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Targeted Protein Degradation – The Story So Far

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1.1 Introduction to Targeted Protein Degradation (TPD)

Protein homeostasis is a pivotal process in cells comprising protein production and removal. The cell has two major pathways to achieve the latter; primarily, the ubiquitin proteasome system (UPS) that degrades the vast majority of all proteins, or alternatively lysosomal degradation and autophagy, capable of degrading whole organelles such as mitochondria [1, 2]. The UPS is centered around the 76 amino acid small protein ubiquitin that is attached or removed from other proteins as a post-translational modification (PTM) and encodes via a complex pattern of ubiquitination the fate of the target protein [3, 4]. Ubiquitination is achieved by the concerted actions of a series of ubiquitin ligases (E1, E2, and E3) that add ubiquitin chains to substrates, thereby marking them for proteasomal degradation. Autophagy is the second major protein degradation pathway and is a self-degrading mechanism in which a cytoplasmic material is sequestered in double-membrane vesicles and delivered to the lysosome for degradation. Using chemical biological tools to control these endogenous degradation machineries is the hallmark of targeted protein degradation (TPD). While conventional small molecules generally block the activity of a protein, small-molecule degraders aim to eliminate or deplete the protein of interest (POI) following the paradigm of event-driven pharmacology [5–7]. This exciting concept not only expands the drug discovery toolbox but also enables broadening of druggable space and has therefore drawn tremendous attention within both the pharmaceutical industry and academia in recent years. The TPD concept is emerging as a new therapeutic modality with many such compounds entering the clinic and a multitude of start-up companies arising with a focus on TPD.

There are essentially two classes of small-molecule protein degraders: monovalent and bivalent degraders. Destabilizers such as small-molecule hydrophobic tags

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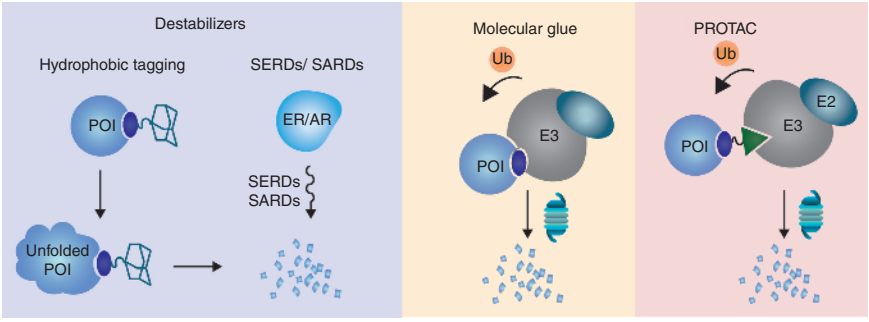


Figure 1.1 Schematic representation of different TPD approaches. While the complete molecular machinery by which HyTags and SERDs induce protein degradation remains unclear, molecular glues and PROTACs act via a E3-ligase-mediated mechanism resulting in proteasomal degradation.

(HyTags) or selective estrogen receptor degraders (SERDs) as well as molecular glue degraders represent monovalent molecules, while proteolysis targeting chimeras (PROTACs®) and related technologies comprise the class of bifunctional degraders (Figure 1.1) [8]. This chapter aims to provide a brief overview of the milestones in TPD achieved to date, including recent clinical advances and the enticing opportunities beyond proteasomal degradation.

1.1.1 What Can Be Expected from this Book?

This book aims to provide a comprehensive overview of the field of TPD. The journey toward controlling protein abundance and function began long before the current excitement around TPD, which has seen a surge in activity during the past 5–10 years. Thus, several techniques modulating protein levels were already established in the early 2000s, consisting mostly of chemical biology tools [9].

To appreciate the rationale behind TPD, one must first familiarize oneself with the cellular machinery controlling protein degradation. The complex cellular processes driving protein degradation, i.e. the UPS and E3 ubiquitin ligases, are highlighted in the contributions of Doris Hellerschmied and Vincenzo D'Angiolella in Chapters 2 and 3, respectively. For a detailed understanding of the molecular mechanisms and structural complexity of the degrader machinery, Morgan Gadd presents Chapter 4 on ternary complexes of small-molecule degraders. Although today we still rely on structural biology to unravel the machinery of ternary complexes, computational approaches are being refined to predict the ternary interaction of a degrader and its protein targets. John Karanicolas in Chapter 5 provides an overview of the current efforts on ternary complex prediction and what we might expect in the future. Switching gears to molecular glue degraders, Cristina Mayor-Ruiz in Chapter 6 explains how the field is progressing from serendipitous discovery to the directed and rational hunt for novel molecular glue degraders. After discussing the general principles of TPD, a specialized discourse on TPD in neurodegenerative diseases and covalent protein degraders is provided by Fleur Ferguson and Xiaoyu Zhang in Chapters 7 and 8, respectively.

Controlling protein levels before the protein is translated from RNA provides another means to achieve TPD. The current approaches to modulate protein abundance by addressing RNA are highlighted in Chapter 9 by Andrei Ursu. The concept of inducing novel protein–protein interactions (PPIs) to facilitate the transfer of a ubiquitin onto a POI has sparked the creative mind of several groups to harness the concept either for additional PTMs or other forms of protein degradation. Thus, George Burslem provides an overview of heterobifunctional molecules beyond PROTACs in Chapter 10.

