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Protein degradation for drug discovery

Targeted protein degradation mechanisms

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Targeted protein degradation mediated by small molecule degraders represents an exciting new therapeutic opportunity to eliminate disease-causing proteins. These molecules recruit E3 ubiquitin ligases to the protein of interest and mediate its ubiquitination and subsequent proteolysis by the proteasome. Significant advancements have been made in the discovery and development of clinically relevant degraders. In this review we will focus on the recent progress in understanding ternary complex formation and structures, ubiquitination, and other critical factors that govern the efficiency of degraders both *in vitro* and *in vivo*. With deeper knowledges of these areas, the field is building guiding principles to reduce the level of empiricism and to identify therapeutically relevant degraders more rationally and efficiently.

Introduction

Small molecules that direct the ubiquitin proteasome system (UPS) to degrade disease-causing proteins have recently grown into a novel and exciting therapeutic modality. These heterobifunctional, or in some cases, homobifunctional molecules [1,2], have been called PROTACs (PROteolysis TArgeting Chimeras), SNIPERs (Specific and Nongenetic IAP-dependent Protein ERasers), or DEGRONIMIDs [3,4]. In this review, we will refer to these molecules as degraders. These degraders can target proteins that are intractable for other modalities, including proteins that either lack a catalytic function or have both catalytic and non-catalytic domains involved in disease biology. In addition, because a degrader molecule binds to a protein to induce its degradation and can then be released to induce more protein degradation in a catalytic fashion, the degrader can target proteins that are not adequately modulated by traditional occupancy-based inhibitors. Understanding the factors listed below will be critical to evolve the development of effective and safe degraders into a rational, effective, and capital-efficient drug discovery process.

1. What is the ligandable proteome?
2. What is the right E3 ligase for the target protein of interest?
3. How can we achieve degradation that is cell-type or tissue-type specific?
4. How do we achieve the maximal efficiency of ternary complex formation?

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5. How do we optimize for the maximal rate of ubiquitination?
6. How do the above parameters correlate to potent and specific target degradation?
7. What is the relationship between the rate of degrader-mediated protein degradation, the rate of re-synthesis, and the effect on translatable biomarkers in cells and more importantly, in *in vivo* systems across different cell types?

Recently, significant progress has been made in understanding the mechanisms of targeted protein degradation, particularly in understanding the kinetics and thermodynamics of ternary complex formation, the structural features of this complex, and other factors that contribute to efficient degradation. This review will focus on how these advancements impact efforts on discovery and development of degraders as a novel therapeutic modality.

Understanding mechanisms of targeted protein degradation

Ternary complex formation

There have been several mechanistic studies focused on elucidating the mechanisms of targeted protein degradation by degraders [2,5–19]. After penetrating a cell membrane, degraders bind the target protein and the selected E3 ligase to trigger subsequent E3-mediated poly-ubiquitination followed by proteasomal degradation (Fig. 1). Spatial proximity of the E3 ligase to the target protein is critical to the initiation of a series of biochemical reactions carried out by the UPS. A necessary, but not sufficient step in the cascade is the formation of a ternary complex among the target protein, the E3 ligase, and the degrader. Ternary complex formation can be confirmed by co-immunoprecipitation [16,19] and size-exclusion chromatography (SEC) [2]. To quantitatively monitor ternary complex formation, several assay formats have been developed. Surface plasmon resonance (SPR) [17,20] and isothermal titration calorimetry (ITC) [2,8,14] have been reported with sufficient sensitivity and accuracy for measuring K_d's, while proximity-based AlphaScreen/AlphaLISA [2,5,8,21–23] and TR-FRET [12,17] methods are used more widely due to their higher throughput. While these assays have been generally adopted to measure the thermodynamics of ternary complex formation, they are not without limitations. For example, AlphaScreen/AlphaLISA and TR-FRET methods rely on recombinant proteins modified with artificial tags to generate fluorescent signals [12,17,23]. To maximize sensitivity of the assay, a particular concentration of protein is selected [8,17,23] (typically between 10 nM and up to μM depending on the intensity of the signal and the K_d value), which may not reflect the endogenous protein level in the cell. Other potential limitations of these assays are the lack of temporal and spatial resolution of the binding events.

