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Targeted Protein Degradation: Elements of PROTAC Design

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Abstract

Targeted protein degradation using Proteolysis Targeting Chimeras (PROTACs) has emerged as a novel therapeutic modality in drug discovery. PROTACs mediate the degradation of select proteins of interest (POIs) by hijacking the activity of E3 ubiquitin ligases for POI ubiquitination and subsequent degradation by the 26S proteasome. This hijacking mechanism has been used to degrade various types of disease-relevant POIs. In this review, we aim to highlight the recent advances in targeted protein degradation and describe the challenges that need to be addressed in order to efficiently develop potent PROTACs.

I. Introduction

Protein conjugation with ubiquitin, a small protein modifier, is essential for regulated protein degradation by the 26S proteasome. Despite delineating the ATP-dependent pathway of protein degradation in the late 1970s [1–6], the first application to exploit this system for targeted protein degradation was reported thirty years later [7]. Proteolysis Targeting Chimeras (PROTACs) are heterobifunctional molecules consisting of: (1) a ligand that binds a POI; (2) a ligand for recruiting an E3 ubiquitin ligase (E3 recruiting element; E3RE) to promote POI ubiquitination; and (3) a linker connecting these ligands (Figure 1A) [7–11]. To date, there are over 100 reports describing the use of PROTACs for targeted protein degradation (Web of Science search: February 14, 2018) and their utility in chemical biology and drug development. In this review, we describe recent advances in the targeted protein degradation field and discuss those principles underlying efficient PROTAC design that remain to be elucidated.

I.i. Mechanistic Overview of PROTAC-mediated Protein Degradation

Ubiquitin is conjugated to a protein substrate via an enzymatic cascade [5,6,12]. First, an E1 activating enzyme primes ubiquitin via an ATP-dependent mechanism forming an E1~ubiquitin conjugate (~; thioester bond) [5,6,13] followed by formation of an E2~ubiquitin conjugate via a transthiolation reaction with an E2 conjugating enzyme (Figure

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1A) [5,6,14]. Finally, one of the ~600 putative E3 ligases mediates the transfer of ubiquitin to a substrate protein [5,6,15].

E3 ligases mediate protein substrate specificity and catalyze this final transfer via a non-covalent or covalent mechanism depending on the E3 type [12,15]. The three major families of E3 ligases include the RING/U-box family [16–18] and the active-site cysteine-containing HECT [19,20] and RING-in-Between-RING (RBR) families [21,22]. Some E3 ligases function by recognizing specific degradation motifs, known as degrons [23,24]. For example, UBR E3 ligases function via the N-end rule pathway, wherein a 'destabilizing' N-terminal amino acid promotes UBR-mediated ubiquitination [23,25]. Meanwhile, the von Hippel Lindau (VHL) E3 ligase recognizes Hypoxia-Inducible Factor 1 α (HIF1-α) whereby hydroxylation of a key proline residue on the HIF1-α degron motif is essential for VHL -recruitment [26–28]. This degron forms the basis of one of the most widely used E3REs for PROTACs (Table 1) [29–31].

By recruiting an E3 to a POI, PROTACs hijack ligase activity for POI ubiquitination and subsequent degradation by the 26S proteasome (Figure 1A) [8–11]. PROTACs induce the ternary complex (POI:PROTAC:E3 ligase) for ubiquitination, after which the POI is committed for destruction. Since the PROTAC is not degraded in this process, it can promote ubiquitination and degradation of multiple POI equivalents, thus operating sub-stoichiometrically [32]. This catalytic, event-driven modality contrasts with the traditional inhibitor paradigm wherein sustained target binding is indispensable for eliciting a desired biological response. In the standard occupancy-driven paradigm of drug development, potency is dependent on binding affinity. For example, POI inhibition likely cannot influence non-catalytic target protein function(s) (Figure 1B). Additionally, sustained target engagement is difficult in cases of target overexpression, the presence of competing native ligand(s), or target protein mutations that result in loss of target engagement and subsequent resistance (Figure 1B) [33,34]. Since PROTACs inhibit protein function via degradation, this event-driven technology can be used to circumvent the common disadvantages of traditional occupancy-driven inhibitors described above.

