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Targeted Protein Degradation by Small Molecules

Daniel P. Bondeson and **Craig M. Crews**

Department of Molecular, Cellular, and Developmental Biology, Department of Chemistry, and Department of Pharmacology, Yale University, New Haven, Connecticut 06511

Abstract

Protein homeostasis networks are highly regulated systems responsible for maintaining the health and productivity of cells. Whereas therapeutics have been developed to disrupt protein homeostasis, more recently identified techniques have been used to repurpose homeostatic networks to effect degradation of disease-relevant proteins. Here, we review recent advances in the use of small molecules to degrade proteins in a selective manner. First, we highlight all-small-molecule techniques with direct clinical application. Second, we describe techniques that may find broader acceptance in the biomedical research community that require little or no synthetic chemistry. In addition to serving as innovative research tools, these new approaches to control intracellular protein levels offer the potential to develop novel therapeutics targeting proteins that are not currently pharmaceutically vulnerable.

Keywords

protein degradation; ubiquitin proteasome system; chemical knockdown; PROTACs; IMiDs

INTRODUCTION: THE LIMITS OF INHIBITORS AND GENETIC KNOCKDOWNS

Much of biomedical research consists of interpreting data gleaned from the functional inhibition of proteins. Chemical inhibitors, genetic knockdown and knockout models, and mutagenesis screens have elucidated many of the complex signaling networks in biology. Furthermore, protein inhibition is a mainstay of drug development and the many beneficial therapeutics that improve human health.

Despite this, modern technologies for perturbing protein function clearly have their limitations. Chemical inhibitors, typically small molecules that bind to an enzyme or receptor active site, function by locking their target protein in a state that prevents it from performing its function. This mode of action has two limitations. First, these inhibitors are active only while bound to the protein of interest (POI), necessitating high levels of compound that may elicit unwanted off-target effects (1). Second, these inhibitors must be able to bind to an active site or allosteric site of a protein to inhibit the protein's function.

DISCLOSURE STATEMENT

C.M.C. is a founder, consultant, and shareholder at Arvinas, LLC.

These requirements exclude the approximately 80% of the human proteome classified as undruggable (2).

Although these limitations hamper chemical inhibitors, knockdown strategies provide an efficacious alternative. RNA interference (RNAi), antisense oligonucleotides, and gene-editing techniques, most notably CRISPR/Cas9, provide means for circumventing these issues. Acting at the genetic level, these techniques lack the limitations regarding a target's druggability. Initial excitement for rapidly developing a genetic knockdown for a target has subsided owing to nucleic acid delivery issues, but efforts are still being made using nanomaterials and other packaging technologies to develop these technologies into therapeutics (3). Additionally, these techniques are typically irreversible (limiting their utility as research tools) and often contain unwanted off-target effects (thus narrowing the therapeutic index and obfuscating interpretation of data).

Here, we review technologies with the temporal control and pharmaceutical properties of small molecules capable of knocking down POI levels. These chemical knockdown strategies provide therapeutic options that extend beyond traditional druggable space but still share similar pharmacokinetic properties with typical small-molecule drugs.

When and How Are Proteins Normally Degraded?

After translation, polypeptides are processed, folded, chaperoned, and modified before attaining their final functional state. Additionally, because many proteins are required only transiently, cells possess elegant systems for the removal of unwanted or damaged proteins.

The largest protein disposal system is the ubiquitin proteasome system, which comprises nearly 1% of cellular mass. This system consists of a cascade of enzymes that activate, conjugate, and ligate the 76–amino acid protein ubiquitin onto a lysine residue of a protein. Because ubiquitin contains several lysine residues itself, a chain of polyubiquitin can be assembled: Chains coupled to different ubiquitin lysine residues act as signaling motifs for various processes. Most apropos, certain linkages send the protein to the 26S proteasome, a large protease complex that recognizes, unfolds, and degrades ubiquitinated proteins. The molecular determinants of which proteins are targeted for ubiquitination are defined by a class of enzymes known as E3 ubiquitin ligases. In this review, we highlight studies using a particular E3 ligase family, the cullin ring E3 ligases (CRLs); these are large scaffolding complexes in which one portion recruits the substrate and brings it into close proximity to the reactive E2 ubiquitin for ubiquitination. Several excellent reviews have been written on this subject (4–6).

Another protein disposal system is autophagy. In this process, the protein to be removed is sent to the lysosome, an organelle that contains an acidic environment and up to 50 different kinds of enzymes focused on degrading and processing these substrates (7). Although ubiquitination can signal autophagy, more commonly, the regulated autophagy system employs specific chaperones to bring targeted proteins to the lysosome (8).

