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Outlook

Unifying Catalysis Framework to Dissect Proteasomal Degradation Paradigms

Frances P. Rodriguez-Rivera* and Samuel M. Levi

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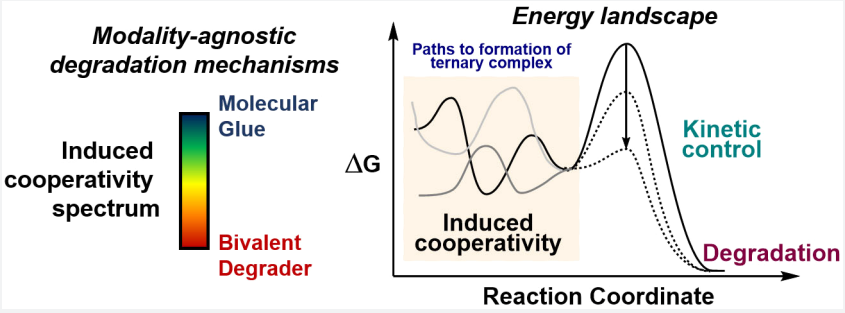

ABSTRACT: Diverging from traditional target inhibition, proteasomal protein degradation approaches have emerged as novel therapeutic modalities that embody distinct pharmacological profiles and can access previously undrugged targets. Small molecule degraders have the potential to catalytically destroy target proteins at substoichiometric concentrations, thus lowering administered doses and extending pharmacological effects. With this mechanistic premise, research efforts have advanced the development of small molecule degraders that benefit from stable and increased affinity ternary complexes. However, a holistic framework that evaluates different degradation modes from a catalytic perspective, including focusing on kinetically favored degradation mechanisms, is lacking. In this Outlook, we introduce the concept of an induced cooperativity spectrum as a unifying framework to mechanistically understand catalytic degradation profiles. This framework is bolstered by key examples of published molecular degraders extending from molecular glues to bivalent degraders. Critically, we discuss remaining challenges and future opportunities in drug discovery to rationally design and phenotypically screen for efficient degraders.

I. INTRODUCTION

Cellular processes are governed by both subcellular compartmentalization and molecular recognition. These molecular interactions in turn provide a template to convey information as cues that can impact signaling, biosynthesis, and degradation pathways in live cells. Quantifying kinetic and thermodynamic contributions inherent to these cooperative interactions is essential to gain a deeper biological understanding and unlock novel biology.[1] Despite remarkable biological complexity, hijacking and reprogramming these molecular recognition patterns with chemically induced proximity (CIP) approaches has been exploited to understand biological mechanisms and leverage this knowledge to identify novel therapeutics.[2−4] In particular, proteasomal protein degradation has recently emerged as a privileged therapeutic strategy that enables the selective destruction of a protein of interest (POI) by reprogramming proteostasis machinery.[5,6]

Productive proteasomal degradation relies on coordinated steps that include (1) the recognition of POI and formation of a ternary complex; (2, 3) charging and modification of POI with ubiquitin (Ub); (4) polyubiquitination of POI; and (5) release of poly-Ub POI followed by proteasomal destruction (Figure 1a). This defined catalytic cycle has been efficient and selectively commandeered by different modalities. Many recent reviews provide detailed accounts on the identification and development of proteolysis targeting chimeras, known as PROTACs, as well as molecular glues.[7−9] At first glance, both degrader entities can showcase different energy landscapes and degradation profiles (Figure 1b,c). However, the distinction between bivalent degraders and molecular glues has blurred over the last several years into a conceptual continuum,[10,11] as researchers build up molecular glues to affect the degradation profile while conversely trimming down PROTACs to optimize degradation and improve pharmacokinetic (PK) properties. As new degradation mechanisms are discovered, the detailed characterization of highly dynamic ternary complexes