II. Current Status of the PROTAC Technology

In the past several years, targeted protein degradation has generated excitement in both academic and industrial settings where POIs ranging in protein class, function, and/or subcellular localization have been successfully degraded (Table 1) [8–11,35–80]. In contrast to this wide range of targeted POIs, relatively few RING E3 ligases have been targeted for recruitment by PROTACs [8–11,35–80].

II.i. PROTAC-mediated Degradation of Epigenetic Erasers

Some PROTACs have been developed to degrade nuclear proteins such as histone deacetylases (HDACs) [44,81]. For example, sirtuin2 (Sirt2), an NAD+-dependent class III HDAC was targeted for degradation [44] by appending SirReal3b, a potent Sirt2 inhibitor [82], to thalidomide via a triazole-based linker to yield a Sirt2-selective PROTAC exhibiting micromolar activity in HeLa cells [44]. This PROTAC retained inhibitory activity against Sirt2 and promoted Sirt2 degradation in a CRBN- and proteasome-dependent manner while

eliciting downstream α-tubulin hyperacetylation [44]. Similarly, degradation of HDAC6, a class IIB Zn^{2+}-dependent HDAC, was achieved using a PROTAC incorporating a hydroxamic-acid-based HDAC6 inhibitor and pomalidomide as its E3RE [81]. Since most HDAC inhibitors bind the active site and/or chelate Zn2+[83], degradation may help characterize the non-catalytic roles of HDACs in disease development and progression.

II.ii. Challenging Localization, Affinity, and Resistance Mechanisms

Recently, an enzalutamide-based PROTAC, ARCC-4, resulted in androgen receptor (AR) degradation at low nanomolar concentrations in VCaP cells, a model for castration-resistant prostate cancer (CRPC) [57]. Surpassing the potency of enzalutamide itself, ARCC-4 also circumvented resistance mechanisms that emerge in patients due to previous therapeutic regimens [57].

The advantages of degradation over inhibition has also been shown when studying PROTAC-mediated degradation of membrane-bound receptor tyrosine kinases (RTKs). As a major drug target group, 47 small-molecule kinase inhibitors have been approved by the FDA. Some of these inhibitors have proven as useful recruiting elements in PROTACs to degrade both serine/threonine and tyrosine kinases[35,41,43,47,49,50,52,55,58,63,65,69–71,84–89]. Despite the clinical success of inhibiting receptor tyrosine kinases (RTKs) [90,91], ‘kinome rewiring’, i.e., the compensatory feedback activation of alternative kinases, is often observed as a resistance mechanism. As previously shown when targeting other POIs (i.e. AR) [55,57,66], degradation can circumvent resistance mechanisms. Appending RTK inhibitors to the VHL-recruiting E3RE afforded PROTACs that degrade membrane-bound WT EGFR as well as disease-relevant EGFR mutants [35]. Moreover, longer-sustained suppression of RTK-downstream signaling was observed in contrast to the kinome rewiring that results from inhibition alone [35] highlighting both the scope of PROTAC target space, and the advantages of degradation over inhibition, respectively.

Interestingly, the use of promiscuous kinase ligands in PROTACs has provided insights into the basis for PROTAC-mediated POI selectivity [35,49,50]. Using foretinib, a kinase inhibitor that binds over 130 kinases at 10 μM, interesting and non-overlapping degradation profiles were observed depending on the E3RE used (i.e., VHL vs CRBN) [49]. Moreover, degradation was observed even with some weak-binding kinases, such as p38α, likely due to positive cooperativity via protein:protein interactions (PPIs) between p38α and VHL in the ternary complex [49]. Similarly, a CRBN-recruiting PROTAC with a promiscuous kinase ligand exhibited different target degradation profiles unrelated to their binding affinities across different cell lines [50], further corroborating the disconnect between target affinity and degradation efficiency. Interestingly, robust TBK1 degradation (>70%) was observed with PROTACs containing VHL ligands displaying up to 3-fold diminution in affinity relative to the typical VHL E3RE (Table 1) [43], further supporting the idea that POI and/or E3 engagement alone does not determine a PROTAC’s efficacy. This diminishes the need for high affinity ligands and expands the potential PROTAC target space.