Ternary complex formation is influenced by several key parameters, including the concentration of the target and

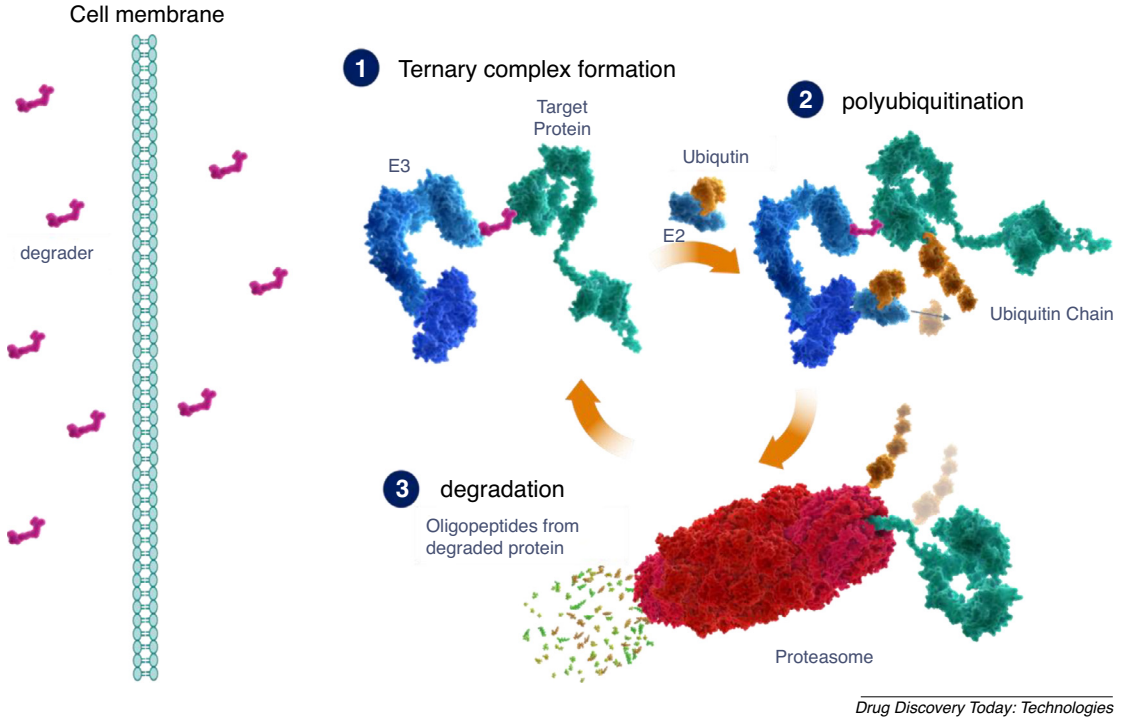

Fig. 1. Schematic representation of the catalytic cycle of the targeted protein degradation. A degrader, depicted in purple, mediates ternary complex formation between the E3 ligase and the target protein, which then leads to poly-ubiquitination of the target protein, and its subsequent degradation by the proteasome. The released degrader becomes available to repeat this cycle.

E3 ligase, the intracellular concentration of the degrader, the affinity that the degrader has for each protein, and protein-protein interactions between the target protein and the recruited E3 ligase (defined as positive or negative cooperativity) (Fig. 2). A typical binding isotherm for these three interacting partners to form a ternary complex has a bell shape, where the maximal level of the ternary complex is reached with the increasing concentration of the degrader followed by an inhibitory phenomenon due to dominant binary interactions, which is often referred to as the ''hook effect''. Therefore, unlike a binary binding isotherm that eventually reaches a plateau, there is an optimal concentration range for maximal ternary complex formation. Douglass et al. [24] developed a more comprehensive mathematic description of ternary complex formation, in which the overall shape of the curve is influenced by the target protein and E3 ligase concentrations, and the affinities of the binary complexes.

