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Protein degradation for drug discovery

Monitoring and deciphering protein degradation pathways inside cells

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A new series of therapeutic modalities resulting in degradation of target proteins, termed proteolysis targeting chimeras (PROTACs), hold significant therapeutic potential with possible prolonged pharmacodynamics, improved potency, and ability to target proteins previously thought of as ``undruggable''. PROTACs are heterobifunctional small molecules consisting of a target binding handle bridged via a chemical linker to an E3 ligase handle which recruit the E3 ligase and ubiquitin machinery to target proteins, resulting in subsequent ubiquitination and degradation of the target. With the generation of small molecule PROTAC compound libraries for drug discovery, it becomes essential to have sensitive screening technologies to rapidly profile activity and have assays which can clearly inform on performance at the various cellular steps required for PROTAC-mediated degradation. For PROTAC compounds, this has been particularly challenging using either biochemical or cellular assay approaches. Biochemical assays are highly informative for the first part of the degradation process, including optimization of compound binding to targets and interrogation of target:PROTAC:E3 ligase ternary complex formation, but struggle with the remaining steps; recruitment of ternary complex into larger active E3 ligase complexes, ubiquitination, and proteasomal degradation. On the other hand, cellular assays are excellent at determining if the PROTAC successfully degrades the target in its relevant setting but struggle as early development PROTAC compounds are often poorly cell-permeable given their high molecular weight. Additionally, if degradation is not observed in a cellular assay, it is difficult to deconvolute the reason why or at which step there was failure. In this review we will highlight the current approaches along with recent advances to overcome the challenges faced for cellular PROTAC screening, which will enable and advance drug discovery of therapeutic degradation compounds.

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Introduction

Target protein degradation using PROTACs has emerged as one of the most exciting and promising new areas for small molecule drug discovery [1–10]. PROTACs are heterobifunctional small molecules consisting of two binding modules; one which binds the target protein of interest to degrade and one which binds an E3 ligase component (Fig. 1), most demonstrated with either von Hippel-Lindau (VHL) or cereblon (CRBN) proteins [2,7,11–20]. To function, PROTACs must permeate the cell, and while they can engage either the target or the E3 ligase component separately as binary complexes, the goal in PROTAC design is ultimately to drive simultaneous engagement of the target protein and E3 ligase component, termed the ternary complex [2,7,21] (Fig. 1). The ternary complex must also exist within the larger context of a functional E3 ligase complex, with optimized structural positioning of the target protein for efficient ubiquitination [2,7,21] (Fig. 1). Afterwards, the ubiquitinated target protein is released from the ternary complex, is trafficked to the proteasome, and is degraded [2,7,21] (Fig. 1). Data support PROTACs function at sub-stoichiometric binding concentrations, suggestive of a catalytic mechanism of action wherein a single PROTAC can cycle through this process, degrading multiple copies of a target [2,7,22]. Given this, it is not surprising then that comparative studies of PROTACs to their inhibitor counterparts have shown them to be more efficacious at lower doses with extended pharmacodynamics due to the longer timescales for new protein synthesis [18,19,23–30]. Additionally, as compared to their parental inhibitor counterparts, PROTACs have shown improved selectively and specificity [18,19,22,31] as well as the ability to overcome resistance mechanisms [32,33]. Lastly since a target binding ligand is sufficient for recruitment of the E3 ligase, the opportunity to target proteins lacking conventional enzymatic binding pockets may soon become a reality. These benefits and the ability to degrade a protein on demand are expected to greatly expand the therapeutic treatment possibilities of this class of compounds.

Initial success with PROTACs against key disease targets [14,18,19,22–24,26,28,33–36] has spurred rapid growth and significant investment in targeted degradation research across all areas of science; academia, biotechnology, and pharmaceutical drug discovery. Chemical PROTAC libraries and degradation tool compounds are growing in number, discovery of E3 ligase recruiters beyond those of VHL and CRBN is underway [7,20], and technologies to deconvolute the intricate biological processes depicted in Fig. 1 required for PROTAC activity are being developed [37]. Together these efforts are expanding the field and creating space for faster discovery, yet they do not come without their challenges. A significant bottleneck in the process of drug discovery has been cellular characterization of PROTAC compounds to efficiently and rapidly rank-order compounds based upon degradation rate, the achievable extent of degradation (Dmax), and the time frame of efficacy. Here we focus on the key steps in the degradation pathway and discuss the different technology approaches for deciphering PROTAC cellular mechanism of action and the applicability of each approach for drug discovery efforts.