Outline

This review is divided into two parts. In the first, we review those chemical knockdown strategies that have the most direct clinical application. These small molecule–based approaches are capable of degrading target proteins without requiring any genetic manipulation. These include selective estrogen downregulators, immunomodulatory drugs (IMiDs) or cereblon binding molecules, proteolysis targeting chimeras (PROTACs) against a variety of targets, and hydrophobic tags for androgen receptor (AR) and Her3.

In the second half, more generalized strategies for manipulating levels of any targeted protein are presented. These techniques require the fusion of a second protein to the POI, which extends the technique into broader applications: for example, when no ligand is available for a POI. Here, auxin-inducible degrons, PROTACs that target modular fusion proteins, and several other techniques are reviewed.

SELECTIVE ESTROGEN RECEPTOR DOWNREGULATORS

Selective estrogen receptor downregulators (SERDs) were among the first class of compounds identified that have the added benefit of inducing degradation of their target protein. Estrogen receptor α (ERα) is a well-known oncogenic driver for metastatic breast cancer (9). Although ERα modulators have been in the clinic since tamoxifen was first approved by the US Food and Drug Administration (FDA) in 1977, spurious ERα activation in various tissues led to a need for pure antiestrogens. Fulvestrant (ICI 182,780 or Faslodex™) was first described in the early 1990s as a pure antagonist capable of overcoming these partial agonistic issues. Its therapeutic mechanism was soon attributed to its ability to decrease intracellular ERα levels (10, 11), but despite approval by the FDA in 2002, fulvestrant suffers from poor bioavailability and is administered by monthly intramuscular injection.

Given these issues, new SERD compounds have been developed recently, with several entering clinical trials. The most advanced compound is ARN-810 (Figure 1*a*), developed by Seragon Pharmaceuticals. With improved oral bioavailability, this compound shows promising preclinical results in mice (12–14) and is now in Phase I clinical trials. Other compounds with oral bioavailability and high potency have also been described by AstraZeneca (15–19), Pfizer (20), and other research groups (21).

SERDs may be oldest application of induced protein degradation, but the mechanism by which ER degradation is achieved is not well understood. It is thought that upon binding, a SERD induces conformational changes of the protein, exposing novel hydrophobic motifs that can be recognized by chaperones and trigger degradation (22, 23). Only one SERD-ERα crystal structure has been solved, but the mechanism by which that compound induces ERα degradation is not shared with other SERD compounds. Because the ligand-ERα structures are thought to differ, clear routes to rationally design SERDs are thus not obvious. Indeed, even in the most recent reports, some groups note surprising differences in degradation with minor structural changes to their SERDs (16). However, high-throughput screening assays have been developed that identify compounds that either unveil the relevant hydrophobic surfaces (23) or assess intracellular levels of ERα rapidly (12, 15).

IMMUNOMODULATORY DRUGS

The phthalimide class of compounds known as IMiDs has a storied history. First discovered and described in the 1950s and 1960s as a sedative, thalidomide was discovered to be a potent teratogen. However, since then, it and its close analogs have been repurposed as potent anticancer agents (24). Given the excitement regarding thalidomide's anticancer activity, particularly for multiple myeloma, medicinal chemistry efforts were undertaken to develop related compounds possessing these beneficial activities. Thalidomide itself is approved for newly diagnosed multiple myeloma, and three other thalidomide analogs are of particular interest. First, lenalidomide (Revlimid, CC-5013, Figure 1*b*) is approved for the treatment of relapsed multiple myeloma, myelodysplastic syndrome (MDS, particularly those genotyped to a chromosomal 5 truncation) (25), and mantle cell lymphoma. It is also in Phase III trials to expand its approval within these diseases as well as for the treatment of acute myeloid leukemia and chronic lymphoblastic leukemia. Second, pomalidomide (Pomalyst, CC-4047) has also been approved for relapsed multiple myeloma (26). And third, a more recently described compound, CC-122, also shows activity as a pleiotropic pathway modifier and is in Phase I trials for multiple myeloma, diffuse large B cell lymphoma, chronic lymphoblastic leukemia, and several solid tumors (27). How these compounds have such potent activity has only recently been elucidated.

In 2010, a major step toward understanding IMiD action was made upon identification of cereblon as a major target of thalidomide teratogenicity. Using a chemoproteomic probe of immobilized thalidomide, the authors identified cereblon as a substrate adapter for the CRL4a ubiquitin ligase complex, whose auto-ubiquitination is inhibited by thalidomide. Importantly, mutations in cereblon that block thalidomide binding also inhibit models of thalidomide teratogenicity in chicken and zebrafish (28). Another major breakthrough in the understanding of IMiD mechanisms was demonstrated in three elegant studies (29–31) that used different profiling techniques to identify the transcription factors Ikaros and Aiolos as proteins whose ubiquitination is increased upon IMiD treatment. Whereas IMiDs decrease cereblon auto-ubiquitination, they also increase Ikaros ubiquitination. Cells expressing an IMiD-resistant Ikaros mutant (Q147H) are resistant to IMiD-induced cytotoxicity (29, 30). More recently, casein kinase 1α (CK1α) was similarly identified as a protein selectively degraded by lenalidomide (27, 32).