II.iii. ‘Ineffectual’ ligands can be used for POI degradation

In addition to not needing high affinity ligands to ensure PROTAC efficacy, ligands which lack the ability to modulate cellular POI function can be utilized in PROTACs for POI degradation, as highlighted by a PROTAC which induced the degradation of a multidomain co-regulator of transcription, tripartite motif 24 protein (TRIM24, originally classified as transcriptional intermediary factor 1α). [80] Potent and selective inhibitors of the TRIM24 bromodomain (BD) showed little to no phenotypic consequences in TRIM24-endogenously expressing cells, suggesting BD-inhibition alone may not be sufficient as an anti-cancer strategy in TRIM24-dependent cancers. [92,93] Interestingly, the ligand exhibiting lower affinity and selectivity towards TRIM24[92] afforded a selective and efficient VHL-recruiting PROTAC (dTRIM24). [80] In parallel with genetic studies, dTRIM24-mediated TRIM24 degradation revealed the functional importance of its RING domain in acute leukemia cellular proliferation while disproving its previously characterized dependency in MCF-7 tumour cells. [80] This application exemplifies “non-functional” selective ligands can be exploited by the PROTAC technology to characterize the functional importance of select POIs in disease development and progression.

II.iv. Current Toolbox for Studying what Determines PROTAC Efficacy

BD and extra-terminal (BET) domain family are attractive therapeutic targets given their role in transcriptional control of key oncogenic driver genes [94–97]. Two BD inhibitors, OTX-015[98] and JQ1[99], have been incorporated into PROTACs to study the biological consequences of BET degradation and to characterize the POI:PROTAC:E3 ternary complex [100]. Crystallographic data of the BRD4:PROTAC:VHL ternary complex revealed the PROTAC promotes protein:protein interactions covering 700 $Å^2$ worth of surface area between $BRD4^{BD2}$ and VHL [100]. Interestingly, the PEG linker forms additional protein-ligand interactions within the ternary complex [100]. In contrast, efficient POI degradation was observed for a selection of CRBN-based PROTACs having either no or negative cooperativity [56]. Therefore, due to its multi-faceted nature, the importance of ternary complex formation remains empirical.

Given the complexity of the cellular environment, technologies that monitor ternary complex formation, POI ubiquitination and degradation *in cellulo* are of great benefit. PROTAC-mediated protein degradation by E3 ligases belonging to other families can be monitored using HaloTag7-E3 ligase fusion proteins and a GFP- $FKBP^{F36V}$ reporter system [101]. Excitingly, GFP- $FKBP^{F36V}$ degradation was observed for select RING, HECT and RING-in-Between-RING (RBR) E3 ligases, revealing the applicability of PROTACs to E3 ligases of other families [101]. Similarly, the dTAG system can be used to evaluate and validate the biological consequences of PROTAC-mediated POI degradation without need for POI ligand development by by monitoring the degradation of $FKBP^{F36V}$ POI fusion proteins [102]. This technique has been used to: (1) selectively induce POI degradation that discriminates the target from similar isoforms [102]; (2) validate preclinical therapeutic targets [103]; and (3) validate previously identified targets in disease progression [104]. However, despite the utility of these systems, E3 ligase efficacy, POI ubiquitination and degradation may be misrepresented using these larger tags [101,102]. Moreover, monitoring the various steps of

PROTAC-mediated POI degradation (Figure 2) in real-time remains difficult using these approaches.

An innovative, modular live-cell platform using CRISPR/Cas9 endogenous tagging, luminescence readouts, and NanoBRET technology has been developed to provide functional real-time characterization of PROTAC-mediated POI degradation [105]. This new system allows for monitoring the kinetics of HiBiT-BRD2, BRD3, and BRD4 ternary complex formation, ubiquitination and degradation using CRBN-recruiting and VHL-recruiting PROTACs [105]. For instance, despite all reaching D_{MAX} >86%, maximal degradation levels and recovery rates were observed at different times for each BET protein, even when treated with the same PROTAC [105]. A direct correlation between degradation rates and ubiquitination rates was observed, as opposed to ternary complex formation rate and stability [105]. Interestingly, a significantly different degradation profile of ectopically expressed NLuc-BRD4, as compared to the HiBiT- BRD4 system is observed. Both degradation rates and levels were reduced, while recovery rates appeared more rapid compared to endogenously-tagged HiBiT-BRD4 [105]. Therefore, initially screening for active compounds using methods lacking real-time capabilities, and/or using reporter systems that rely on ectopic expression may overlook: (1) PROTAC-mediated degradation levels, (2) the potential for degrading select POIs, and (3) whether new E3 ligases can be hijacked. Altogether, systems such as NanoBRET can help with PROTAC development and optimization.