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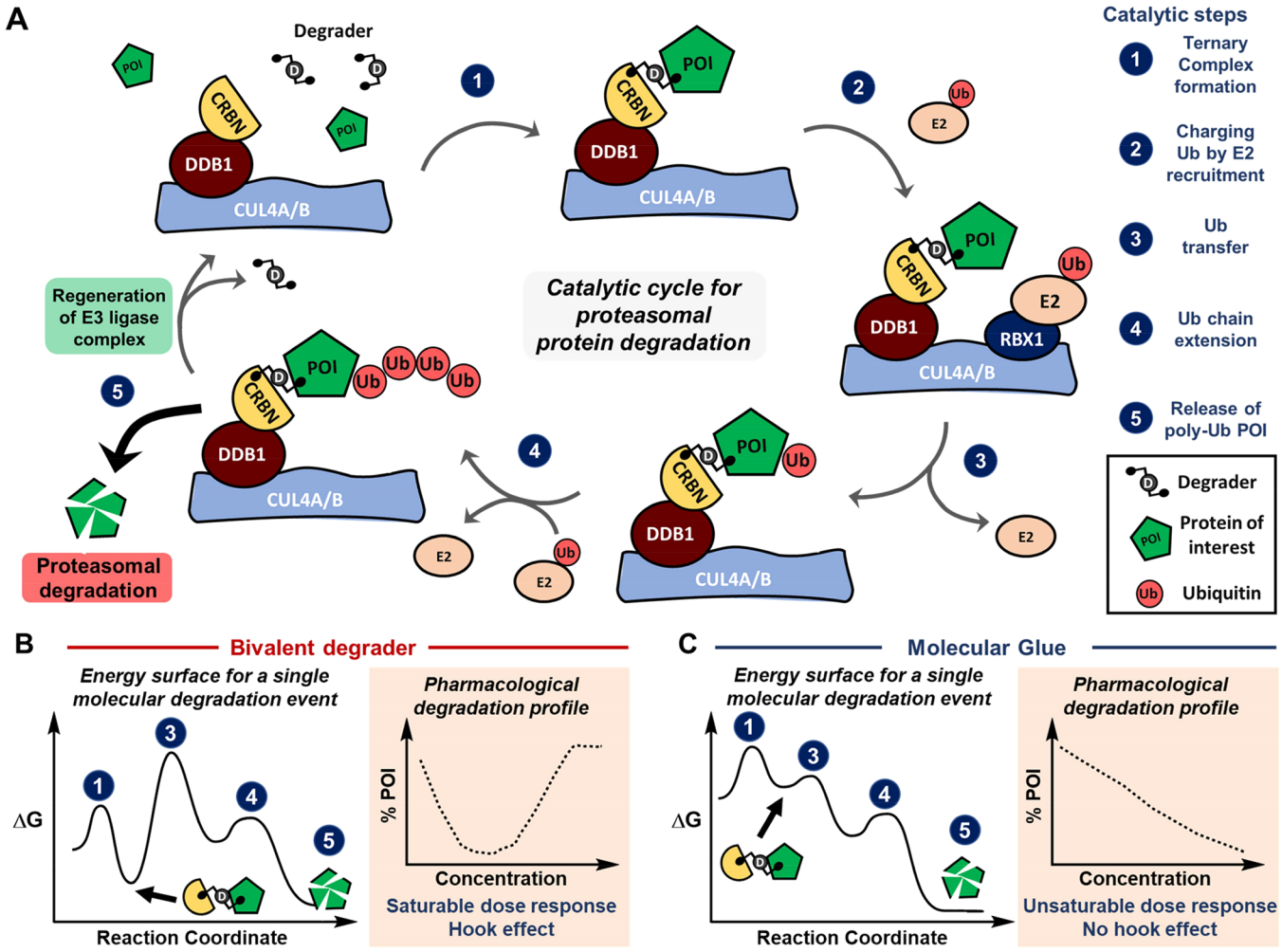

Figure 1. (A) The catalytic cycle for proteasomal protein degradation can be hijacked by unnatural degrader entities. This catalytic cycle is orchestrated by multiple enzymes, where the CUL4A-DDB1-CRBN complex is illustrated here. Sequence of events including (1) ternary complex formation, (2) charging Ub by E2 recruitment, (3) Ub transfer, (4) Ub chain extension, and (5) release of poly-Ub from the ternary complex are required for proteasomal degradation. (B, C) Molecular degraders exhibit different energy landscapes, which ultimately impact the degradation profile and saturability for degrader dose−response curves. Energy diagrams represent reaction coordinates for a single molecular degradation event, whereby the formation of binary complexes is omitted for clarity. The pharmacological degradation profile describes the average population of molecular degraders with corresponding equilibria that can be empirically measured.

and corresponding catalytic implications will enable an improved mechanistic understanding and selection of opportunities for future investment.

With exponential growth in targeted protein degradation reports across academic and industry research,[12] we believe that this area presents a novel opportunity to employ concepts from catalysis (chemical catalysis, enzymology, and pharmacology) that enable the development of innovative chemical probes and medicines. Here, we introduce the concept of induced cooperativity to provide a framework by which molecular glues and monovalent and bivalent degraders exemplify a spectrum of degradation mechanisms. In Section II, we highlight key examples from the literature with a focus on leveraging the importance of kinetics to pursue privileged ternary complexes and inform future degrader design. Section III weaves these catalytic considerations into cellular contexts to capitalize on cellular screening technologies and identify improved starting points to develop efficient degraders. In this Outlook, we use a catalysis lens to help contextualize opportunities and tackle challenges in order to enable the future discovery and development of efficient degraders.