Although these results collectively implicated cereblon and Ikaros/CK1α as the target of IMiD action, the mechanism of this cytotoxicity is unclear. Ikaros family members activate the *IRF4* locus, a gene to which myeloma cells are addicted (33, 34), although not all IMiD-sensitive cell lines have decreased IRF4 levels, indicating IRF4 might not be necessary for this cytotoxicity (30). In del(5q) MDS, haploinsufficiency of CK1α leads to hyperproliferation, whereas homozygous loss leads to apoptosis, a finding that helps explain lenalidomide sensitivity in CK1α (32, 35).

From a biochemical standpoint, recent studies have also led to an understanding of how IMiDs recruit these new substrates to cereblon. Crystal structures of the cereblon-IMiD complex (36, 37) and the ternary complex between cereblon, lenalidomide, and CK1α (38) have confirmed that the IMiD glutarimide moiety binds to a hydrophobic cavity in cereblon,

whereas the phthalimide ring is free to form contacts with the substrate. The phthalimide ring, in combination with local residues from cereblon, creates a surface that binds to a remarkably small beta hairpin loop on CK1α. Furthermore, this hairpin shares structural, but not sequence, homology to Ikaros, providing mechanistic data for the selectivity of the different IMiD compounds for their respective targets.

In conclusion, IMiDs are an intriguing class of compounds with surprising mechanisms of action. Because minor differences among family members affect substrate binding, it will be interesting to identify other proteins that may be targeted by related compounds. In addition, given the recently discovered structural information on CK1α recruitment, will it be possible to design IMiD-like compounds that recruit particular hairpin motifs?

PROTACS

Although IMiDs and SERDs have found clinical success, the applicability of the system is currently limited. For example, rationally designing a thalidomide analog to target a specific protein for degradation would be difficult given the small structural determinant on the potential substrate that would be challenging to predict and exploit.

Previous Generations of PROTACs

For the past 15 years, our lab has developed the PROTAC technology, which lacks these limitations and is able to induce selective protein degradation without the need for genetic manipulation. PROTACs are heterobifunctional molecules that have discrete binding moieties for the substrate of interest and for an E3 ligase connected by a chemical linker. The first PROTAC, developed in collaboration with the Deshaies group at CalTech (39), consisted of the natural product ovalicin and a peptidic ligand for the CRL1 F-box protein β-TRCP. This initial PROTAC demonstrated ternary complex (substrate–PROTAC–E3 ligase) formation, ubiquitination activity, and limited degradation of its target protein in *Xenopus* extracts (40). Since this first publication, our group and others have published approximately 30 papers validating this technology.

These studies have explored both the limitations and potential of the PROTAC technology, and several key lessons have been learned. First, different E3 ligases can be hijacked by PROTACs for selective protein degradation. β-TRCP, MDM2 (41), CIAP (42), and von Hippel–Lindau (VHL) (43) have all been employed for induced protein ubiquitination using a heterobifunctional dimer approach. Although they are not technically PROTACs, other bifunctional peptides have been used to direct POIs to the lysosome for degradation (44). Second, small molecules have been employed for either binding moiety. The MDM2 inhibitor Nutlin (41) or the IAP ligand bestatin (45–47) have both been used in PROTACs to engage their cognate E3 ligases. Likewise, small-molecules have also been used as substrate-targeting ligands [e.g., small-molecule agonists of the retinoic acid receptor (42), fumagillin and ovalicin for methionyl aminopeptidase 2 (48)].

Third, and disappointingly, these compounds have been very limited in their potency. Most of these early-generation compounds are, at best, active in the low-micromolar range with only partial degradation of the POI. Because these compounds are large and charged (or at

least highly hydrophilic), cell permeability is a key contributor to this lack of potency, although the low affinity of these peptides for their targets is also likely a contributing factor. Another issue, which is only now being appreciated, is the role that proper linker geometry has in PROTAC potency. This is discussed in more detail below.

From a technological standpoint, four papers published in May and June of 2015 made significant advances toward the therapeutic application of PROTACs.