Degrader-mediated cooperativity of binding is another parameter that impacts the thermodynamics of ternary complex formation, and as a result, the optimal concentration range for the degrader. The cooperativity, which reflects the energy difference before and after ternary complex formation, is represented by a scaling factor termed as α, defined mathematically as K_d(binary)/K_d(ternary). If the degrader facilitates novel interactions between the E3 ligase and target protein, the affinity of the ternary complex will be higher than that of the binary interactions between the degrader and each protein, and thus, α is > 1. If the degrader does not stabilize interactions between the E3 ligase and the target protein, the binding affinity in the ternary complex remains the same as in the binary complexes and as a result, α is equal to 1. If ternary complex formation is disfavored, which may be due to steric clashes or electrostatic repulsions when the two proteins are forced into proximity by a degrader, a weaker binding affinity will be observed for the ternary complex compared to the binary complex and α will be less than 1. ITC [2,8,14], TR-FRET [12,17], and SPR [20] have all been utilized for determining α, either by direct measurement (ITC and SPR), or by fitting experimental data to a mathematic model (TR-FRET). However, it is not clear whether there is a positive correlation between the cooperativity and the potency of degrader-mediated protein degradation. For example, one study reported [12] a series of CRBN-BRD4 degraders that showed negative cooperativity ($\alpha < 1$) yet led to effective degradation. Among them, dBET6 (Fig. 3) has an $\alpha = 0.6$ for $BRD4_{BD1}$ (Bromodomain 1), and $\alpha = 0.2$ for $BRD4_{BD2}$ (Bromodomain 2) (Table 1), which indicates K_d of dBET6 binding in the ternary complex is 2-fold to 5-fold weaker than the K_d measured for the binary complex. Nonetheless, dBET6 mediates potent cellular BRD4 degradation with $DC_{50} = 10$ nM [12]. Conversely, MZ1, a von Hippel Lindau (VHL)-based BRD4 degrader (Fig. 3) is reported [8] to have a strong positive cooperativity ($\alpha = 2.3$ for $BRD4_{BD1}$, 17.6 for $BRD4_{BD2}$, Table 1) by Gadd et al. Although MZ1 and dBET6 are not tested in the same assay, their cellular degradation potencies are estimated to be similar using dBET1 as a reference (Table 1) [9,12]. Therefore, high degradation potency can be achieved, even for degraders with negative cooperativity, suggesting that other factors, such as permeability and ubiquitination, could also contribute to the cellular potency [7]. Recently, the Wang lab reported [25] a series of BRD4 degraders with DC_{50} in the 1–100 picomolar range. Profiling of these potent degraders in ternary complex assays to obtain cooperativity information could help elucidate the role of cooperativity in improving degradation efficiency.

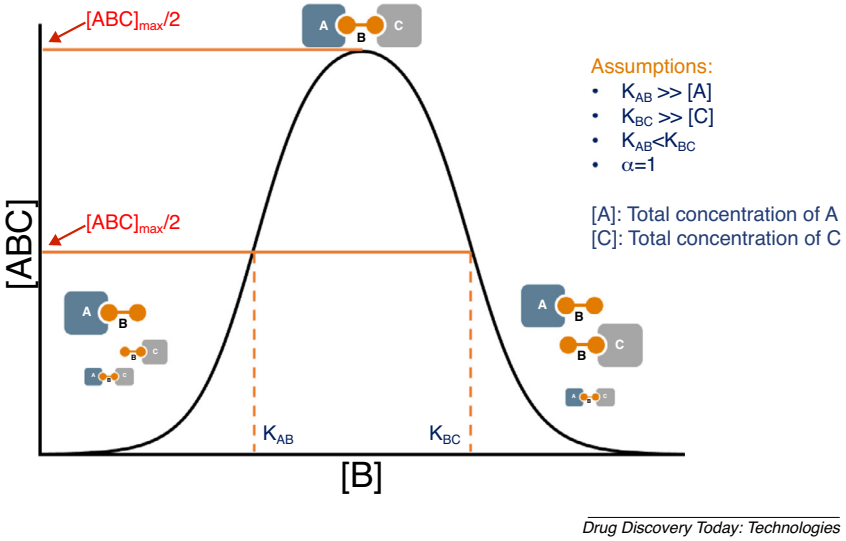

Fig. 2. Bell-shaped binding isotherm of ternary complex formation in response to the degrader concentrations.

