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Targeted protein degradation: current and future challenges

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Abstract

Traditional approaches in the development of small-molecule drugs typically aim to inhibit the biochemical activity of functional protein domains. In contrast, targeted protein degradation aims to reduce overall levels of disease-relevant proteins. Mechanistically, this can be achieved via chemical ligands that induce molecular proximity between an E3 ubiquitin ligase and a protein of interest, leading to ubiquitination and degradation of the protein of interest. This paradigm-shifting pharmacology promises to address several limitations inherent to conventional inhibitor design. Most notably, targeted protein degradation has the potential not only to expand the druggable proteome beyond the reach of traditional competitive inhibitors but also to develop therapeutic strategies of unmatched selectivity. This review briefly summarizes key challenges that remain to be addressed to deliver on these promises and to realize the full therapeutic potential of pharmacologic modulation of protein degradation pathways.

Keywords

Targeted protein degradation; PROTACs; Molecular glues; Chemical biology; E3 ligase

The framework of chemically induced protein dimerization

Proximity and interactions between proteins underpin most cellular processes. This leaves their dynamic modulation as an important goal in chemical biology and ligand discovery. Several strategies to induce protein–protein interactions (PPIs) have been innovated and are summarized in an excellent recent review [1].

Historically, the concept of inducing novel PPIs through small molecules goes back to immunosuppressive natural products, such as rapamycin and FK506. Mechanistically, both bind the protein FKBP12, thus inducing novel interactions with the mechanistic target of rapamycin kinase (mTOR) and the phosphatase calcineurin, respectively. As a consequence of this drug-induced neoassociation, several activities of mTOR and calcineurin are modulated [2–4]. Thus, both small molecules act as ‘chemical neomorphs’ that achieve

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their cellular effect by inducing PPIs that otherwise do not occur in nature. Another example of such a chemical neomorph is the plant hormone indole-3-acetic acid (IAA, auxin). Auxin induces molecular proximity between the ubiquitin ligase substrate receptor TIR1 and a family of transcriptional regulators called Aux/IAAs [5,6]. As a consequence, IAAs get ubiquitinated and degraded by the proteasome. Thus, auxin modulates the function of the SCF^{TIR1} ligase, enabling molecular recognition and degradation of a protein it would not recognize in its absence. The notion that small molecules can artificially change the target spectrum of cellular effectors of the ubiquitin—proteasome system, in particular of E3 ubiquitin ligases, represents the core concept of targeted protein degradation (TPD) [7].

Monovalent molecular glues and heterobifunctional degraders

Conceptually, there are two chemical strategies to alter the substrate spectrum of an E3 ubiquitin ligase (Figure 1). First, E3 ligases can be reprogrammed by monovalent small molecules, often referred to as ‘molecular glues’ (MGs). Auxin is a prime example of how a MG connects an E3 ligase (SCF^{TIR1}) and the target protein. Ligand binding to the E3 ligase alters protein interface properties, leading to dimerization with a neosubstrate. Similarly, the clinically approved drug thalidomide and its analogs, collectively called ‘immunomodulatory drugs’ or IMiDs, were found to act via a MG-type mechanism, namely, by reprogramming the target spectrum of the E3 ligase CRL4^{CRBN}. After the initial identification of the substrate receptor Cereblon (CRBN) as the cellular target of thalidomide, seminal studies uncovered that IMiD binding to CRBN leads to recruitment and proteasomal degradation of zinc finger (ZF) proteins IZKF1 and IKZF3 [8–10]. In general, MGs such as IMiDs orchestrate molecular recognition between the E3 ligase and the neosubstrate in a highly cooperative manner (Figure 1A). This means that the MG typically induces novel PPIs between the E3 ligase and the target protein which contribute to the formation of a trimeric E3:MG:target protein complex. Notably, IMiDs *per se* have no measurable binding affinity to their degraded targets but hijack an entire surface patch on CRBN to induce productive dimerization [11]. While this outlines the exciting possibility of degrading unligandable proteins, the discovery of E3 modulating MGs has so far mostly been serendipitous and cannot be easily generalized for other targets and target classes.