Next-Generation PROTACs

To develop potent PROTACs, high-affinity small-molecule E3 ligase ligands had to be developed. The E3 ubiquitin ligase $CRL2^{VHL}$ is responsible for the regulated ubiquitination of hypoxia inducible factor 1α (HIF1α). This interaction is very specific: A specific hydroxylation event on a single proline residue is sufficient to mediate the VHL-HIF1α interaction (49). Given this concise molecular determinant for binding, our lab sought to develop a small-molecule VHL ligand for use in PROTACs based on the hydroxyproline residue. Using a combination of in silico and fragment-based screening, an initial VHL ligand with low micromolar affinity was further developed into a high-affinity ligand with a K_d of 180 nM (50, 51).

With this VHL ligand in hand, three different classes of VHL-targeting PROTACs were made to target the bromodomain-containing protein 4 (BRD4), the receptor interacting serine/threonine protein kinase 2 (RIPK2), and the nuclear hormone receptor estrogen-related receptor α (ERRα) (52, 53). BRD4 is a reader protein of epigenetic marks, and although it is not mutated in cancers, inhibition of BRD4 has been shown to decrease expression of the oncogene *CMYC*, leading to selective killing of cancers addicted to c-Myc (54, 55). RIPK2 is strongly implicated in autoimmune diseases such as Crohn's disease as well as cancer, and it functions through a combination of enzymatic and scaffolding roles (56, 57). ERRα is known as a master regulator of metabolic homeostasis (58) and some cancers (59). Based on the importance of these targets, VHL-based PROTACs were synthesized targeting RIPK2 (PROTAC_RIPK2; Figure 1*c*), ERRα (PROTAC_ERRα), and BRD4 (MZ1) to VHL.

Each of these ligands was chosen for their high affinity and selectivity, as well as for their known protein-ligand structural data. In designing a PROTAC, a key decision is the attachment point of the linker; solvent-exposed surfaces of the ligand are necessary. For RIPK2, the amino-quinoline-based kinase inhibitor ligand bound with near 100-fold selectivity over RIPK3, and unpublished structural data indicated a solvent-exposed region that could be modified. Although several ERRα ligands are known, one compound has nearly 100-fold selectivity over the closely related ERRγ, a K_d of approximately 40 nM, and the appropriate structural data for the design of the linker attachment point (60). Similarly, the choice of OTX015 as the BRD4 ligand was due to selectivity for BRD2/3/4, high potency, and a known solvent-exposed region for linker attachment (61). Linker attachment at a solvent-exposed region on the target protein ligand is critical, but the optimal linker length and composition is more difficult to discern, as discussed below. As these PROTACs demonstrate well, no one linker is optimal for every target protein: ERRα was degraded with a 6-atom linker, RIPK2 with a 14-atom linker, and BRD4 with a 10-atom linker. In each

case, near-complete removal of the protein was achieved at PROTAC concentrations as low as 5 nM (53).

Whereas the VHL ligand on which these PROTACs are based was generated through a traditional structure-based drug design approach, other ligands with selectivity and high affinity for an E3 ligase were discovered recently: IMiDs. The available structural data for these IMiDs (36, 37) was used to generate the PROTACs ARV-825 (62) and dBet1 (63), which target BRD4 for ubiquitination by the E3 ligase $CRL4a^{CRBN}$.

ARV-825 and dBet1 are based on the BRD4-selective inhibitors OTX-15 and JQ1, respectively (55), coupled to an IMiD phthalimide. Linker-wise, the two compounds differ significantly; ARV-825 contains a 14-atom PEG linker, whereas dBet1 has a 7-atom, primarily alkyl linker. These linker differences may be responsible for the significant differences in intracellular potencies between these two PROTAC molecules: Greater than 90% BRD4 degradation is observed at 1 nM and 0.5 μM for ARV-825 and dBet1, respectively.

On Choosing PROTAC Warheads and Linkers

Although the differences between these compounds are certainly interesting, hard and fast principles for effective PROTAC design remain elusive. To aid in our understanding of so-called linkerology, our lab recently explored the effects of different variables on PROTAC efficacy and target protein selectively, using two E3 ligands, three targeting ligands, and four different linkers spanning a diversity of chemical space (64). The goal was the design of a PROTAC capable of degrading the oncoprotein BCR/Abl, with the hope that BCR/Abl degradation would possibly eliminate kinase-independent functions of the protein (65, 66).

This study of BCR/Abl PROTAC development yielded several interesting conclusions regarding the effects of the targeting warhead, E3 ligase, and linker. First, the choice of protein-targeting ligand has a large influence on PROTAC selectivity and degradation activity. The Abl tyrosine kinase inhibitor imatinib was unable to degrade Abl or BCR/Abl when incorporated into a PROTAC, whereas bosutinib-based PROTACs gave the most profound degradation of Abl, and dasatinib-based PROTACs were best at degrading BCR/Abl. These differences might arise from differences in affinity, as imatinib has a much lower affinity than do the other compounds. However, because bosutinib and dasatinib bind with similar affinities, perhaps subtle structural changes in BCR/Abl change the efficiency of ubiquitin transfer. More studies are required to illustrate conclusively why this might be. Second, the PROTAC linker may influence cell permeability more than target-protein degradation; although linker changes did not influence whether BCR/Abl or Abl was degraded, they did affect the efficiency with which the substrate was degraded.