II. INDUCED COOPERATIVITY AND PURSUIT OF PRIVILEGED TERNARY COMPLEXES FOR PROTEASOMAL DEGRADATION

Induced cooperativity through proximal recruitment of artificial protein−protein interactions (PPIs) is essential for effective catalysis to take place in a protein degradation paradigm. A fundamental understanding of the kinetic and thermodynamic parameters that contribute to cooperativity can inform on the degrader design and optimization process. As a result, this understanding could drive efficient protein degradation and expedite the identification of key chemical matter through rational drug design and tailored screening approaches. To conceptualize the induced cooperativity spectrum, we can think of how small molecule degraders can toggle through both cooperativity and binary affinity axes (Figure 2a). In the leftmost side of the spectrum, a molecular glue theoretically may not have any detectable binary affinity toward either protein surface by itself but forms a highly cooperative ternary complex. Conversely, at the rightmost end of the spectrum, a bivalent degrader can exhibit a maximum

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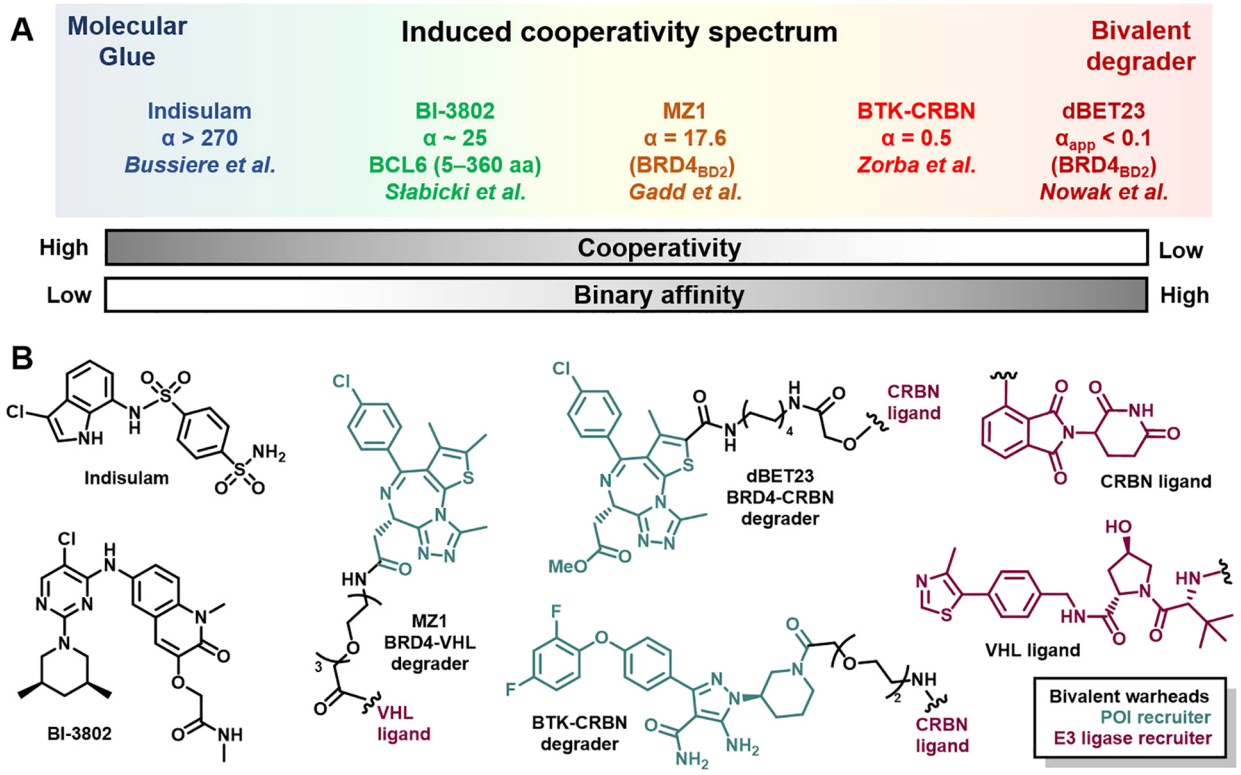