As the main goal of this book is to provide a summary of the TPD field from all angles (including both academia and the industry), the transition of small-molecule protein degraders from chemical biology tools to drug discovery and clinical applications is an essential part of the story. Proof-of-concept (PoC) studies that often originated within academic groups provide the foundation to generate excitement and ultimately produce convincing preclinical data. However, to develop viable clinical candidate degraders, many additional parameters need to be optimized and other challenges must be addressed. To shed light on the path towards clinical candidate development, a team from Boehringer Ingelheim provides an overview of TPD in drug discovery in Chapter 11, while a team from Roche digs deep into the drug metabolism and pharmacokinetic (DMPK) properties and hurdles associated with small-molecule protein degraders in Chapter 12. Finally, in Chapter 13, a team from Arvinas shares their views on their exciting journey of TPD from a chemical biology tool to a clinical candidate.

1.2 Development of Targeted Protein Degradation: Chronology and Milestones

Despite the recent explosion in interest in TPD, the first protein degrader drugs appeared more than 30 years ago with the development of selective estrogen receptor degraders (SERDs) for the treatment of breast cancer (Figure 1.2). Shortly before the launch of fulvestrant as the first SERD drug, the first paper describing PROTACs was published in 2001. However, it was not until 2004 with the advent of cell-permeable PROTACs that the current "gold rush" slowly began to take shape. Since the late 2000s, advances have been reported with increasing frequency, beginning with the recruitment of new E3 ligases (cereblon [CRBN], von Hippel–Lindau [VHL], and cellular inhibitor of apoptosis protein [cIAP] binding warheads) and the burgeoning development of new modalities for TPD such as HyTags and IMiD molecular glues. In 2015, PROTACs took a leap forward toward drug-like therapeutics with the first *in vivo* efficacy experiments. Just four years later in 2019, ARV-110 became the first PROTAC to enter human clinical trials for degradation of the androgen receptor (AR) to treat prostate cancer. The following year, the first non-oncology PROTAC entered the clinic (KT-474), an IRAK4 degrader for immune-mediated inflammatory diseases, and today, there are more than 20 PROTACs and molecular glues in ongoing clinical trials. It is clear from the ever-increasing pace of development that we are still in the expansion phase for this promising class of therapeutic agents, and we expect many more advances in the coming years.

1.2.1 Milestone 1: Early Monovalent Protein Degraders – The Surprising Biology of Fulvestrant

In the early 1990s, researchers discovered a strange phenomenon while studying the anti-estrogen breast cancer drug today called fulvestrant (known then as ICI 164,384, Figures 1.2 and 1.3). In a radioligand displacement experiment, fulvestrant showed twofold weaker binding when compared to the endogenous agonist ligand estradiol (K_d values of 1.9 and 0.9 nM, respectively). Despite this fact, cells treated with fulvestrant showed a profound reduction in their response to stimulation with estradiol [10]. From the standpoint of classical pharmacology, this posed a conundrum; as an antagonist, fulvestrant would be expected to exert its effect through an occupancy-driven mechanism. A competitive antagonist would occupy the binding site and therefore block the binding and stimulatory effect of estradiol, while an allosteric modulator could bind to an orthosteric site to modify protein dynamics and modulate the pharmacological response. However, fulvestrant did something else; somehow, the compound elicited a significant reduction in estrogen receptor (ER)

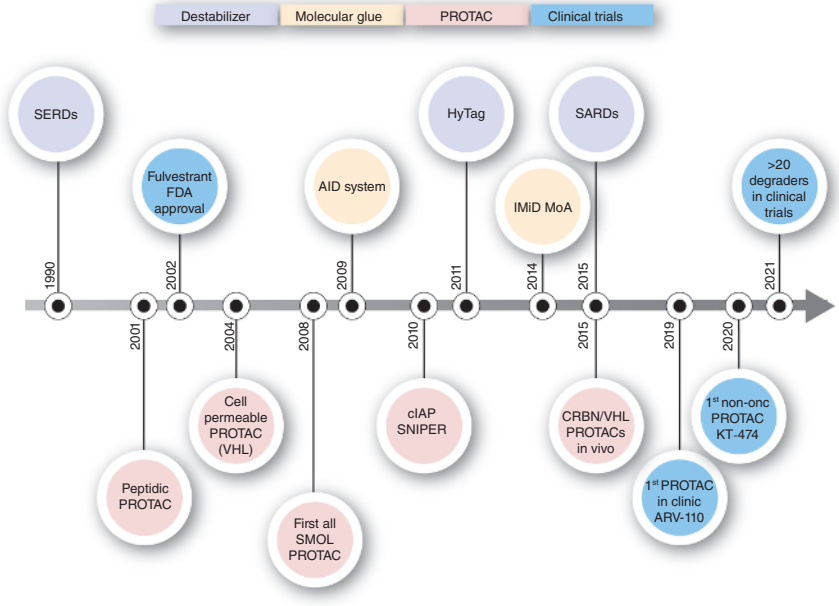
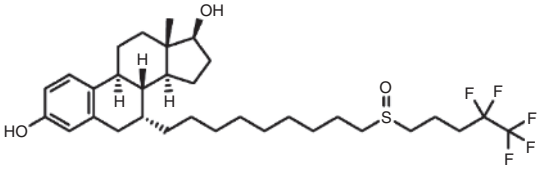


Figure 1.2 Timeline highlighting key achievements of the TPD field. SMOL, small molecule.