Third, the E3 ligase being recruited can also significantly influence the PROTAC's ability to degrade different substrates. In this study, only cereblon was able to degrade both BCR/Abl and Abl, whereas VHL was only able to degrade Abl efficiently. Several hypotheses could explain this rather curious result. For example, owing to steric differences between BCR/Abl (approximately 210,000 Da) versus c-Abl (110,000 Da), perhaps only Abl can fit into VHL complexes. Alternatively, whereas VHL seems to have the larger endogenous substrate

(HIF1α = 95,000 Da versus MEIS2 = approximately 50,000 Da), perhaps $CRL2^{VHL}$ has a less flexible cullin domain than does $CRL4a^{CRBN}$ (67). The need for proper presentation of lysines on the target protein to the recruited E3 ligase may also play a key role. This hypothesis of ubiquitination zones for selecting particular lysine residues on the substrate has been reviewed recently (68).

HYDROPHOBIC TAGGING

Given the clinical success of fulvestrant, which mediates ERα degradation by exposing a hydrophobic patch on the surface of the protein, we hypothesized that a ligand for a POI could be functionalized similarly into a hydrophobic tag to mimic a partially unfolded state. In this way, a hydrophobically tagged protein would be recognized by the same cellular quality control that recognizes and discards terminally misfolded or unfolded proteins. Although we have published this strategy in model systems before, it has been employed recently to degrade endogenous proteins, [i.e., the pseudokinase Her3 (69) and the AR (70)] without the need for fusion protein genetic engineering. A similar system based on appending a large Boc_3Arg motif to a POI ligand has also been reported to induce selective degradation (71). Recently, however, it was shown that the Boc_3Arg motif inhibits global translation by blocking the mammalian target of rapamycin complex 1 pathway (72). How this data reconciles with the purported degradation caused by this ligand is unclear.

Her3 Degradation

Although Her3 is a member of the ErbB family of receptor tyrosine kinases, sharing high sequence similarity with the archetypical epidermal growth factor receptor, it differs from family members in that it lacks key catalytic residues in its active site, leading to loss of detectable kinase activity. Thus, researchers have proposed that Her3 functions primarily as a pseudokinase (i.e., a scaffolding protein rather than an active kinase). Given the difficulty of pharmacological targeting of pseudokinases using current small-molecule approaches, these proteins make attractive targets for strategies based on induced protein degradation. As a first step, a Her3 ligand was identified by screening a library of ATP-competitive compounds in a competitive time-resolved fluorescence energy transfer assay, and its potency was improved via the addition of an acrylate, thus generating a first-in-class, highly selective covalent ligand to Her3 (69, 73). Subsequent coupling of a hydrophobic adamantyl moiety produced a Her3 degrader compound that abrogated almost all Her3-dependent signaling in cultured tumor cells.

Androgen Receptor Degradation

Hydrophobic tagging technology has also been employed to degrade the AR, a well-defined oncology target (74). Like ERα, the AR drives the growth of many hormone-responsive tumors, particularly in prostate cancer, which is responsible for the second-highest cancer-related mortality rate for men in the western world. Although aromatase inhibitors and AR antagonists have been largely successful in treating early stages of prostate cancer, resistance develops in ways similar to ERα-targeting selective estrogen receptor modulators. By appending the alkylfluoryl chain of fulvestrant onto dihydrotestosterone, the first selective androgen receptor downregulator (SARD) compound was discovered (75). Other

compounds that decrease AR expression have been reported to decrease AR by inhibiting its synthesis (76, 77).

As a parallel strategy to rationally design a SARD, we appended the adamantyl moiety to the AR agonist RU59063 (70). Gratifyingly, we found that this addition switched the agonist into a pure antagonist capable of degrading AR protein (half-maximal degradation at 1 μM; maximal degradation of 95%). Moreover, this SARD was also able to inhibit proliferation of a model castration-resistant prostate cancer cell line resistant to enzalutamide.

FUSION-BASED DEGRON TECHNOLOGIES

SERDs, IMiDs, and specific PROTACs are interesting compounds for their ability to degrade disease-relevant proteins, but these tools must be custom designed and synthesized for each target protein. Thus, for a particular protein to be degraded, a sizeable synthetic effort must be made [e.g., the conjugation of the VHL ligand to a ligand of the targeted POI (if such a ligand is available)]. This latter issue is particularly problematic because many attractive proteins to be targeted using degradation strategies are currently undruggable targets for which no ligand exists.