The major alternative to MGs is heterobifunctional degraders, which consist of one ligand binding to a ubiquitin ligase and a second ligand designed to engage a protein of interest (POI). Both ligands are connected by a flexible linker of suitable length to allow simultaneous binding to the E3 and the POI, leading to molecular proximity (Figure 1B). Productive ternary complex formation ultimately leads to ubiquitination and degradation of the POI. Such heterobifunctional degraders are often referred to as proteolysis targeting chimeras (PROTACs) [12]. First PROTAC concepts relied on peptidic agents with limited cellular efficacy. However, over the past couple of years, significant progress has been made with nonpeptidic heterobifunctional molecules [13–15]. These efforts enabled the targeted degradation of a range of proteins with well-defined ligand binding sites, such as bromodomain proteins and kinases [16,17]. Among a small group of E3 ligases that can be harnessed for PROTAC development, CRBN and the von Hippel-Lindau tumor suppressor (VHL) stand out in terms of their versatility and *in vivo* compatibility [13,18,19]. Other accessible E3 ligases include MDM2 and cIAP1 [20,21]. The modular nature of PROTACs

comes with tangible upsides but also considerable challenges. First, it allows a rational and straightforward design simply by exchanging the target-binding warhead. On the other hand, their molecular weight (typically above 800 Da) poses a challenge for pharmacokinetic optimization [22]. Furthermore, as PROTACs are required to individually bind both the target protein and the E3 ligase, the associated degradable space is limited to proteins that can efficiently be liganded with small molecules. In the following, we want to highlight some of the recent discoveries and trends in the field of PROTACs and MGs and put emphasis on some of the remaining key challenges.

Exploiting selectivity of TPD

Conventional pharmacologic inhibition of a protein typically depends on ligand binding at a functional site, which often is conserved throughout enzyme families. This poses a challenge for selective inhibitor design, as showcased, for instance, by the field of kinase inhibitors [23]. In contrast, TPD requires not only compound binding but also positioning of the E3 and the POI in a configuration conducive to ternary complex formation.

Finally, tripartite binding must occur in a manner that ensures accessibility of lysine residues within the ubiquitination zone of the ligase. Notably, recent studies have uncovered that these requirements can provide an avenue toward the design of highly selective degraders starting from more promiscuous targeting ligands. First observations of target discrimination through TPD were made with MZ1, a PROTAC that prompted preferential BRD4 degradation even though using a pan-bromodomain and extraterminal (BET) protein binding targeting ligand [14,24]. Moreover, when the multikinase inhibitor SNS-032 was conjugated to thalidomide to create a putative multikinase degrader, the resulting compound selectively degraded CDK9, despite retaining a multikinase binding spectrum [25]. Further studies have systematically addressed this phenomenon by generating CRBN- or VHL-based degraders based on additional multikinase inhibitor warheads [16,17]. Although the degraders would still bind to hundreds of kinases, degradation was achieved for only 10–20% of the targets across different cellular backgrounds. In addition, it was shown that the degradable target spectrum was dependent on the hijacked E3 ligase. Subtle changes in linker configuration were sufficient to generate isoform-specific degraders of the p38 mitogen-activated protein kinase (MAPK) family [26]. Together, these studies also highlighted that binding affinity to the POI was not correlated to its degradability. Further supporting the notion that degraders can elicit unprecedented selectivity, structural dissection of BET PROTACs led to the first rational development of a BRD4-selective degrader. Mechanistically, selectivity was dictated by protein–protein interface determinants outside of the ligand binding pocket on the POI [27,28]. Furthermore, subtle modification of the E3 ligase binder allows controlling for residual inhibitory consequences of the PROTAC [29].