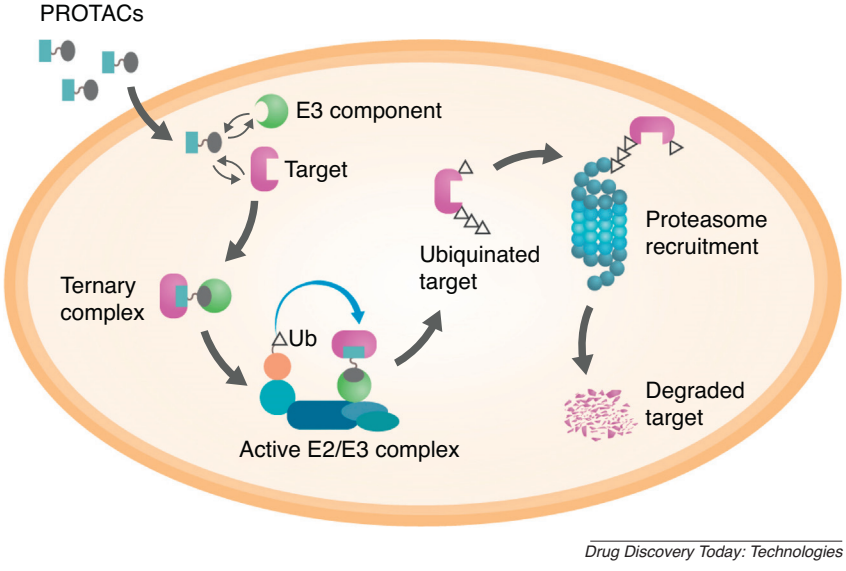

Fig. 1. Schematic of the targeted degradation pathway mediated by PROTAC. Heterobiofunctional PROTAC compounds, permeate the cell where they can interact in several states; as binary complexes with the degradation target, termed Target, or to an E3 ligase or protein within an E3 ligase complex, termed E3 component, and in a Target:PROTAC:E3 recruiter ternary complex. For the target to be ubiquitinated, designated by the Ub triangles, the ternary complex must be formed as part of a larger Active E2/E3 ligase complex which can include many other accessory proteins required for activity depending on the E3 component chosen for the PROTAC development. The ubiquitinated target is then trafficked to the proteasome where it binds and is degraded.

Measuring target protein levels

As the goal for any PROTAC compound is to degrade a specific target or class of targets, the most traditional and longstanding approach to study intracellular function has been to treat cells with the degradation compound, then measure target protein levels. Historically this was done most often using antibodies and western blotting [14,16–19,23,34,38], but more recently global mass spectrometry has been employed to look at not only specific target loss, but any potential off-target effects [18,39] (Table 1). Both approaches detect endogenous proteins and their response to the degradation compound but require lysis and are difficult to configure for high-throughput analysis of larger compound libraries. The success for western blot is also very dependent on the availability of a high-quality and specific antibody, which can significantly impact dynamic range for accurate quantitation of protein levels. Mass spectrometry on the other hand is highly quantitative, but compared to western blotting, is more labor intensive, adds significant cost, and requires technical expertise for analysis of data.

Given the dynamic nature of the degradation process, the end-point lytic assays mentioned above must be conducted at multiple time points to establish degradation responses over time. This is both tedious to perform and granular in detail when trying to determine a multi-phasic degradation profile consisting of the initial degradation to Dmax, the time at Dmax, and then recovery. In attempts to monitor protein degradation in real time, experiments were performed using ectopic expression of target proteins fused to fluorescent proteins; GFP, EGFP, or mCherry, coupled with confocal imaging following PROTAC treatment [15,19,38]. These experiments elegantly provided the first images of target protein loss at different times and PROTAC concentrations, allowing for real-time assessment of degradation within the cell [18,19,38]. For PROTACs with high activity, the initial loss could often be detected within 30–90 min, indicating rapid and efficient ternary complex formation, ubiquitination, and degradation once the compound is within the cells [18,19,38].