Fortunately, to address this need for model systems for controlling intracellular protein levels, the chemical biology community has developed more generalizable approaches to controlling protein levels using small molecules. Here, we review several techniques in which a small molecule recruits a ligand-mediated degron to an E3 ligase, summarized in Table 1.

Auxin-Inducible Degron

In plants, small-molecule auxin hormones control many different growth and cell cycle functions. The mysteries of this system were elucidated in the past 15 years when it was shown that auxins act as a molecular glue, similar to IMiDs, between $CRL1^{Tir1}$ and indole-3-acetic acid (IAA)/auxin transcription factors (78, 79). This natural protein degradation system has been exploited to recruit exogenous substrates to an E3 ligase for ubiquitination and subsequent degradation. For example, the auxin-binding domain [a.k.a. auxin-inducible degron (AID)] has been fused to GFP and other POIs to induce the efficient and rapid degradation proteins from yeast and mammalian cells (80, 81).

Although the system requires genetic engineering, the fusion proteins and small molecules used are bioorthogonal and interface well with endogenous machinery. The minimal AID domain must be fused to the POI but is small (44 amino acids) (82). Additionally, the Tir1 F-Box protein must also be expressed exogenously but interfaces with the endogenous CRL1 complex within cells. This is unlikely to cause inhibition of the endogenous degradation machinery. Moreover, the small molecule IAA is also thought to be inert in eukaryotic cells, although possible metabolic byproducts may be toxic. Overall, this system is bioorthogonal and enables rapid ($t_{1/2}$ = 20 min) depletion of the POI.

Several studies have highlighted the usefulness of this system in addressing biological questions, such as in the study of centrosome formation in human cells (83, 84) and

calcineurin function in the malarial parasite *Plasmodium berghei* (85). The use of CRISPR/Cas9 to introduce AID into the endogenous genomic locus of the gene of interest for conditional protein depletion in *Caenorhabditis elegans* has proved to be particularly useful (86). Zhang et al. (86) first confirmed that the AID system is active in all development stages of the worm and that degradation is rapid: A 20-min half-life in the presence of auxin was observed. Furthermore, the authors compared RNAi-and auxin-mediated depletion and found that whereas RNAi demonstrated a particular phenotype (e.g., 2% progeny arrested in development), the auxin was capable of a much more pronounced phenotype (100% arrest). This indicates that despite RNAi's limited ability to affect a phenotype owing to tissue distribution, cell penetrance or incomplete knockdown, the chemical genetic tool enables a more robust phenotype. Finally, the authors show the use of the AID system in the germ–line, a tissue understudied because of a complete lack of conditional protein degradation tools. The authors clearly demonstrate complete protein knockdown within 45 min and show that within 6 h of knockdown, meiotic nuclei are disfigured (86). These data more closely match knockout rather than knockdown phenotypes, an important corroboration of the chemical and genetic tools.

PROTAC-Recruiting Degrons

Our lab has used the modified bacterial dehalogenase HaloTag as a model system for induced protein degradation for several years, given the ease of generating chloroalkane-based HaloTag ligands synthetically. The hydrophobic tagging approach was first developed using the HaloTag system and was shown to induce efficiently the degradation of cytosolic as well as transmembrane HaloTag fusion proteins in cell culture and in vivo (87–89). The ability to induce selectively the degradation of a single HaloTag fusion protein proved valuable in the study of the unfolded protein response (UPR) of the endoplasmic reticulum. In this case, an endoplasmic reticulum–localized HaloTag fusion protein was unfolded upon addition of a hydrophobic tag, thus inducing the UPR (90). Moreover, because this unfolded protein represented only a small portion of total protein within the endoplasmic reticulum, the cell was able to resolve and adapt to the stressor. Thus, more specific transcriptional changes could be studied using the destabilized HaloTag compared to other globally acting agents typically used to study the UPR (90).

We next sought to develop potent PROTACs able to degrade the more-stabilized HaloTag7. These compounds are similar to the VHL-based PROTACs presented above: The heterobifunctional molecule binds to the E3 Ligase VHL, while the chloroalkane simultaneously forms a covalent bond with the HaloTag receptor protein (91). From a panel of different compounds, we found that HaloPROTAC3 was the most efficacious. Interestingly, extending the linker by one PEG unit led to an approximately 20% reduction in activity, and shortening the linker by one PEG unit abrogated any activity completely. A similar HaloTag-based degradation system has also been presented that uses the E3 ligase IAP instead of VHL (92). Although this system is not as efficient, the authors show that nuclear proteins can also be degraded. To aid in the use of HaloTag, GeneCopoeia has made available 20,000 human and 15,000 mouse open reading frames fused to HaloTag7 (http://www.genecopoeia.com/tech/halo-tag/).