Figure 2. The induced cooperativity spectrum encompasses multiple degrader entities. (A) Theoretical ends of the induced cooperativity spectrum are flanked by molecular glues (left) and bivalent degraders (right). This spectrum can be further dissected into contributions arising from cooperativity (α value) and binary affinity for either POI or E3 Ub ligase recruiter, or a combination thereof. Selected degrader examples are described based on cooperativity values reported or estimated from experimental data from indicated references. (B) Chemical structures for small molecule degraders from part A, with bivalent warheads corresponding to the POI recruiter in teal and E3 ligase in purple.

binary affinity toward either POI or E3 ligase, with negative cooperativity arising from steric clashes within the ternary complex. To support these notions, we discuss selected examples of empirically discovered protein degraders and key considerations for ternary complexes across the induced cooperativity spectrum.

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In less than two decades, researchers have advanced PROTACs from basic science to the clinic, where a handful of molecules have entered phase I trials.[13] PROTACs typically feature an E3 ubiquitin ligase recruiting end, a linker, and a ligand for the POI (Figure 2b). Colocalization of the E3 ligase complex with a protein of interest can lead to productive polyubiquitination and proteasomal degradation. This approach, however, is not without several challenges including extensive medicinal chemistry to identify suitable E3 ligase recruiters and optimized linkers that afford bifunctional small molecules with improved cell permeability and efficient degradation. To access robust protein degradation, these challenges can be addressed by a careful balance of target binding potency, cellular permeability, and judicious choice of E3 ligase for a successful ubiquitination outcome. Beyond cellular degradation, developing efficient degraders also requires tackling unique hurdles to achieve the *in vivo* pharmacological phenotype with improved pharmacodynamics (PD) and PK.[14] However, this drug discovery phase can be prohibitively long, and guidelines for accessing the desired degradation profile remain largely empirical.[5]

To highlight a single target class, kinase inhibitors have provided a ripe entry point to develop bivalent degraders against a highly characterized kinome with corresponding chemical ligandability.[15,16] Several reports have demonstrated that degraders based on pan-kinase inhibitors could exhibit an exquisite degradation selectivity of a single kinase, which was unprecedented for the parent kinase ligand.[17−19] Furthermore, Crews and co-workers developed a selective degrader for the p38-gamma isoform of the MAPK family by linking a foretinib warhead to a VHL recruiter.[19] Collectively, these studies demonstrated that key design elements such as the E3 ligase recruiter and linker composition were required to achieve the desired target selectivity and degradation. However, these efforts also revealed that target engagement alone was insufficient to result in protein degradation. For this reason, significant work in the protein degradation field has gravitated toward identifying key determinants required for the productive formation of a ternary complex that lead to efficient degradation.

The recombinant expression of ternary complex components has facilitated the evaluation of the binding affinity of degraders to both POI and E3 ligase, as well as the determination of cooperativity (α value). Isothermal calorim-

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etry (ITC), fluorescence polarization (FP), size exclusion chromatography (SEC), and luminescent proximity assay (i.e., aLISA) are among popular techniques that allow the measurement of these thermodynamic parameters under steady state conditions.[20] In addition to the recent understanding emerging from crystal structures,[21] these biophysical methods have helped define interactions at the protein−protein interface that can display both cooperative behavior and recently appreciated plasticity. Notably, bivalent degrader design can capitalize on protein−protein interactions (i.e., BRD4-VHL,[22] BRD4-CRBN,[23] BTK-CRBN[24]) to develop more efficient degraders that can also benefit from cooperative behavior (Figure 2b). This work was followed up by the cyclization of the MZ1 degrader to produce macroPROTAC-1, where Ciulli and co-workers demonstrated that reducing entropic cost can increase cooperativity and maintain cellular degradation efficiency.[25] Harnessing cooperativity within ternary complexes can also have a positive impact in minimizing the hook effect from catalysis and safety perspectives.[26,27] In addition to routine ternary complex characterization, prospective mathematical models such as three-body equilibria can also inform iterative degrader design.[28−30] Furthermore, more recent in silico methods can also guide the prioritization of degraders as well as linker design that benefit from increased protein surface complementarity to reduce the time for exploring structure−activity relationships (SARs).[24,31−33] Combining experimental and modeling approaches to prioritize stable ternary complexes has been productive[11] but may also bias discovery of degraders toward molecules that primarily access static and long-lived populations. Thus, opportunities where sufficiently fast protein degradation occurs, such that a small fraction of ternary complex is present, could go unrecognized.