III. What does the Future hold for PROTACs?

Irrespective of the mechanistic insight acquired, the full potential of PROTAC technology remains untapped. We have learned a lot from using tool compounds that target kinases and BET proteins, but it remains to be determined how transferable these discoveries are to other protein families and/or classes.

In addition to exploring other protein types, it is imperative that we explore the “PROTACability” of other E3 ligases given observed discrepancies in POI degradation depending on which E3 ligase is recruited. Only <1% of the putative E3s in the human proteome have successfully been hijacked, specifically those belonging to the RING family (Table 1) [8–11,35–80,101]. We have yet not recruited other E3 ligases such as U-box, HECT, and RBR E3 ligases using small-molecule ligands specific for these E3s[101]. Since degradation efficiency is unaffected by attenuating E3 ligase affinity with small changes to the E3RE [43], one could target other E3s, hypothetically even in a tissue-specific manner, potentially without laborious efforts to optimize E3RE affinity. Alternatively, significant contributions are more likely to be made by exploring E3RE vector attachment point(s) (Table 1) and other features important for optimal ternary complex geometry.

Techniques enabling the classification and inhibition of active E2/E3 pairs may afford the identification of novel E3 modulators [106–108]. Meanwhile, some E3 ligases are auto-inhibited via post-translation modification dependent mechanisms[109]. For example, phosphorylation of NEDD4–2, a HECT-type E3 ligase, via GPCR-mediated activation of c-Src kinase activity mediates its activity [110]. Therefore, one could hijack these activation

mechanisms to design conditional PROTAC-mediated POI degradation. Given that some E3 ligases bind viral proteins [111], characterizing such binding interactions could inform the design of nature-inspired E3REs. Finally, complimentary to N-terminal degrons, recent reports of C-terminal degrons for the Cullin2-RING ligases suggest there are likely degrons for other E3 ligase families [112,113]. As was done with for VHL[29–31], we could potentially use degrons to develop E3REs [101], increasing the repertoire of 'hijackable' E3s in the PROTAC toolbox.

Excitingly, technologies such as NanoBiT system [105] allow for the characterization of the dynamic nature of PROTAC-mediated degradation (Figure 2). In the occupancy-driven archetype of drug development, efforts are shifted towards optimizing lead candidate target-residence time (i.e. increasing 1/kOFF) as this has proven efficacious in optimizing for pharmacokinetic and pharmacodynamic drug properties [114]. Using current and new technologies, can we identify which key kinetic steps along the mechanistic coordinate of targeted protein degradation should be optimized? If so, are these target protein- and/or E3 ligase type-dependent?

In addition to characterizing the kinetics of the basic steps required for an effective PROTAC (Figure 2), we should also evaluate what occurs between ubiquitination and degradation. Are there additional cellular systems that can be hijacked or potentiated for the PROTAC technology. For example, p97 prepares protein transport from E3 ligase machinery to the 26S proteasome including with CRBN neosubstrates [115,116]; should this function be further characterized in PROTAC design? Moreover, mechanisms of degradation escape, such as chaperone protein(s) [117,118] and deubiquitinating enzymes [119], should be further characterized for tuning PROTAC efficacy.

IV. Conclusions

Most research efforts highlighted here demonstrate that we have yet to furnish a plug-and-play approach for PROTAC development. However, we can now appreciate that binary target engagement affinities are not indicative of degradation efficiencies for PROTACs [49,50,56]. Meanwhile, the importance of POI ubiquitination versus ternary complex formation and stability for efficient degradation has been uncovered, using well-established target proteins and tool compounds [105]. Given these significant findings, the focus should shift to surveying the broad utility of PROTACs by probing new protein targets and capitalizing on new E3 ligases from other E3 families, including those which are auto-regulated. In parallel, advancing current and new technologies to understand and even predict PROTAC-mediated degradation via computational, biochemical and cellular methods is essential for the field to flourish. These efforts will enable understanding the underlying plastic nature of the PROTAC technology and help truly establish its therapeutic potential.