A final system based on PROTACs and a fusion protein-binding domain was highlighted alongside the cereblon-based PROTACs against BRD4 (see above). This system uses a FK506 binding protein 12 (FKBP12) fusion protein and its high-affinity ligand conjugated to Thalidomide. This compound was also able to degrade free FKBP12 with high potency (63).

Small-Molecule Modulation of Protein Activity

Two other techniques requiring genetic manipulation allow control over protein levels but are more nuanced in their mechanism of action. The first, small molecule–assisted shutoff (SMASH), produces native protein in the absence of compound (93). Upon compound binding, however, a tag is prevented from self-excising, which exposes a hydrophobic degron, thus causing the protein to be degraded quickly. Therefore, compound addition does not degrade the protein but inhibits its processing into a functional protein. This limits the technique to instances in which a protein is short-lived (and so protein produced prior to compound addition does not interfere with the experiment) or in which removal of drug and accumulation of protein can suffice. Another report used this technology to boost the statistical power of a dual-reporter screen by tuning the expression of one of those reporters using SMASH (94).

The second system is a modification of the popular Shield-1 (Shld-1) drug-on technology. This system makes used of a destabilized mutant of FKBP12 that is stabilized in the presence of the compound Shld-1 and has proved useful in controlling fusion protein stability in parasites (95), worms (96), and medaka (97) as well as to potassium channel biology (98) and the cytosolic UPR in mammalian cells (99). Several technological advancements are worth noting: By combining Shld-1 stabilization with induced dimerization of a split-ubiquitin system, release of the native protein is achieved in response to a small molecule (100). In addition, this system can be further combined with Tet-On transcriptional control to produce a 130-fold dynamic range of POI levels (101).

OUTLOOK

Comparison of the Technologies: What Tool Should One Use?

As outlined in this review, there are several options for controlling intracellular protein levels using a small-molecule approach, each with its own advantages and disadvantages. The choice of a particular protein degradation system depends on the specific biological problem to be addressed and the properties of the desired protein degrader compound.

Currently, PROTACs provide the best means of designing and generating a modular compound that can degrade a POI directly without fusing it to a degron. Although a powerful protein degradation system, PROTAC generation requires a known ligand for a POI. As discussed above, important considerations such as linker geometry, attachment point, and choice of the particular E3 ligase engaged must be considered. Typically, a panel of different PROTACs should be developed and then assayed for their ability to degrade the POI selectively and potently.

If no known ligand for the POI exists, then small molecule–based protein degradation systems using protein fusions are available. Because the POI is likely available as a fusion to the HaloTag protein, HaloPROTACs can provide a robust tool for specific degradation. Alternatively, AID also provides very rapid degradation of the POI, allowing for more granular temporal questions to be answered, and has been demonstrated to be efficacious in vivo.

In those cases for which genetic manipulation of the POI is unacceptable and no known ligand is available, modern screening technologies can help develop a high-affinity ligand for the POI. Differential scanning fluorimetry (a.k.a. the thermal shift assay) (102), NMR-based fragment screens (103), competitive binding screens when a weak ligand is available (69), and affinity-based selection are all popular techniques (104). Other techniques to identify cryptic binding sites may also identify novel pockets on protein surfaces that could be targeted by degradation technologies (105, 106).

What Do the Next Several Years Hold for Targeted Protein Degradation?

The past two years have seen an exciting number of novel strategies to target a particular protein for degradation. Importantly, these technologies have expanded beyond the academic chemical biology arena: IMiDs are already FDA approved, and PROTAC-based clinical candidates are currently being developed. Given the fast pace of this field, the next several years will surely be an exciting time for those interested in small-molecule control of intracellular protein levels.

The IMiD class of degraders offers many opportunities for new discoveries in this field; undoubtedly, novel protein substrates that can be degraded through structurally diverse IMiDs will be identified. Furthermore, given the recent discovery of the binding motif of CK1α and Ikaros to the cereblon-IMiD complex, it will be exciting to see whether rational substrate design can be performed to either maximize the efficacy of existing IMiDs or create new IMiDs with novel activities. However, given the challenges of stabilizing protein-protein interactions (107), this rational approach to IMiD design may prove difficult.