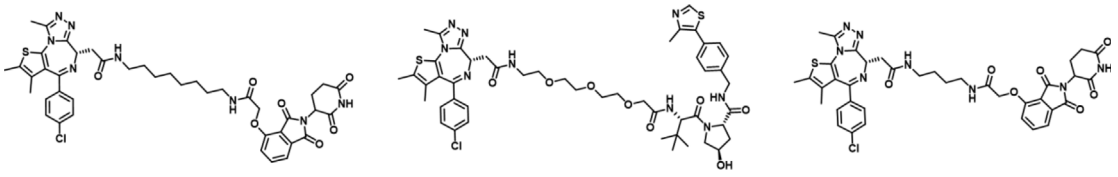

Fig. 3. Structures of dBET6, MZ1 and dBET1.

Table 1. comparison of cooperativity and degradation potency of dBET6, dBET1 and MZ1.

Compound	DC_{50}	α for $BRD4_{BD1}$	α for $BRD4_{BD2}$
dBET6	10 nM [12]	0.6 [12]	0.2 [12]
dBET1	500 nM [12]	0.2 [12]	0.1 [12]
	34 nM [9]		
MZ1	2 nM [9]	2.3 [8]	17.6 [8]

Ternary complex structures

Structural biology has proven valuable in understanding how degraders influence the ternary complex structure and the interactions between the E3 ligase and the target protein. In 2017, Gadd et al. reported [8] the first ternary complex crystal structure of VHL-Elongin BC (VCB), $BRD4_{BD2,}$ and a degrader (MZ1). More recently, Nowak et al. [12] reported several ternary complex structures of CRBN-DDB1, $BRD4_{BD1}$ with a set of degraders. Structural information derived from these sets of ternary complexes illustrates that, while binary binding interactions of degraders within each protein binding pocket remain unchanged (Fig. 4), the interaction patterns are different at the protein-protein interface [12]. In the ternary complex structure of {VCB-MZ1-$BRD4_{BD2}$} [8], several hydrogen bonds, as well as hydrophobic and electrostatic interactions between VHL and $BRD4_{BD2,}$ have been observed. Furthermore, back-folding of the linker provides additional contacts with both the E3 ligase and the target protein. In total, these interactions induce formation of a buried surface area of 1933 $Å^2$, more than three times as large as that observed in the ternary complex of {CRBN-dBET-$BRD4_{BD1}$} (~550 $Å^2$) [8]. Conversely, in the ternary complex structure of {CRBN-dBET-BRD_{BD1}}, no obvious hydrogen bonds were detected by computational calculations. Single amino acid point mutagenesis studies indicate that each dBET degrader has its own interaction signature, likely due to the plastic

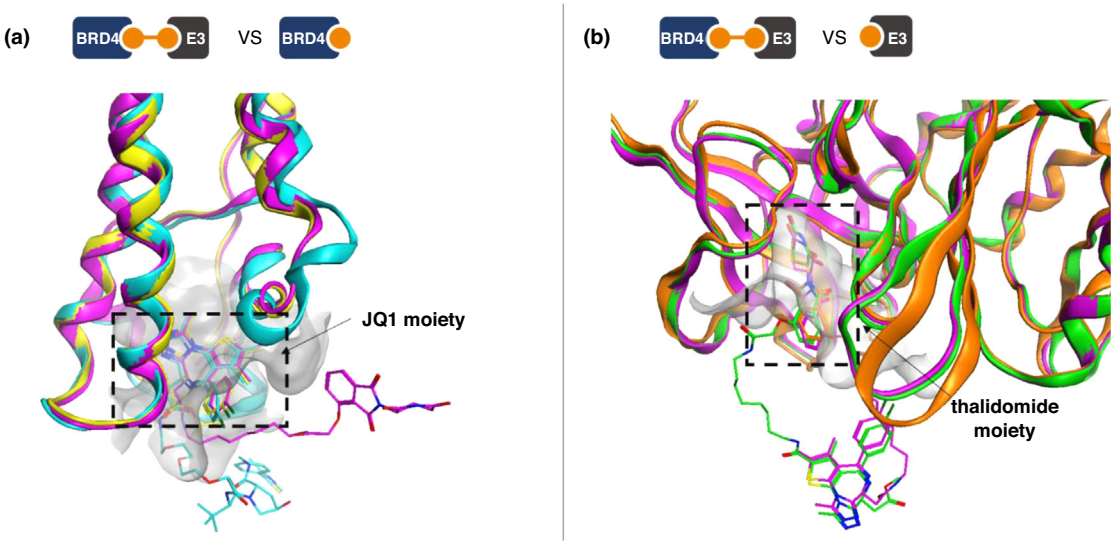

Fig. 4. (A) Overlay of the $BRD4_{BD1}$ binding pockets in the ternary complex and in the binary complex; (B) Overlay of the CRBN binding pockets in the ternary complex and in the binary complex.