Collectively, these studies have shown how selectivity of otherwise promiscuous scaffolds can be achieved through TPD and lead to highly specific degraders. This was ultimately exploited in a disease-relevant context by generating PROTACs capable of selectively degrading CDK6 over CDK4 [29,30]. Selective CDK6 degraders could emerge as valuable therapeutic options in hematologic malignancies and will enable addressing kinase-independent scaffolding functions [31,32].

Expanding the scope of E3 ligases via covalent ligands

Rational identification of novel E3 ligands is a challenging task and most discoveries so far have happened serendipitously. The covalently acting natural product nimbolide is an example of such a finding. To elucidate the mechanism of action of nimbolide, activity-based proteomic profiling was applied, leading to the identification of the E3 ligase RNF114 as the primary target [33]. Other studies have explored the rational identification of covalent interactors of the E3 ligases. Gel-based activity-based proteomic profiling was used to screen for covalent binders of RNF4, identifying several probes with varying specificity for RNF4 over other proteins [34]. In another approach, broad cysteine-reactive probes were conjugated to an FKBP12 ligand and tested for their ability to degrade FKBP12 [35]. This led to the identification of a covalent ligand of the CRL4 substrate receptor DCAF16.

An important question was if the different covalent E3 binders could further be developed as E3 recruitment elements in a heterobifunctional degrader design. All of these studies assayed degradability of BET proteins by synthesizing PROTACs that connect the novel covalent E3 ligands to the known BET bromodomain antagonist JQ1 [24]. RNF114- and DCAF16-based degraders were selective for BRD4. The RNF4-based PROTAC however caused destabilization of a broader range of proteins including BRD4. Although effects of covalent binding of a POI through PROTACs have been studied previously, potential advantages and disadvantages of degraders that covalently engage the E3 ligase still remain to be experimentally validated [36]. Conceptually, these could include the reduction of the requirement for a ternary binding event to a binary interaction. Further ligand optimization will be required to develop second-generation, *in vivo* compatible degraders.

Exploring the limits of CRBN modulation with MGs

Although first PROTAC molecules recently entered clinical investigations, MG degraders, such as the aforementioned IMiDs, are already routine treatment options for different B-cell neoplasms. Generalizing and rationalizing the discovery of novel MGs thus marks a key future challenge. This could be achieved by further exploring the limits of the 'degradable space' in reach of the CRL4^{CRBN} ligase complex or by chemically unlocking novel ligases. The first evidence that the CRL4^{CRBN} ligase complex can be hijacked for the degradation of proteins other than the initially identified IKZF1/3 surfaced when CK1a was identified as an additional target of lenalidomide [37]. Soon, several additional IMiD targets such as GSPT1, ZFP91, and SALL4 were described [38–41]. This growing list motivated the first comprehensive assessment of the spectrum of neosubstrates that can be degraded by reprogramming CRL4^{CRBN} with IMiD-like MGs [42]. Since the Cys₂-His₂ (C2H2)ZF domains of known substrates were sufficient for drug-induced recruitment, it was reasoned that IMiDs may degrade other proteins containing this feature. Indeed, four novel neosubstrates were identified using a scalable protein stability reporter assay. Structural and sequence homology analysis revealed that the overall ZF fold, rather than a particular linear amino acid sequence, is required for IMiD-based recruitment to the CRL4^{CRBN} interface. This enabled a computational prediction of neosubstrates via a holistic docking approach, leading to the identification of several additional proteins that can biochemically be recruited to CRBN after IMiD treatment. Further chemistry efforts will be required to

develop chemical matter that can expand on these efforts. Forthcoming studies will likely take advantage of these modern-day docking strategies to predict 'glue-able' interfaces between POIs and E3s, which might enable the prioritization of novel MG scaffolds.