The PROTAC strategy has the advantages of modularity, potency, and in vivo efficacy and will likely be the system of choice for those interested in exploring the undruggable proteome via small-molecule modulation of protein levels. Given the hundreds of E3 ligases in the cell, as ligands to ligases are identified, they will likely be incorporated into novel PROTAC degraders, thus expanding the rapidly growing number of known E3 ligases that can be hijacked to induce targeted protein degradation (108–112). Overall, targeted protein degradation offers strategies for asking and answering new biological questions as well as targeting functions of hitherto undruggable proteins.

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Glossary

POI	protein of interest

CRL	cullin ring E3 ligase
IMiD	immunomodulatory drug or cereblon binding molecule
PROTAC	proteolysis targeting chimera
AR	androgen receptor
SERD	selective estrogen receptor downregulator
ERα	estrogen receptor α
CK1α	casein kinase 1α
BRD4	bromodomain protein 4
SARD	selective androgen receptor downregulator
AID	auxin-inducible degron
UPR	unfolded protein response
SMASH	small molecule–assisted shutoff

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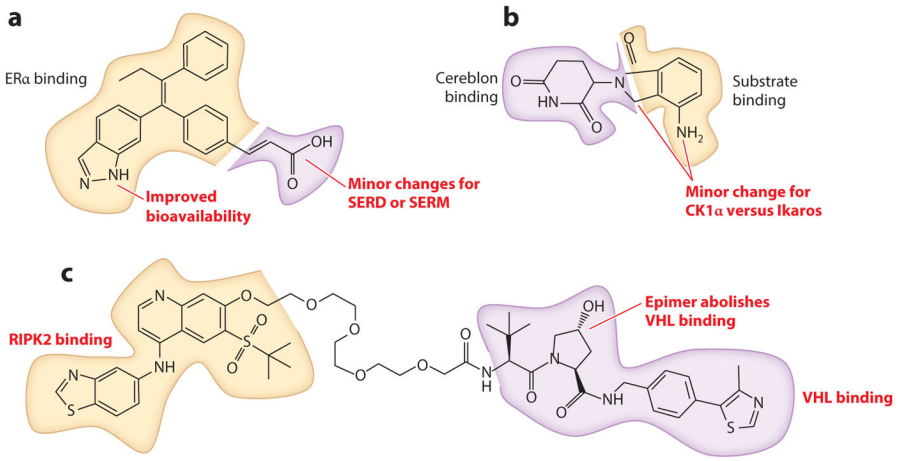

Figure 1.

Compounds that degrade their respective targets without requiring any genetic manipulation. The substrate binding portion is highlighted in yellow, whereas purple dictates parts of the compound that induce target protein degradation. (*a*) ARN-810, a bioavailable SERD that causes degradation upon rearrangement of hydrophobic portions of ERα by the vinyl carboxylic acid. Minor changes at the carboxylic acid position can create ERα agonists. (*b*) Lenalidomide, an immunomodulatory compound that causes degradation of Ikaros and CK1α by binding to the E3 ligase cereblon and creating a novel surface for their interaction. Minor structural changes in the compound abrogate CK1α binding but maintain Ikaros binding. (*c*) A PROTAC induces degradation of RIPK2 by recruiting it to the E3 ligase VHL. The two binding motifs are separated by the linker, allowing enhanced modularity. Abbreviations: CK1α, casein kinase 1α; ERα, estrogen receptor α; PROTAC, proteolysis targeting chimera; RIPK2, receptor interacting serine/threonine kinase 2; SERD, selective estrogen receptor downregulator; SERM, selective estrogen receptor modulator; VHL, von Hippel–Lindau.

Table 1

Summary of small-molecule-inducible degrons

Fusion tag	Compounds	N or C terminal?	Mass of tag	DC_{50}[a]	D_{Max}[b]	$t_{1/2}$[c]	Reference(s)
HaloTag	HaloPROTAC3	Both	37 kDa	19 nM	90%	4–8 h	91
HaloTag	Bestatin 1b	Both	37 kDa	1 μM	39%	1 h	92
SMASH[d]	Asunaprevir	C terminal	NA	1 nM	100%	NA	93–94
AID[e]	IAA	Both	5 kDa	50 μM	100%	20 min	80–86
FKBP12	dFKBP12	Both	13 kDa	10 nM	95%	ND	63

Abbreviations: AID, auxin-inducible degron; FKBP12, FK506 binding protein 12; IAA, indole-3-acetic acid; NA, not applicable; ND, not determined; SMASH, small molecule–assisted shutoff.

[a] DC_{50}, concentration to achieve half-maximal degradation; does not correspond to 50% protein remaining.

[b] D_{Max}, maximal percentage of protein degraded; 100% indicates no protein remaining.

[c] $t_{1/2}$, half-life of degradation; time at which half-maximal degradation is observed.

[d] SMASH is autocleaving and thus not present in the expressed protein.

[e] AID also requires coexpression of the Tir1 F-Box protein.