nature of the interaction surface [12]. These results also point to the impact of the linker length and the linking vector on the interactions between the E3 ligase and the target protein. While obtaining ternary complex X-ray structures remains challenging, especially with CRBN/DDB1, additional methods such as cryo-EM will also be able to provide valuable structural insight into ternary complex formation. The current and future structural studies will help rationalize the design of degraders that increase cooperativity by promoting productive target protein-E3 ligase interactions and accelerate lead optimization in drug discovery. Importantly, in the absence of structural biology data, computational effort to model protein-protein interaction interfaces is likely to have a significant impact on the design of degraders that induce productive ternary complex interactions [12,13,15,17,23,26].

Ubiquitination

Poly-ubiquitination of the target protein following the ternary complex formation is essential for marking the target protein for degradation by the proteasome. Ubiquitination studies at Kymera have shown a strong correlation between efficient ternary complex formation and ubiquitination (Chen *et al.,* unpublished). Mass spectrometry as a label-free system [27,28], should allow mapping of the lysine ubiquitination with high resolution [27–30]. The target ubiquitination mediated by the degrader and the ternary complex formation can be mapped at single amino acid resolution by identifying di-glycine modified peptides using mass spectrometry [8,31]. While K48 is often considered as the lysine ubiquitination that favors protein degradation, there is a growing list of lysine position modifications (K6, K11, K27, K29, K33, K36), several of which appear to also mediate the target protein to the proteasome [29,32–34]. These types of mapping studies will potentially reveal 'guidelines' for which additional types of ubiquitination events are likely to lead to the targeted protein degradation.

Kinetics of ternary complex formation, protein degradation and re-synthesis

The last step in the catalytic cycle of the targeted protein degradation is the actual degradation of the protein by the proteasome. The timing of degradation has been part of the characterization of degrader effects. Some degraders have been shown to mediate rapid degradation of the target proteins, such as MetAP2 [10] and RIPK2 [19]. MetAP2 is almost completely degraded within 30 min, and RIPK2 level is reduced by half within one hour of degrader treatment in THP-1 cells [10,19].

The kinetics and dose-dependent degradation induced by degraders is frequently monitored by Western blot. However, Western blot-based quantitation has many limitations. It requires specific antibodies, provides only semi-quantitative protein measurements, may have a limited dynamic range for proteins of lower abundance, and necessitates collection of endpoint samples rather than allowing monitoring changes of protein levels in real time. Other methods, such as capillary electrophoretic separation [35] and mass spectrometry, have improved the quantitation and dynamic range. However, like Western blot, these methods do not allow monitoring of changes in protein levels in real time.

Newer technologies exploit split enzyme complementation systems to quantify tagged target proteins in cells. Such systems include β-galactosidase tagged BRD4 [36], which relies on exogenously expressed proteins and detection of the target in cell lysates [5]. In a recent paper, Riching et al. [9] describe the use of a split luciferase system to circumvent some of these limitations. The protein of interest (POI) is tagged with an 11-amino acid peptide, called ''HiBiT'', via gene editing. The other portion of luciferase, called ''Large BiT'' can be co-expressed in the same cell, and a stable substrate can then be added to quantify the amount of luciferase, which corresponds to the amount of POI (Fig. 5) [9]. The ability to detect the luminescent signals generated by luciferase abolishes the need for an antibody specific for the target protein. Furthermore, the luminescent signal can be quantitated over a large dynamic range and can be monitored in cells in real time [9]. In this manner, the rate of degradation of HiBiT-tagged bromodomain proteins induced by the degraders, dBET1 and MZ1, is determined. The kinetic measurements reveal nuances in action of these degraders for the different BRD proteins. For example, the dose-response curves of degradation rates show that MZ1 mediates equal rates of degradation for BRD4 and BRD2, however the dose-response curves of maximum degradation show that MZ1 selectively degrades BRD4 over BRD3 and BRD2. This system