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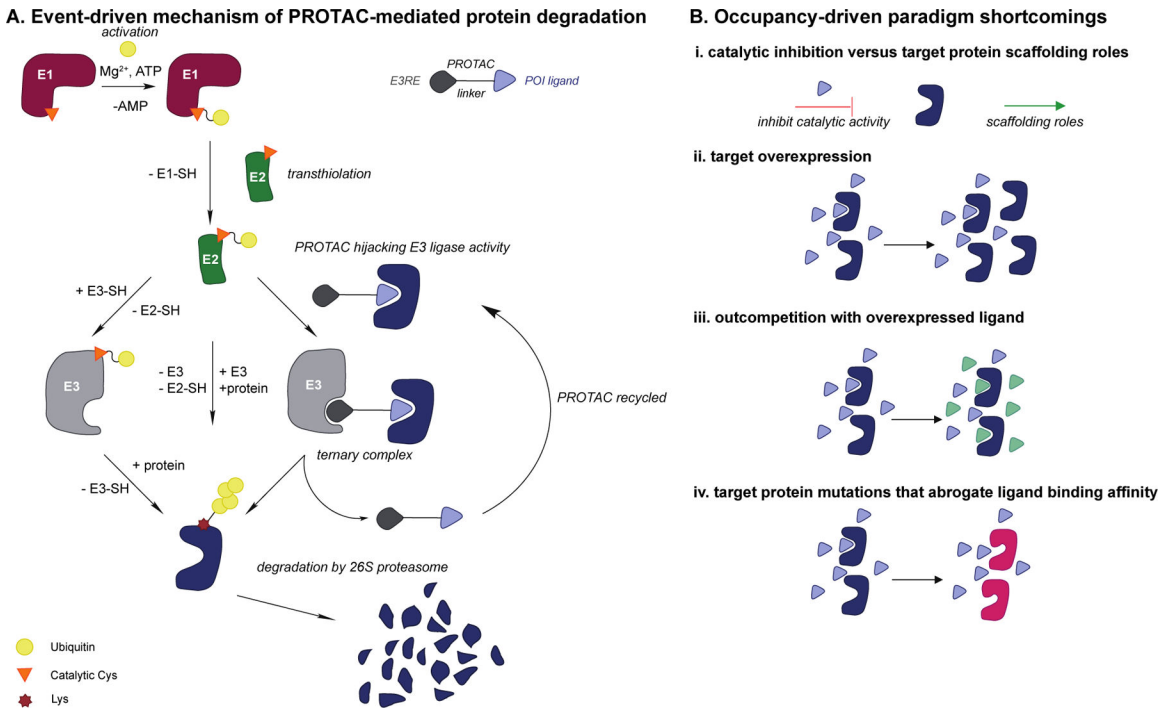


Figure 1A.

Mechanistic overview of PROTAC-mediated POI ubiquitination via the ubiquitination enzymatic cascade, and POI degradation via the 26S proteasome. **B.** Potential shortcomings of occupancy-driven paradigm of small-molecule/drug-target binding wherein sustained target engagement is limited due to: i. catalytic inhibition offers cannot potentiate non-catalytic and/or scaffolding roles of target-protein, ii. target protein overexpression, iii. competition with overexpressed native ligand for same binding site, and iv. target protein mutations potentiate small-molecule/drug binding.