The use of GFP or any other fluorescent protein to monitor degradation in real time provides significant advantages compared to western blotting, however the examples published have relied upon ectopic expression which might not reflect the true degradation and regulation of the endogenous target [37]. Of greatest concern is that fusion proteins typically utilize constitutive promoters lacking native epigenetic and transcriptional regulation and resulting in constant production of the target protein inside the cell. To address this challenge yet preserve the ability to monitor endogenous protein levels in live cells, genome editing using CRISPR/Cas9 technology [40,41] has become an attractive approach to tag endogenous proteins to study native biology. Recently, CRISPR/Cas9 was employed to endogenously tag PROTAC targets with HiBiT, an 11 amino acid peptide that results in bright luminescence following spontaneous complementa-

Table 1. Cellular Assays for characterization of PROTAC activity and degradation. Listed in the table represent different cellular assays for key steps in PROTAC development and screening. Indicated are the different applications and related technologies to study these applications. Listed in the rows are the features and/or requirements of each technology; live cell, lytic, endogenous protein, ectopic expression, fusion tag, antibody, and throughput screening capabilities (Low, Medium, and High). The following acronyms are defined as: cellular thermal shift assay (CETSA), NanoLuc bioluminescent resonance energy transfer (NanoBRET), target engagement (TE), and protein:protein interaction (PPI).

	Live Cell	*Lytic*	*Endogenous*	*Ectopic*	*Fusion tag*	*Antibody*	*Throughput*
Protein Degradation							
Western blot		✓	✓			✓	Low-Med.
Fluorescent fusion tags	✓			✓	✓		High
HiBiT endogenous tagging	✓		✓		✓		High
PROTAC binding and permeability							
CETSA		✓	✓			✓	Low-Med.
NanoBRET TE	✓			✓	✓		High
Ternary Complex Formation							
NanoBRET PPI	✓		✓	✓	✓		High
Ubiquitination							
Mass spectrometry		✓	✓				Low-Med.
NanoBRET Ub Live	✓		✓	✓	✓		High
NanoBRET Ub Lytic		✓	✓	✓	✓	✓	Low-Med.
Degradation Phenotype							
HaloPROTAC		✓		✓	✓	✓	Low-Med.
dTAG	✓	✓	✓	✓	✓	✓	Low-Med.

tion with is cognate, LgBiT protein [42]. Using this system, it was demonstrated in live cells that continual measurement of luminescence over 24 h could be performed immediately after cellular PROTAC treatment, resulting in distinct target-specific degradation and recovery profiles. In addition the system allows for quantitation of key parameters including degradation rate, DC_{50} value, and time at Dmax [37]. Calculation of these parameters allowed for accurate rank ordering of compounds in terms of rate and potency and now provide a means for high-throughput, plate-based cellular screening on a luminometer [37].

The HiBiT endogenous study also directly compared degradation of the same target expressed either as an endogenous or ectopically tagged fusion [37]. As might have been predicted, the transiently transfected fusion protein showed slower degradation rates, right shifted DC_{50}s, and the maximal degraded fraction was significantly reduced [37]. Additionally, the recovery profiles did not correlate with those of the endogenous protein [37]. Therefore, fluorescent or luminescent expression systems reliant upon ectopic expression which do not reflect endogenous target protein homeostasis should be cautiously applied to quantitative degradation studies.

Assessing PROTAC permeability and binding

All the approaches discussed to measure target protein levels after PROTAC treatment are reliant on the compound being cell permeable to some extent. These compounds however consist of two small molecules and a linker between them [2,7,13], challenging the classic notion of a small molecule. Compared to their parental inhibitors or binding ligands, they often have higher molecular weight, many more combinations of charge, rigidity, and/or hydrophobicity, and could show impaired permeability in the early stages of development. The reduced permeability may be due to these changes or impacted by abundant plasma protein binding properties. Determination of cellular permeability and binding to either the target protein or the E3 ligase component in the cell therefore is one of the more critical early steps in evaluation of new PROTAC compounds (Fig. 1).