MG approaches to other ubiquitin ligases

Recent research revealed that the molecular mechanism of IMiDs might not be a unique phenomenon. The arylsulfonamide indisulam and structurally related analogs were shown to induce the degradation of the splicing factor RBM39 by chemically reprogramming the substrate receptor DCAF15 [43,44]. Three recent independent studies have provided the structural and biochemical workup that unequivocally confirmed that indisulam and related aryl sulfonamides indeed function via a MG mechanism analogous to the IMiD pharmacology [45–47]. All three studies characterized a complex of DCAF15:DDB1:DDA1 together with an aryl sulfonamide recruiting RBM39. Of note, these compounds bind to DCAF15 with a significantly lower affinity than IMiDs bind to CRBN. Consistent with a model of high cooperativity, RBM39 binding greatly improves complex stability. In unbiased proteomics experiments, RBM23 is the only other destabilized protein after indisulam treatment [45]. Mechanistically, this is explained by high sequence conservation between their RRM domains involved in indisulam-induced molecular recognition. This remarkable selectivity likely stems from the buried surface area between DCAF15 and RBM39, which is approximately twice as large as that of CRBN and its neosubstrates (Figure 2). Furthermore, indisulam binding involves significant side chain interactions in the ternary complex resulting in increased specificity. This leads all three studies to the conclusion that the DCAF15:indisulam surface will likely not allow the same promiscuous target spectrum as the CRBN:IMiD interface. However, new chemical matter could potentially adopt some of the RBM39:DCAF15 interactions and thereby allow other targets to be degraded.

In addition to developing MGs that recruit true neosubstrates, another promising approach is to chemically reinforce known substrate—E3 interactions that are altered in the disease. One such example is β -catenin, a Wnt signaling effector protein. β -Catenin is often mutated at Ser33 and Ser37 [48]. This disrupts a phosphodegron recognized by the SCF $^{\beta}$ -TrCP cullin-RING ligase, leading to stabilization of β -catenin and associated oncogenic consequences [49]. A recent study developed several biochemical screens coupled to structure-informed lead optimization to identify a compound that can re-enhance binding and ubiquitination of mutant β -catenin [50]. This marks the first successful study with the rationale of finding MGs for a specific ligase:substrate pair and provides a blueprint for similar future endeavors.

Collectively, the field of TPD has matured to a level where its generalizable nature is widely accepted. We will increasingly learn how to leverage some of the challenges that are associated with degrader design to our advantage to develop small molecules with unprecedented selectivity and potency. We believe that key challenges in the field will be to chemically unlock a larger number of ligases and to further rationalize the development of monovalent MGs. In addition, with the first heterobifunctional degraders in clinical trials, initial promising results on safety and druglikeness will have to be solidified (NCT03888612 and NCT04072952). This also leads to important considerations on anticipating, detecting,

and circumventing associated resistance mechanisms. Along these lines, recent studies have shown that mutations in E3 ligase complexes or regulators thereof can lead to resistance to different types of degraders [51–53]. Similarly, CRBN mutations/loss of functions have been linked to the clinical mechanism of IMiD resistance, but their relevance have not fully been resolved [54,55]. Importantly, IMiDs are known to also act via a multiple indirect mechanisms, including T-cell and NK-cell modulation [56,57]. It thus remains to be seen how tumors will cope with the selective pressure of TPD therapies that function primarily via cell-autonomous mechanisms.