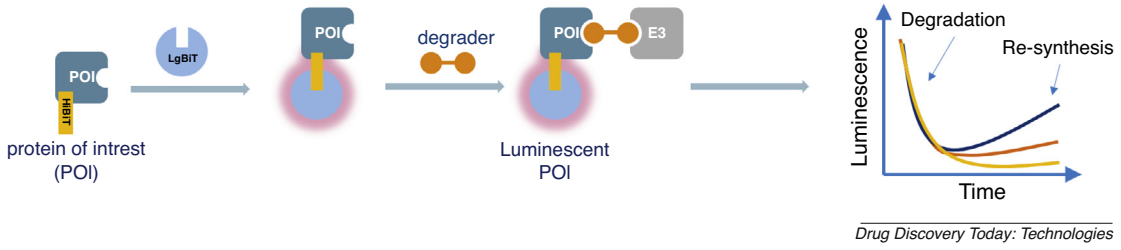

Fig. 5. Scheme of HiBiT system for monitoring targeted protein degradation. The protein of interest (POI) is tagged with a small fragment of luciferase, referred to as ``HiBiT''. Co-expression of the larger fragment of luciferase (``LgBiT'') reconstitutes a functional luciferase and allows the quantitation of POI.

also illustrates that MZ1 sustains BRD4 degradation for 20 h, whereas the effect of dBET1 appears to diminish at 10 h. The recovery of the response is likely due to a combination of re-synthesis of the protein and metabolism of the degrader in the cell. The question then remains as to which of these parameters, rate of degradation, maximum degradation, time to maximum degradation, and durability of the response, will more accurately predict the duration of a degrader's biologic effect.

Riching et al. [9] also extended the HiBiT-LargeBiT system to investigate ternary complex formation and ubiquitination kinetics inside the cell, by transfecting Halo-tagged-E3 ligase or Halo-tagged ubiquitin, respectively (NanoBRET). Degrader-mediated interactions between the bromodomain protein and the E3 ligase, or ensuing ubiquitination of the bromodomain protein, is indicated by a BRET signal. Interestingly, this assay showed that there is a substantial difference between the VHL-based MZ1 degrader- and CRBN-based dBET1 degrader-mediated kinetics of ternary complex formation and duration. For example, the dBET1-containing ternary complex forms more rapidly than the MZ1-containing ternary complex does, but the complex is not maintained over time. In contrast, an MZ1-containing complex forms more slowly, but continues to increase over time. It would have been interesting to observe the durability of the complex over a longer period of time, since MZ1 mediated a longer duration of BRD4 degradation than BRD2 over a time course of 22 h. On the other hand, decreasing of the CRBN-containing ternary complex in the cell could be potentially explained by the intrinsic chemical instability of the IMiD moiety in the degrader. Overall the more stable VHL-based ternary complex is shown to result in higher cellular degradation potency and longer duration of BRD4 degradation compared to the CRBN-based ternary complex. The influence of stability of the ternary complex on cellular degradation efficiency on other proteins will require testing of additional combinations of the E3 ligases, the degraders, and the target proteins.

The ubiquitin NanoBRET assay shows that MZ1 and dBET1 induce substantially different levels of total complex formation. A detailed time course study also shows that both degraders induced ubiquitination and reached a steady state within 30 min of degrader treatment. Although HiBiT is one of the smallest tags, compared to EGFP [12] and dTAG [5], one potential drawback of any tagged system is that tags can alter the endogenous protein expression, location, or function, and therefore studies must be done to ensure the tagged protein behaves as the native version.