A technology used for cell-based small molecule binding studies and established in drug discovery pipelines is the cellular thermal shift assay, CETSA [43–45] (Table 1). CETSA relies upon compound binding to target to stabilize the protein during heating, resulting in an observed shift in the thermal melt curve as compared to the protein without compound present [43–45]. CETSA has been demonstrated to monitor PROTAC engagement [46], yielding information on the binding of compounds to the separate binary complexes, target:PROTAC and E3:PROTAC (Fig. 1). The process of CETSA requires lysis of cells, heating, and antibodies for detection of the stabilized protein [43–45], therefore high-quality antibodies must be available (Table 1). While CETSA offers a label-free approach for interrogating target:PROTAC and target:E3 interactions, enabling mechanistic studies in native cellular systems (Table 1), engagement results are not direct measurements of target affinity, nor fractional occupancy [43–45]. Furthermore, CETSA exploits the dynamics of protein stability. Thus, use of CETSA for assessing engagement of target:PROTAC interactions may be complicated by the opposing effects of PROTAC-mediated target degradation.

A complementary live cell, kinetic technology showing significant adoption for small molecule drug discovery is a high-throughput NanoLuc bioluminescent energy transfer technology (NanoBRET) target engagement assay [47] (Table 1). NanoBRET [48], similar to BRET [49], is a proximity assay, but it differs in the use of NanoLuc luciferase [50] as a luminescent energy donor and a cell-permeable fluorescent small molecule tracer, as an energy acceptor [47] (Table 1). Upon binding of the NanoLuc fusion protein to the tracer, energy transfer occurs, yielding a NanoBRET signal, which is ratiometric and functions in real-time [47]. The ratiometric nature of the assay gates out potential interferences due to target turnover, which may offer a significant advantage over those techniques measuring target stability as an engagement proxy [37]. Furthermore, it is less sensitive to expression level, and can be performed with either endogenous or ectopically expressed NanoLuc or HiBiT/NanoBiT luciferases. NanoBRET target engagement can be performed to rank order compounds, quantify binding affinity and occupancy, and measure compound residence time [37,47,51,52]. This approach has been successfully applied to quantifying both target:PROTAC and E3:PROTAC interactions, and by running the assay in both live and lytic assay format can yield important information on PROTAC permeability. Even highly active PROTAC compounds (DC_{50} values in nM range) were shown to have reduced permeability as compared to their parental compounds, irrespective of pairing with either VHL or CRBN chemical handles [37]. While somewhat unsurprising, this information on permeability can provide further context for observed PROTAC activities at presumably much lower concentrations within cells and serve as a critical point of optimization for rational PROTAC design.

Ternary complex formation

While the above technologies yield insights into the permeability and cellular binding of PROTACs to either the target protein or E3 ligase component, the formation of the target:PROTAC:E3 ternary complex is a necessary precursor for facilitating downstream ubiquitination (Fig. 1). Since this is a small-molecule tethered complex, any small or subtle changes to linker composition in the PROTAC could significantly favor, destabilize, or could prevent ternary complex formation [12,13,15,18,19]. Also playing a role in ternary complex stability is the induced electrostatic surface interactions the PROTAC will create between the target and E3 ligase

component, which do not natively interact in the absence of the PROTAC. Recent structural studies have shed light into this phenomenon showing both examples of induced positive cooperativity with BRD4-MZ1-VHL [12], and non-cooperative binding between BRD4-dBET23-CRBN [15]. As the PROTAC will bind both target protein and E3 components in the context of much larger complexes inside the cell, understanding the formation efficiency and longevity of ternary complexes will be valuable in guiding structure-activity relationship (SAR) development of PROTAC compounds for improving degradation.