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* of special interest

* * of outstanding interest

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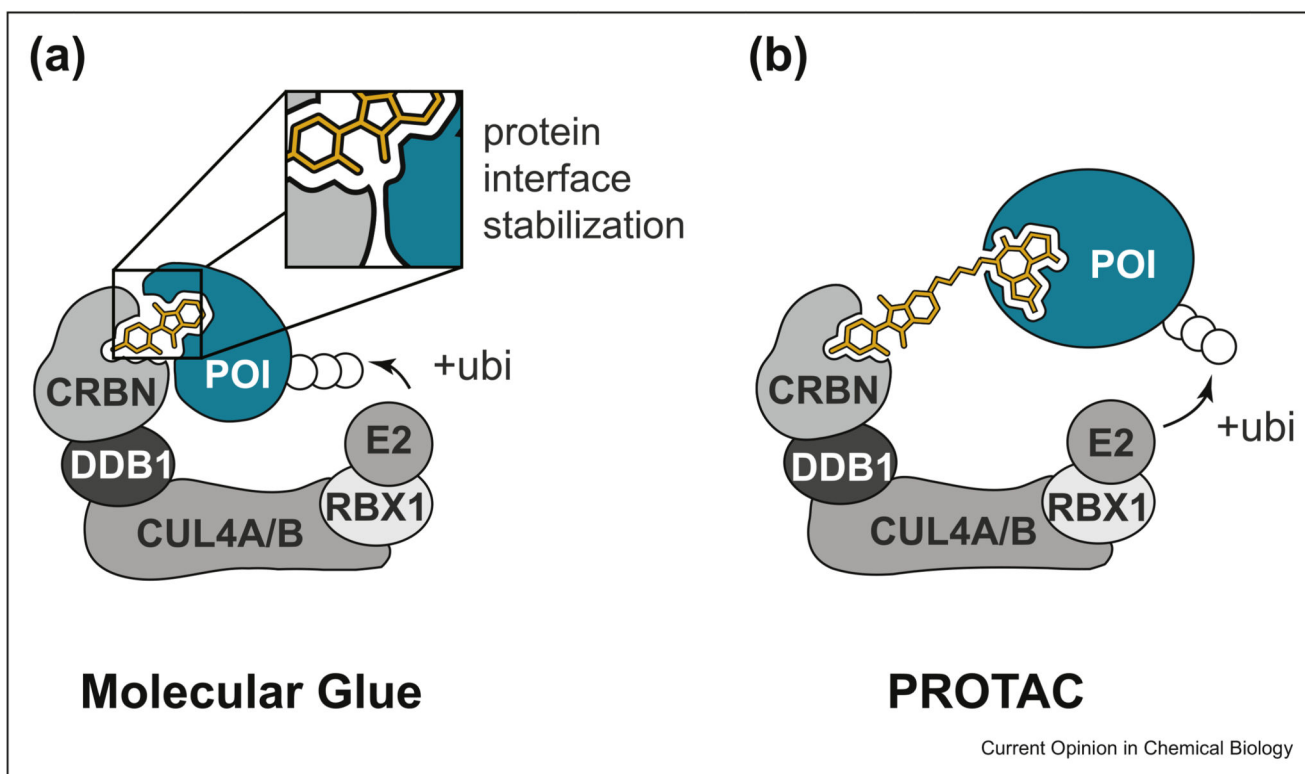


Figure 1. Schematic comparison of molecular glues and PROTACs.

(a) Molecular glues are monovalent compounds that induce the dimerization of two proteins (here: an E3 ligase substrate receptor and a neosubstrate). Compound-induced proximity is often characterized by multiple interactions between the two proteins, resulting in high binding cooperativity. **(b)** PROTACs are heterobifunctional degraders that can individually bind to the E3 ligase and the protein of interest. Simultaneous binding induces molecular proximity and ensuing ubiquitination and degradation of the POI. In contrast to MGs, PROTAC-mediated dimerization is less dependent on compatible protein surfaces and associated binding cooperativity. PROTAC, proteolysis targeting chimeras; MG, molecular glue; POI, protein of interest.