Another parameter that can impact the degrader effect is the target protein re-synthesis rate. In cellular studies, degrader treatment to achieve target protein removal, followed by washing out the degrader, is one method used to follow the return of the target protein levels. Of note is that the protein half-life does not always appear to correlate with the re-synthesis time indicated by these wash-out experiments. In the case of RIPK2, the reported protein half-life is $\sim$60 h [37], while the wash out study showed that RIPK2 protein returned to 50% of the original levels by 8 h and to original levels by 24 h post-wash out, albeit that protein half-life and the wash out experiment were not conducted in the same cell line [19]. Nonetheless, this discrepancy may not be surprising, as protein half-life is usually measured at a steady state, when protein removal is in equilibrium with protein synthesis. In the case of degraders that accelerate protein removal, the system might then be signaled to increase the rate of synthesis, as part of a potential feedback loop. The dynamics of protein level changes are likely to be even more complex *in vivo*, where cells are proliferating or dying and being replaced. Methods using dynamic SILAC labeling [38] and tandem mass tag (TMT) spectrometry [39] now allow for more precise quantitation of mature proteins and nascent proteins, as well as monitoring the changes that occur upon degraders treatment over time. The study by Savitski et al. [38] indicates that a JQ1-based degrader mediates reduction of BRD2, BRD3, and BRD4 protein levels, without accelerating re-synthesis of the proteins, at least for 24 h.

Conceptually, a full catalytic cycle of targeted protein degradation consists of a series of binding and catalytic steps that can be defined in kinetic and thermodynamic terms, including but not limited to ternary complex formation, mono-ubiquitination of the target protein, processive synthesis of polyubiquitination chain, possible recognition by unfoldases such as VCP/p97, engagement of the proteasome regulatory domain, and proteolytic degradation by the proteasome catalytic domain. In addition, ubiquitination can also be reversed by cellular de-ubiquitinating enzymes. Understanding the kinetic mechanism of targeted protein degradation will help to identify rate-limiting step(s) and develop strategies to optimize degraders to improve degradation efficiency. Recently, Fisher et al. has proposed a mechanistic framework for degrader-mediated ubiquitination by applying the concept of transition state stabilization in classic enzymology [7]. This mechanistic framework provides two likely scenarios in which either ternary complex formation or ubiquitination is the rate-limiting step. However, given the presence of de-ubiquitinating enzymes and cellular cofactors such as p97, accurate depiction of kinetic steps leading to protein degradation remains challenging. Our observation at Kymera on one particular degrader series (Chen et al., unpublished), as well as Riching et al. study of BRD degraders [9], indicate a positive correlation between efficiency of ternary complex formation, ubiquitination, and cellular degradation. This correlation suggests the presence of a steady-state level of the ternary complex (or its ubiquitinated form) and a rate-limiting step leading to proteasomal degradation. In this case, optimizing degraders with better binding affinity and cooperativity for efficient ternary complex formation and target protein ubiquitination should directly translate

into better cellular degradation of that target protein. On the other hand, a disconnect between efficiency of ternary complex formation and cellular targeted protein degradation would indicate a rate-limiting step occurring earlier in the catalytic cycle and absence of a steady-state level of the ternary complex. In practice, during optimization of degraders, the rate-limiting step can be altered dramatically, where the binder moieties and the linker composition can have a significant impact on the kinetics of ternary complex formation, and/or on the kinetics of ubiquitin transfer. Therefore, a detailed mechanistic understanding during degrader optimization will not only guide the focus on rate-limiting step(s) and help to identify proper assay formats, but also prioritize chemical series that have potential to achieve more efficient degradation.

Conclusions

The field of targeted protein degradation is growing exponentially in academic research and in industry. Several biotechnology companies have been created with the goal of applying this transformative modality towards drug discovery and clinical evaluation, while several large pharmaceutical companies are either engaging in discovery collaborations or building degradation drug discovery efforts internally. It is clear that there is a concerted industry-wide effort on driving targeted protein degradation towards an established new modality in the drug discovery toolbox; with such an effort and investment, it is natural to expect advancement in the understanding of the technology. New assays, new technologies, new screening modalities are being built to enable efficient and rational approaches.

It is important to highlight, however, that much remains to be understood. Efforts towards rational E3 ligase/target pairing, impact of compound structural changes to ternary complex kinetics with impact on selectivity, potency across cell types, and steady state degradation kinetics are areas that still require more elucidation. Understanding the relationship of *in vivo* degrader exposure to targeted protein degradation across tissues, and time (the Pharmacokinetic-Pharmacodynamic (PK-PD) relationship) is one of the most important aspects, as the field advances towards clinical proof of concept. It will be essential for practitioners in the space to continue to share some of these fundamental findings to accelerate clinical evaluation of drugs based on this therapeutic modality.

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