Fundamentally, the ternary complex is a chemically induced protein:protein interaction (PPI), and there are numerous technologies for studying protein:protein interactions (PPIs) inside cells, including protein complementation, energy transfer (FRET or BRET), co-immunoprecipitations, and affinity tag pulldowns [53]. The application of these technologies to monitoring a PROTAC-induced ternary complex has been challenging given that (a) the complex is highly transient (b) its formation results in immediate loss of the target protein being measured, and (c) most PPI technology approaches will show artefacts if one component is actively being degraded. As mentioned in the discussion of NanoBRET target engagement, the ratiometric nature of BRET in general is refractory to target turnover within the range of detection [48,49], allowing for loss of the donor without impacting readout of complex formation [37]. Taking advantage of this aspect, NanoBRET PPI assays were configured in a similar format as target engagement, utilizing a luminescent energy donor fusion protein, but a HaloTag fusion protein as a fluorescent energy acceptor [37,48,54] (Table 1). NanoBRET PPI experiments allow for simultaneous measurement of degradation in the luminescence channel, and ternary complex formation in the fluorescence channel [37] (Table 1). Furthermore, to study the kinetics and stability of ternary complexes, NanoBRET PPI experiments performed in the presence of the proteasomal inhibitor, MG132, can potentially enhance the signal window of ternary complex and give the ability to study the prolonged stability of the complex without concern of loss of target [37].

Ubiquitination

While formation of the ternary complex is important, perhaps the defining step of PROTAC success is recruitment of the target protein into an active E3 ligase complex for ubiquitination (Fig. 1). This requires ternary complex formation to exist within the context of the larger E3 complex consisting of multiple protein components, as well as proper positioning of the target within the complex for ubiquitination (Fig. 1). Ubiquitination of proteins as a post-translational modification has been studied for decades and can readily be assayed by using western blots or mass spectrometry [55] (Table 1). For PROTAC activity studies, cells can be treated with compound, lysed, and then the target of interest can be enriched by affinity tag pull-downs or co-immunoprecipitations [53]. Detection of ubiquitination, including linkage type, could then be performed with antibodies or by mass spectrometry [55] (Table 1). These approaches can assess endogenous ubiquitination levels on endogenous targets (Table 1), but for reasons presented earlier are difficult to transition to high-throughput screening platforms and laborious if studying multiple time points.

To characterize ubiquitination kinetics in live cells, NanoBRET technology was again deployed using HiBiT endogenously tagged proteins complexed with LgBiT as energy donors and ectopically expressed HaloTag-Ubiquitin as an energy acceptor [37] (Table 1). Similar to ternary complex assays, real-time kinetics of target ubiquitination was readily visualized following treatment with PROTACs [37]. This technology was further adapted to monitor ubiquitination of endogenously tagged HiBiT fusions in cellular lysates utilizing primary anti-ubiquitin and a fluorescent Alexa594-conjugated secondary antibody serving as the energy acceptor instead of HaloTag-Ubiquitin fusion [37] (Table 1). While this assay necessitates use in lytic format, strong agreement in ubiquitination pattern was found, both in terms of the time frame and relative extent compared to the kinetic assay with an ectopically expressed HaloTag-Ubiquitin fusion, and both approaches in the study were linearly correlated with the measured degradation rate [37].

Characterizing protein degradation phenotype

The benefits of targeted degradation related to inhibition have been previously shown [18,19,23–30], but as discussed, the conversion of inhibitors to PROTAC degraders requires significant investment in terms of chemistry synthesis and number of compounds tested to find something permeable and functional. The efforts and time investment would be even greater if the goal were to develop PROTACs for protein targets with unknown binding ligands, like those included in the list of ''undruggable'' proteins. Prior to making these expensive commitments which are not guaranteed to succeed, it would be very beneficial to test if the target of interest, degraded in the same temporal fashion as a PROTAC would do, yielded the desired phenotypic response or outcome. Having this ability would allow for informed decision making about which targets should be chosen as PROTAC targets and whether degradation will have advantages as compared to inhibition.

To this end and to provide a general and broad approach for targeting proteins for degradation, two technologies have independently been developed utilizing different fusion proteins, HaloTag [38,54] and $FKBP12^{F36V}$ [56], which can be degraded by their respective PROTAC degraders, HaloPROTAC [38] and dTAG [56] (Table 1). The HaloPROTAC systems utilizes a chloroalkane handle, which binds covalently to

HaloTag [54], and a VHL recruiter for the E3 ligase handle [38]. HaloPROTAC has been shown to degrade a variety of ectopically expressed HaloTag fusions inside cells and have used antibodies to confirm loss of the target fusion protein [38] (Table 1). More recently, other groups have shown success using CRISPR/Cas9 to endogenously tag proteins with HaloTag and study their localization using imaging [57]. Combining the advancement of HaloTag CRISPR insertion with the HaloPROTAC provides a platform for readily studying phenotype after loss by degradation of any endogenous HaloTag fusion protein, and CRISPR clones could be readily enriched or imaged using the HaloTag fluorescent ligands [54,57].