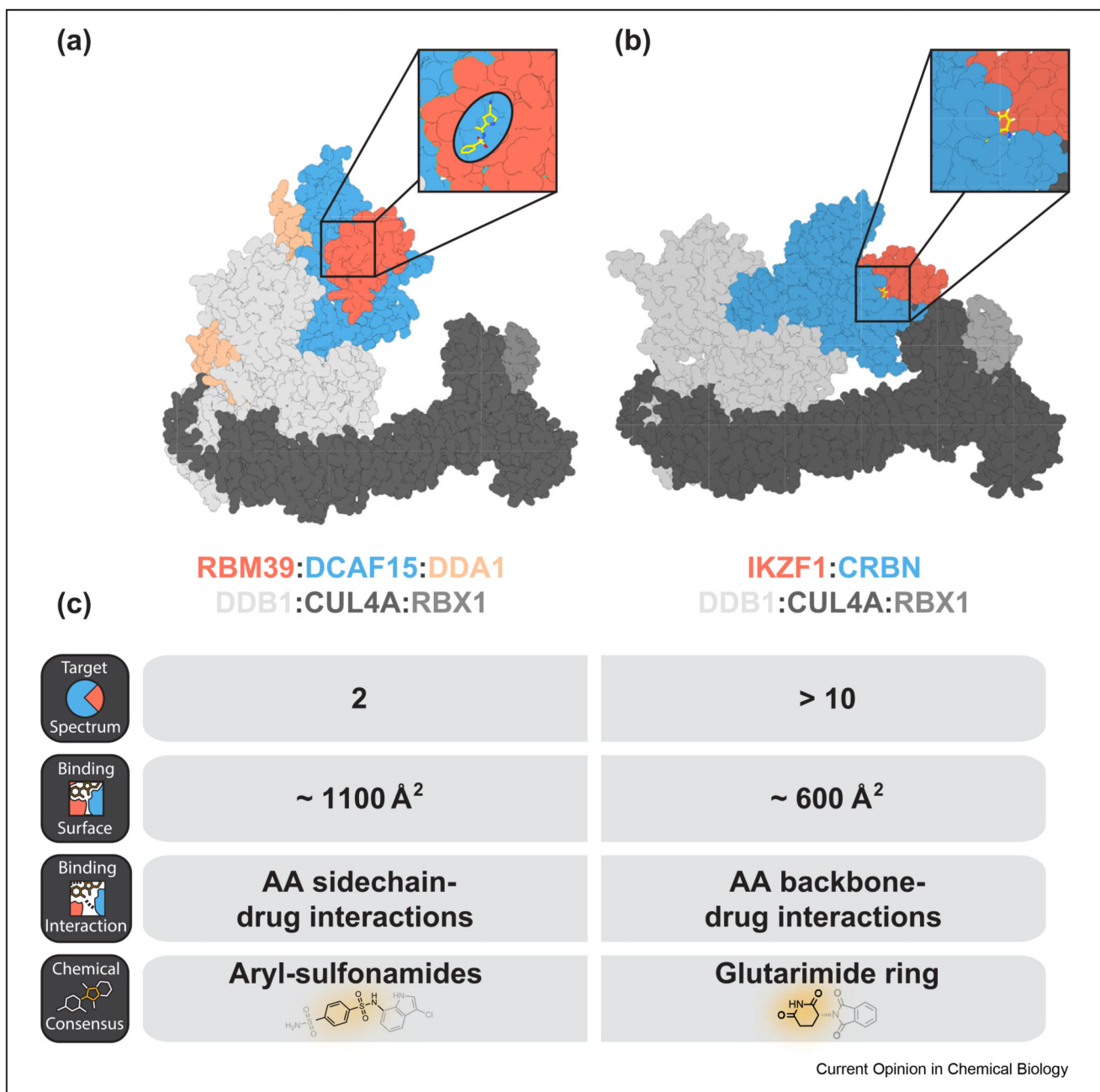


Figure 2. Comparison of DCAF15- and CRBN-based molecular glue degraders.

(a) A structure of DCAF15 (PDB: 6PAI) bound to indisulam, the RRM2 domain of RBM39 and DDB1 aligned with a structure of DDB1:CUL4A:RBX1 (PDB 6PAI). **(b)** A structure of CRBN (PDB: 6H0F) in complex with pomalidomide, the ZF2 domain of IKZF1 and DDB1 aligned with a structure of DDB1:CUL4A:RBX1 (PDB 6PAI). **(c)** Comparison of general properties of DCAF15- and CRBN-based molecular glues.