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In contrast to the notion that stable ternary complexes can enhance degradation efficiency, Pfizer scientists found that increased rigidity and stability may negatively impact BTK degradation mediated by the recruitment of cIAP1.[34] With a suite of biochemical, biophysical, and structural studies, they showed that in solution ensembles can lead to ternary complex conformations with different degradation profiles upon bivalent degrader recruitment, perhaps stemming from rigidification of the degrader linker or intrinsic rigidity of the ternary complex, or a combination of both.[34] In this scenario, visualizing high-stability ternary complexes from an energy landscape perspective can illustrate the steep activation requirement for productive degradation (Figure 3a). In line with recognizing unique attributes of a given ternary complex, Donovan et al. conducted a tour de force effort to map the degradable kinome and answer fundamental questions regarding kinase tractability and degradability. Interestingly, this comprehensive study revealed (1) that high potency binders can be ineffective starting points for degraders, (2) that degradation efficiency is not predicted by the formation of stable ternary complexes, and (3) that catalytic degradation can result from transient and unstable ternary complexes (degrader example depicted as a reaction coordinate in Figure 3b).[35] Both collective works highlighted above point out considerations for overstabilizing ternary complexes as well as opportunities to exploit degradation outcomes based on dynamic yet productive ternary complexes. Furthermore, plasticity in ternary complexes has been previously appreciated for bivalent degraders[22,23] as well as next-generation immunophilins that engage a malleable FKBP12 surface.[36] Therefore, a mechanistic understanding of dynamic protein complexes with refined methods as well as the role of privileged conformations and populations could provide untapped opportunities in protein degradation workflows.[37,38]

Beyond target selectivity and interprotein contacts, the premise of event-driven pharmacology, where a substoichiometric amount of a small molecule catalytically degrades POI, is a unique pharmacological attribute of degradation mechanisms. Indeed, Bondeson et al. demonstrated that bivalent degraders at substoichiometric concentrations can catalyze ubiquitination rates in vitro.[39] The catalytic degradation of long-half-life proteins, such as AKT[40] and RIPK2,[41] has demonstrated profound and prolonged PD. Furthermore, electrophilic molecules can facilitate protein degradation at fractional E3 ligase (i.e., DCAF16) occupancy[42] and with nanomolar doses,[43] both viable strategies for the longer durability of POI removal. Reactivity profiling to engage nucleophilic residues has enabled the discovery and development of novel covalent warheads to pursue previously inaccessible E3 ligases.[44−46] Using a covalent warhead to reprogram substrate recognition by a modified E3 ligase is an elegant strategy to enhance both selectivity and catalysis of degradation. Indeed, Nomura and co-workers achieved selective disruption of RNF114-substrate recognition with a nimbolide warhead thereby accessing the desired efficacy and on-target mechanism of action (MOA).[43] Covalent degraders could also have the unique potential to address unresolved challenges of improved physicochemical properties and PD/PK relationships.[46]

Despite productive strides to harness thermodynamic cooperative interactions, fewer efforts have intentionally pursued kinetically driven ternary complexes that could address the hook effect, overly stable ternary complexes, and lack of catalysis. To do this successfully, a mechanistic understanding of kinetic parameters and enzymology of the protein degradation cycle is imperative.[47] Biophysical methods, including surface plasma resonance (SPR) and biolayer interferometry (BLI), are better positioned to qualify kinetic parameters and have been utilized to inform bivalent degrader design.[34,48] Complementary to measuring k_{off}/k_{on} rates and dissociation half-lives, native mass spectrometry can provide additional granularity of ternary complex formation and intermediate conformational states in a single label-free experiment.[49] Future approaches to further explore degradation opportunities may require an in-depth kinetic analysis of protein complexes as those used for dynamic transcription factors,[37] which microfluidics and single molecule studies may also be well positioned to address.[50] Drawing from enzymology principles, a deeper appreciation and mechanistic understanding of energetics for protein ensembles could provide new avenues for protein degradation paradigms.[51−54] Productive integration of these molecular contributions will continue to refine kinetic degradation models and translational frameworks to successfully develop bivalent degraders.[30,55]