The dTAG degraders, similar in principle, consist of a non-covalent inhibitor which specifically recognizes the mutant form of $FKBP12^{F36V}$ and not the wild-type FKBP12, and then utilizes a CRBN recruiter for the E3 handle [56]. Several targets have been have shown to have very fast degradation kinetics, within 30–90 min, both ectopically and endogenously tagged with the $FKBP12^{F36V}$ fusion, suggesting this system might be excellent for studying phenotype in situations that require fast clearance of a target from the cell [56]. Like HaloPROTAC, loss of $FKBP12^{F36V}$ fusion proteins after dTAG treatment require cellular lysis and confirmed using western blots and antibodies [56] (Table 1). As it is highly desirable to monitor protein levels in live cells, the pairing of these degradation tags with either a luminescent detection system, such as HiBiT [42] or a fluorescent tag, which has been demonstrated using GFP with $FKBP12^{F36V}$ fusion [58], would facilitate kinetic degradation studies inside cells for both technologies. Adoption of HaloPROTAC and dTAG systems will likely need optimization for any given target to obtain maximal degradation, and some proteins, despite optimization, might show very limited degradation for a variety of reasons. These approaches however are excellent strategies and viable solutions for understanding the phenotypic consequences of degrading a target via a PROTAC.

Discussion

Therapeutic targeting of proteins for degradation using PROTACs is an area of drug discovery in its early days and is expected to exponentially grow in the coming years both in terms of successful targets and number of degradation compounds. To facilitate and enable this growth, rapid profiling and triaging of PROTACs for activity is required. A significant number of in vitro, biochemical, and biophysical assays for initial analysis of compounds is currently available, but existing functional and mechanistic assays are fewer in number. Monitoring ternary complex formation or ubiquitination of targets, particularly in formats amenable for high-throughput screening, has proven to be very challenging in a biochemical setting due to difficulties in reconstituting the complexes and/or networks required for function. In cellular studies, the use of western blot analysis to qualify PROTACs as active or inactive often leads to more questions than answers, especially when no degradation is observed. In these cases, one is left asking at what point in the pathway the failure occurred. Was the PROTAC impermeable, incapable of binding targets or forming the ternary complex formation, or did the ternary complex form, but not result in productive ubiquitination? If these questions cannot be answered, decision-making on further PROTAC chemical optimization or rational design of new compounds will be hindered significantly. Additionally, many compounds that have the potential to be highly efficacious and potent degraders with perhaps minor chemical modification will be missed in the screening process.

As compared to other areas of drug discovery, the list of available cellular technologies for PROTAC characterization is short at this time. This is in part due to the newness of this field but should also be credited to the complexity of the biology and the many steps which must be monitored to understand the larger functional picture. To study degradation and obtain quantitative metrics, it has been shown endogenous expression is necessary [37]. Traditionally this has meant use of antibodies and cellular lysates, but with the advances of CRISPR/Cas9 technologies [40,41], endogenous tagging of proteins allows for new detection methods of proteins in live cells without disrupting their endogenous expression or regulation. Examples were discussed here with HiBiT, which is small in size and has high endogenous insertion efficiency [42]. More recent efforts however have demonstrated endogenous insertion success with larger fusion tags, including GFP [59] and HaloTag [57], so it is expected that the list of tools to study endogenously labelled proteins and their degradation will expand as methods improve. Directly related to this is the further use of fusion proteins, HaloTag [54] and dTAG [56], which can themselves be degraded by their respective PROTACs [38,56] for phenotypic studies and determination of which specific targets are best for therapeutic PROTAC development.

The historical and current technologies discussed in this review, as well as future possibilities of next generation cellular assays will be essential for understanding the important processes for PROTAC-mediated degradation and will enable more high-throughput screening approaches. They will also provide the ability to rank order compounds for key cellular parameters, such as degradation rate, efficacy, and potency, in a highly precise and quantitative fashion, bridging the gap between chemical/biochemical analysis to mechanism of action of PROTACs in the cellular context.

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