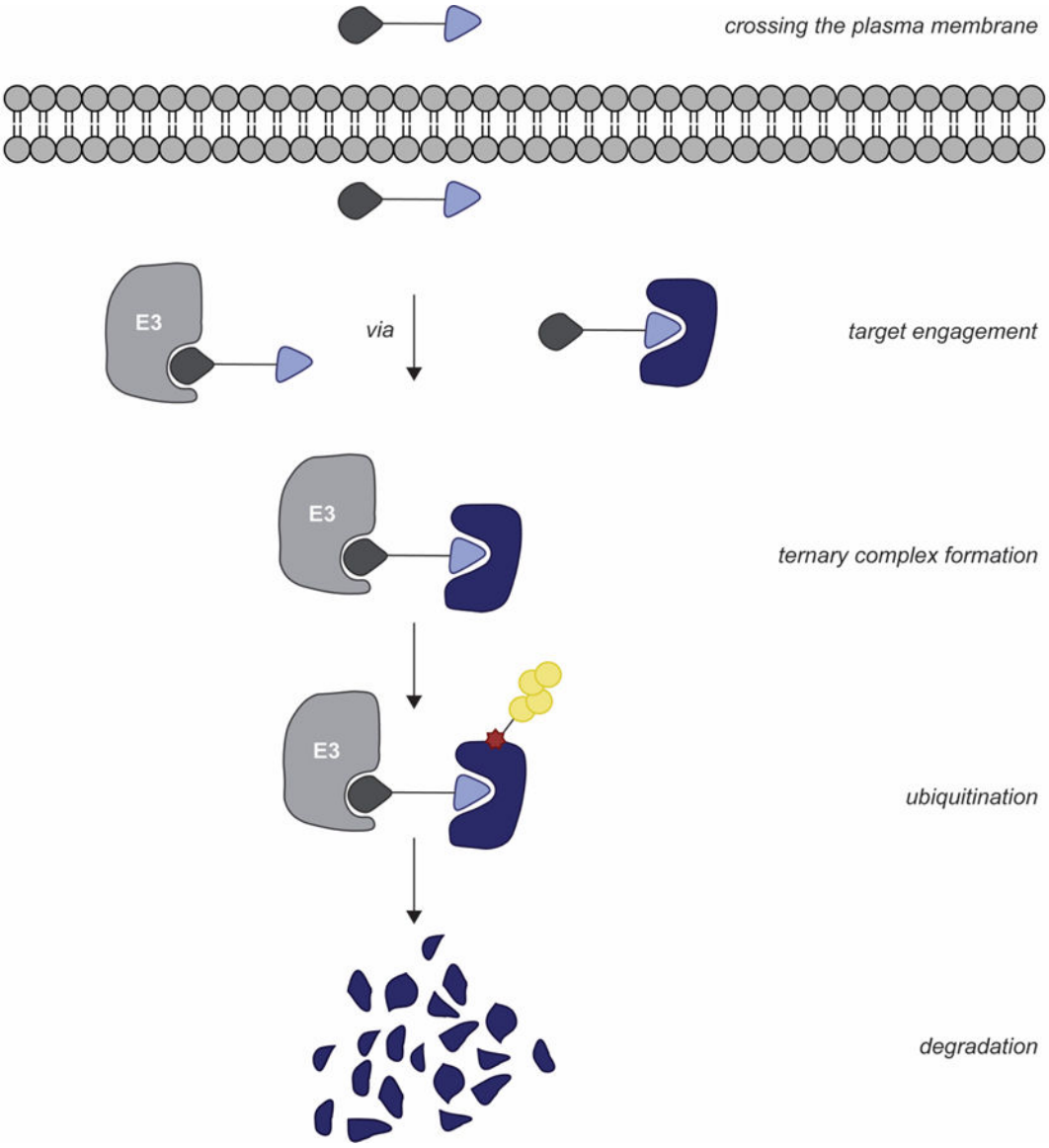
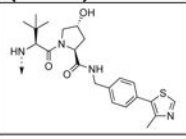
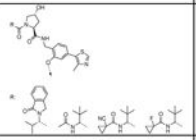
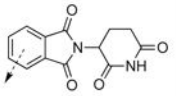
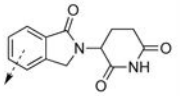
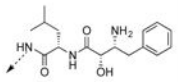
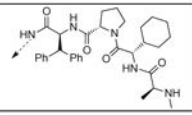
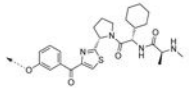
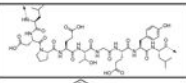
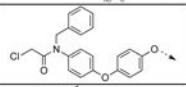
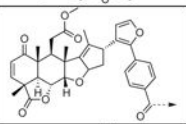
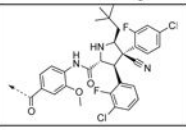


Figure 2.
Overview of steps for PROTAC-mediated POI degradation in a cellular context.

Table 1.

Examples of E3 recruiting elements and respective E3 ubiquitin ligases employed in recent years (~2) for PROTACdevelopment [8–11,35–80]. Dashed arrows represent vectors used for linker attachment in PROTAC synthesis.

E3 ubiquitin ligase	E3 recruiting element (E3RE)			target protein type
VHL*				kinases (cytosolic & receptor), transcription factors, epigenetic readers, E3 ubiquitin ligases
CRBN				kinases (cytosolic & receptor), transcription factors, epigenetic readers & erasers, E3 ubiquitin ligases
XIAP & cIAP*				kinases, transcription factors, epigenetic readers, E3 ubiquitin ligases
Keap1				microtubule-associated protein (tau)
RNF4				epigenetic reader
RNF114				epigenetic reader
MDM2				epigenetic reader

* Notable examples