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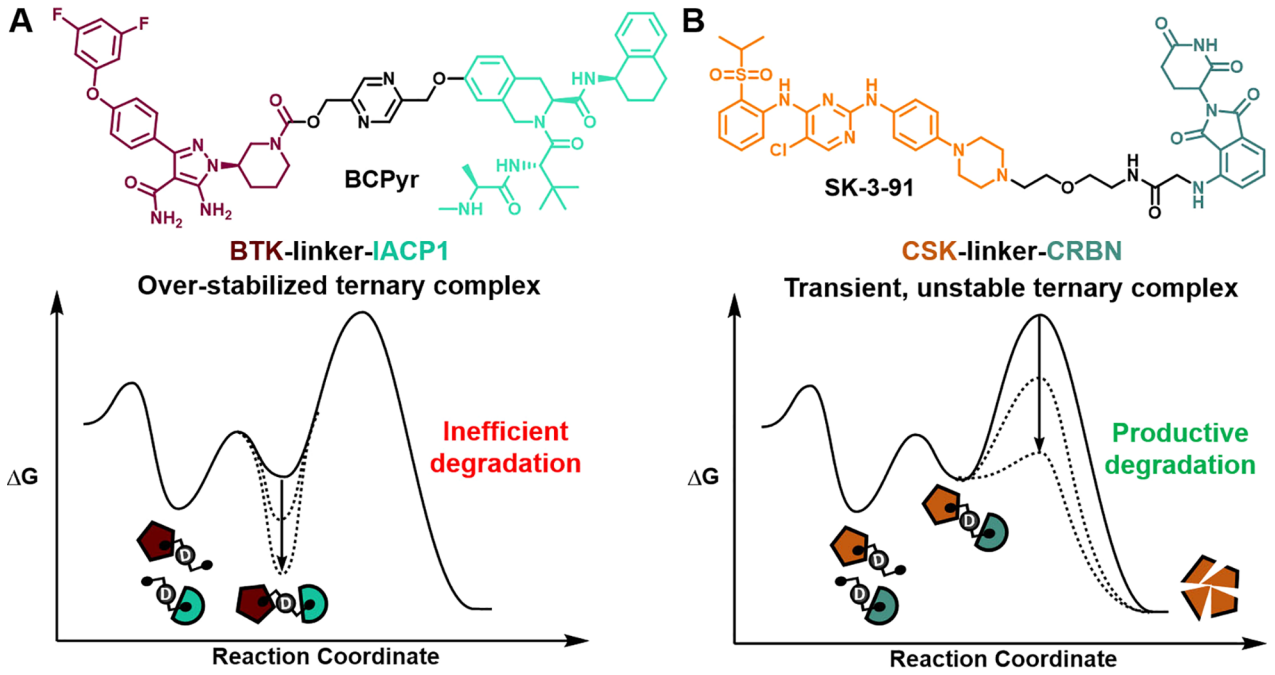

Figure 3. Energy landscapes for specific examples where stable ternary complex formation leads to inefficient degradation (A), and unstable complexes can lead to productive degradation (B). Chemical structures of degraders BCPyr and SK-3-91 are color-coded by POI ligand, linker (black), and E3 ligase.

Following the induced cooperativity spectrum, monovalent degraders offer a distinct degradation profile from bivalent degraders.[56] A recent emergence in identifying monomeric degraders may be a result of both improved MOA deconvolution frameworks for molecules of a desired phenotype and more sensitive detection methods. These degraders can offer distinct physicochemical properties from bivalent counterparts with improved solubility and cell permeability, which are critical for selecting the dose and route of administration.[56] Often serendipitously discovered, molecular perturbagens modulating B-cell lymphoma protein 6 (Bcl6), an oncogenic transcription factor, are great examples of how small changes in chemical structure can induce target inhibition or degradation.[57,58] Recently, Ebert, Fischer, and co-workers unraveled the molecular MOA of BI-3802 (Figure 2b), a monomeric degrader that initially triggers the polymerization of Bcl6, followed by entrapment in cellular foci and, finally, destruction by the proteasome.[59] Notably, a bivalent degrader derived from a structurally different Bcl6 inhibitor was not superior to the parent warhead and exhibited mild anti-proliferative properties.[60] This latter study raises important considerations when repurposing inhibitor warheads as degrader starting points, specifically, untangling mechanistic contributions of target inhibition from degradation to understand cellular phenotypes. Collectively, Bcl6 inhibition and degradation studies exemplify the need for rigorous MOA deconvolution for chemical matter of interest with an eye toward target dynamics that are governed by kinetic and thermodynamic processes.

