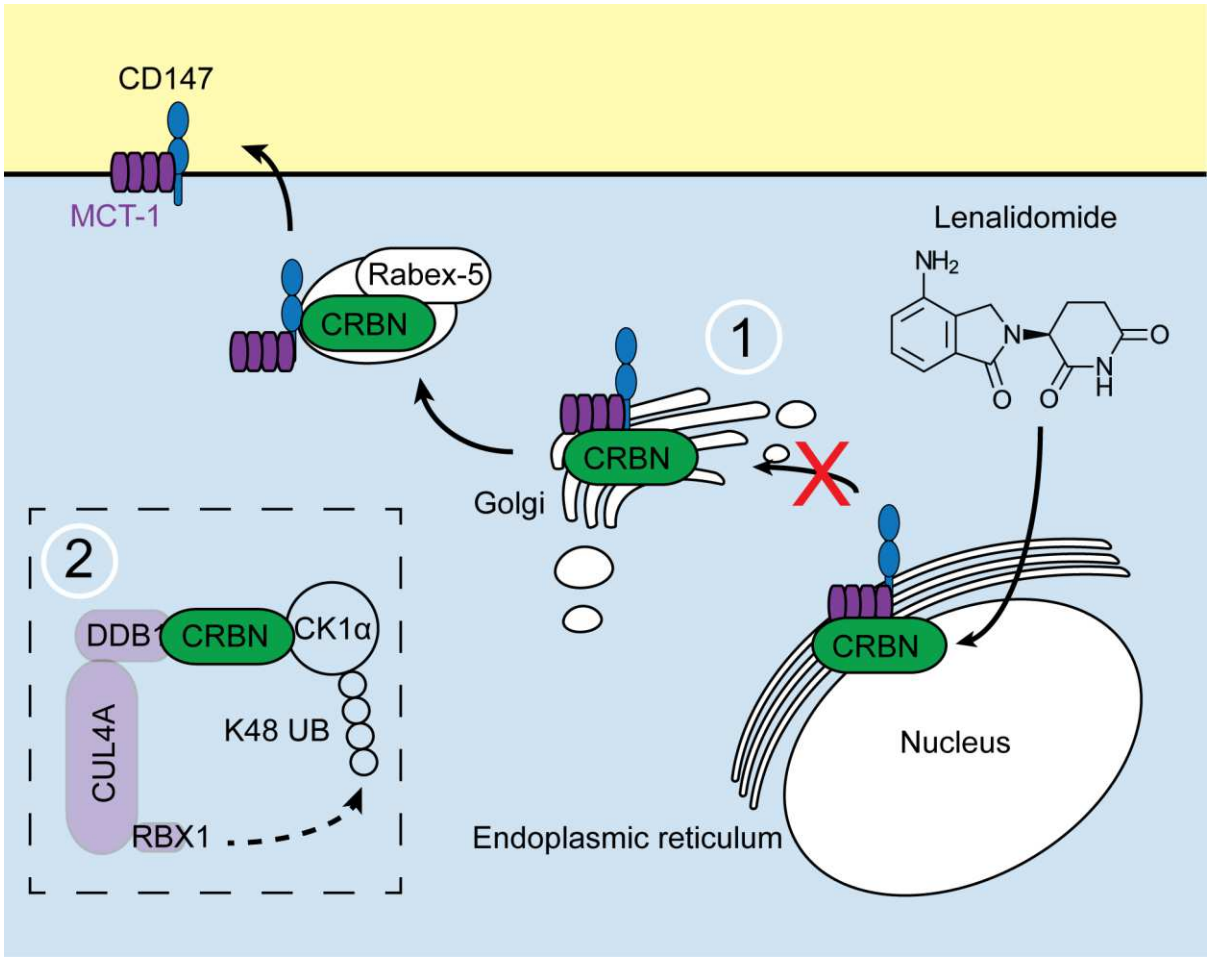
CD147
MCT-1
Rabex-5
CRBN
Lenalidomide
NH2
1
CRBN
Golgi
2
DDB1
CRBN
CK1α
CUL4A
K48 UB
RBX1
CRBN
Nucleus
Endoplasmic reticulum