Harnessing an occupancy-driven mechanism, PPI stabilizers, such as cyclosporin A, FK-506, and rapamycin, benefit from stable ternary complexes for driving immunosuppressive pharmacology.[61] Conversely, event-driven pharmacology could advantageously exploit conditions where ternary complex formation is transient. Thus, kinetically processing this intermediate could lead to rapid and selective degradation of the protein substrate. The immunomodulatory (IMiD) drugs demonstrate this principle and will also be considered through the lens of the induced cooperativity spectrum. Often identified by empirical methods, molecular glues can create a neomorphic surface that can, in turn, selectively engage neosubstrates and funnel them for proteasomal degradation.[8] This surface programmability has enabled drug discovery efforts to hone selectivity further for bespoke neosubstrates by diversifying chemical scaffolds of IMiDs.[10] Remarkably, CRBN has been exploited as a privileged E3 ligase that can recognize structural degrons from over 100 Zn finger substrates.[62−64] Moreover, this programmed complementarity has recently been exploited to modulate CAR-T cell activity.[65,66] On the other hand, anticancer drugs with an arylsulfonamide scaffold (e.g., indisulam, Figure 2b) engaging DCAF15 also serve as molecular glues but only engage a handful of targets, as crystal structures have revealed a highly conserved peptide sequence in degradable neosubstrates.[7,67−69] In a prospective effort to leverage structure-based drug design, scientists at Nurix Therapeutics were able to mimic a native phosphoepitope in β-catenin as a molecular glue that enhances association with $SCF^{\beta\text{-TrCP}}$, its cognate E3 ligase, to afford successful proteasomal degradation.[70] Similarly to bivalent degraders, the development of molecular glues requires a long discovery phase with extensive medicinal chemistry campaigns and remains empirical.

As our structural understanding of matching the POI and E3 ligase continues to improve, the SAR cycle time to develop degraders that stabilize ternary complex formation and reduce entropic cost will also shorten. However, with this approach, biased attention is concentrated on an early step of the catalytic degradation cycle, which may not be the rate-limiting step for all degradation mechanisms across the induced cooperativity spectrum. Furthermore, we may not uncover novel starting points to kinetically alter the degradome of a ligand. Going forward, ligand screening platforms that could be

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adapted to select for catalytic degraders and provide new footholds for challenging targets will be important. In the next section, we focus on intrinsic attributes from the cellular milieu that can affect catalytic degradation and how those can be leveraged to identify efficient degraders.

III. CELLULAR CONTEXT IS CRITICAL TO FINDING EFFICIENT DEGRADERS

When considering degrader catalytic efficiency in live cells, any biological factor that decreases saturation could diminish efficient degradation. Of potential factors, global protein abundance levels of POI or E3 ligase may not correlate with efficient degradation, perhaps in part due to a smaller labile or degradable pool.[35] In addition to expression levels, interrogating relevant cellular contexts that recapitulate key biology is far more important. From a historical research perspective spanning the past decade, dissecting thalidomide's toxicity mechanism epitomizes the necessity to thoroughly examine the molecular MOA of degraders in different cellular contexts. After the initial discovery of CRBN engagement in zebrafish,[71] multiple neosubstrates have been identified,[10] of which SALL4 degradation phenocopies have observed teratogenicity.[72,73] Furthermore, our understanding of ligandable and recruitable E3 ubiquitin ligases has significantly expanded along with degradable neosubstrates.[74,75] Leading this front, carefully defined phenotypic screens present unique advantages to discover and develop E3 ligase modulators.[76] With growing multiomic data sets generated from cellular, preclinical, and clinical studies, future targeted protein degradation efforts could leverage systems biology approaches to nominate degradation hypotheses with greater confidence.[77,78] In practice, the success of such strategies will be highly dependent on the available cellular and chemical tools to validate them.

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Within recent years, several cellular technologies have emerged as valuable tools to interrogate protein degradation in live cells. Chemical genetic approaches such as the HaloPROTACs[79] and dTAG platform[80] have democratized access to visualize the degradation of your favorite POI in lieu of available small molecule ligands, including orphan cell surface transporters.[81] Genetic tools such as haloTag and GFP fusions have enabled the early assessment of Ub ligase compatibility,[82] while biodegraders have enabled swift scanning of POI degradation fitness for a number of E3 ligases[83] and targeting specific conformational states of KRas.[84] Collectively, these technologies offer a diverse menu to select tractable degrader starting points in live cells.

In order to drive SAR optimization of degraders, low- to high-throughput quantitative methods are routinely used.[85] Of these methods, luminescence-based workflows developed by Promega surfaced as very powerful tools to kinetically characterize protein degradation events.[86] In these experiments, POIs can be labeled with HiBiT tags at endogenous levels to interrogate competing biosynthesis and degradation rates.[87] Furthermore, the portability of HiBiT constructs helps paint a crisp picture by reporting on specific events of the catalytic degradation cycle: cytosolic access, target engagement, ternary complex formation, Ub transfer, and proteasome processing.[86] Of note, deconvolution of TL12-186, a pan-kinase PROTAC, illuminated the mechanistic profile with the required selectivity and temporal resolution to identify degradation of POI subpopulations.[88] Collectively, these reports showcase that a detailed mechanistic understanding of degradation profiles for bivalent degraders can be achieved in relevant cellular contexts. Given the breadth and depth afforded by HiBiT tagging, this approach is also well positioned to characterize and exploit kinetically favored degradation mechanisms across the induced cooperativity spectrum.

In contrast to bivalent degraders, the prospective identification of privileged small molecule degraders remains challenging. In this context, functional genomic screens have unveiled degradation dependencies that accelerate the identification of novel molecular glues. For example, three independent studies identified distinct chemical scaffolds that stabilize DDB1-CDK12 interaction and thus lead to the enhanced degradation of Cyclin K, a CDK12 interactor.[89−91] These findings represent a novel mechanism by which a molecular glue can induce POI degradation by a distant PPI rather than directly reprogramming the POI-E3 interface. Phenotypic screens that capitalize on degradation nodes, such as hyponeddylated cells,[89] or exploit cancer vulnerabilities[92−94] are elegant strategies to discover and enhance the druggability of novel degrader biology in relevant cellular contexts. Notably, Koduri et al. devised a creative screening strategy for novel IKZF1 degraders, which led to the identification of Spautin-1, a novel molecular glue that does not require CRBN, and subsequently deployed this strategy to uncover CDK2's role in regulating the abundance of the oncogenic transcription factor ASCL1 pertinent to small cell lung cancers.[93] Additionally, genetic screens have also provided early insights into potential resistance mechanisms emerging from multiple proteasomal degrader modalities.[92,94,95] This collection of studies has embraced the tractability of genetic screens and translatability of phenotypic approaches to intentionally identify novel molecular glues and deconvolute underlying mechanisms of proteasomal degradation. Therefore, functional genomics will continue to play an important role in deconstructing desired phenotypes in relevant cellular contexts.

Finally, the complexity of the ubiquitin proteasome system (UPS) should not go unrecognized. As the understanding of proteostasis machinery accumulates across cellular environments, we will have a better appreciation for the intricacies of Ub code,[74,96] redundancies of 600 Ub ligases,[97] or lack thereof, regulation of CRL4 network dynamics,[98] and protein turnover rates.[99] Going forward, the identification of underlying mechanisms that amplify POI destruction upon degrader recruitment, such as potentiating proteasomal flux[100] or enhancing Ub-chain elaboration,[101] can unlock unique synergies to complement degrader approaches. Equally exciting, the degradation of disease-relevant protein aggregates[102] and polymerized Bcl6[59] have already shifted naive perceptions on processing and unfolding activities by the proteasome. Consequently, converging the elucidation of degradation mechanisms with compelling cellular contexts presents bright prospects to access therapeutically relevant human biology with catalytically efficient molecules.

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IV. CONCLUSIONS AND OUTLOOK

Capitalizing on catalytic considerations for the induced cooperativity spectrum can advance future degrader discovery campaigns. Importantly, exploiting kinetically privileged protein degradation and leveraging phenotypically relevant cellular contexts present untapped opportunities. Of broader significance, the concept of using catalytic molecules to kinetically control cellular processes extends beyond the area of proteasomal degradation, including pioneering studies where novel modalities can hijack autophagy and lysosomal recycling mechanisms.[103−105] Theoretically, any cellular process can be modulated with an approach that colocalizes cellular machinery to a target of interest, where applying induced cooperativity principles is critical. Though the vast majority of examples to date have focused in targeted protein degradation, early results for controlling both installation and removal of posttranslational modifications, like phosphorylation[106,107] and glycosylation,[108,109] have also been reported. In the pursuit of innovative chemical tools and medicines, the growth and success of this budding field could be significantly advanced by experimental workflows leveraged by catalysis researchers. We hope that this Outlook contextualizes the opportunities and challenges for this field under the lens of catalysis and brings forward new ideas that will ultimately benefit patients.

AUTHOR INFORMATION

Corresponding Author

Frances P. Rodriguez-Rivera − *Discovery Chemistry, Merck & Co., Inc., Kenilworth, New Jersey 07033, United States;* orcid.org/0000-0003-0651-8639; Email: frances.rodriguez-rivera@merck.com

Author

Samuel M. Levi − *Pfizer Worldwide Research and Development, Pfizer, Inc., Cambridge, Massachusetts 02139, United States;* orcid.org/0000-0003-0973-3991

Complete contact information is available at: https://pubs.acs.org/10.1021/acscentsci.1c00389

Notes

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