Figure 1.3 Structure of the SERD fulvestrant, a marketed breast cancer drug.



Fulvestrant

levels as confirmed by Western blot analysis, immunoprecipitation, and radiography [11]. Here was a drug that abrogated protein function not by merely occupying the active site and blocking its function but by directly reducing the quantity of protein within the cell. The disappearance of the receptor led to an insufficient quantity of protein to elicit an agonist response, thereby halting ER signaling by a new mechanism of action. Researchers dubbed these new compounds SERDs, and SERD molecules are still among the foremost treatments for ER-positive breast cancer today.

Since these early discoveries, there has been significant progress in our understanding of SERDs and related molecules. Researchers have demonstrated that proteasomal degradation of ER is linked to receptor dimerization and nuclear localization [12], and new ER receptor degraders have been designed through improved understanding of protein–ligand interactions at the molecular level (see 1.2.6) [13]. However, despite over two decades of research, the molecular biological cascade by which fulvestrant exerts its mechanism of action has not yet been fully elucidated.

Nonetheless, research teams took inspiration from the growing understanding of proteasomal degradation to create new methods of targeted degradation. In 2009, Nishimura et al. described the auxin-inducible degron (AID) system by which small-molecule plant hormones ("Auxins") could be harnessed to induce degradation (Figure 1.2) [14]. In these studies, researchers transplanted the plant-specific auxin-dependent degradation to other eukaryotic cell lines, including yeast cells and engineered cell lines derived from higher organisms including mouse, monkey, and human. The method was later adapted using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing system to enable endogenous tagging of human and mouse cells, thereby expanding the utility of the AID system for the functional analysis of POIs [15].

In 2011, Crews and coworker described another new approach to targeted protein degradation (TDP) using hydrophobic tagging (HyTag). In its original incarnation, the target protein for the HyTag approach was a bacterial dehalogenase (HaloTag), which could be fused to a protein of interest, initially using green fluorescent protein (GFP), which enabled the facile quantification of protein levels (Figure 1.1). The tagged fusion protein could then be engaged using a chloro-alkyl bearing small molecule (the HyTag), which reacted specifically with the HaloTag protein. The HyTag also includes a linker moiety (derived from polyethylene glycol, PEG) and terminates in a lipophilic chemical group, such as an adamantyl residue. Treatment of these engineered cells with HyTag molecules enabled specific degradation of the protein of interest via the proteosome, presumably through recognition of the HyTag-bearing target as a misfolded protein (Figure 1.1). To illustrate the therapeutic potential of this modality, the authors also extended the method to the degradation of tagged $HRAS^{G12V}$ (GTPase HRas protein), a mutant protein that is a key driver in several tumor types [16, 17].

Inspired by the earlier discovery of fulvestrant as an ER degrader, in 2015, the Crews laboratory combined their HyTag approach with a ligand that engaged the AR, a key pharmacological target in the treatment of prostate cancer. The high-affinity AR ligand RU5906324 was combined with a PEG-based linker and the hydrophobic tag to create a new series of selective AR degraders termed SARDs, by analogy with the earlier SERD acronym. The administration of SARDs to LNCaP

prostate cancer cells showed dose-dependent degradation of the AR and a concomitant arrest of cell proliferation. SARD molecules remain a potential source of new therapies for the treatment of prostate cancer.

1.2.2 Milestone 2: The First PROTACs – From Peptidic Degraders to Chemical Biology Tools

Looking back into the development of TPD, the birth of PROTACs can be identified with the 2001 PNAS publication by Crews and Deshaies describing chimeric molecules targeting the POI protein methionine amino peptidase 2 (MetAP2) in tandem with the E3 ligase Skp1–cullin–F box (SCF) complex to elicit ubiquitination and degradation [18]. Why did this initial publication not create the wave of excitement around TPD, which we see today, and instead flew under the radar for almost 15 years? The answer may lie in the structure of the first PROTAC molecule (Figure 1.4). Despite its seminal role in the development of TPD, the compound was not in the least drug-like and incorporated many chemical features that severely limited its range of applications. To engage the F box protein β-TRCP, a doubly phosphorylated 10 amino acid peptide was used, while the MetAP2 protein was engaged using the covalent natural product ovalicin, with both entities tethered together using a polyglycine suberate linker. Consequently, the first PROTAC was covalent on the target site (and therefore not substoichiometric) and not cell permeable, restricting the PoC experiments to be carried out *in vitro* in *Xenopus* egg extracts. Nevertheless, this publication provided the first example of small-molecule-induced protein degradation without the need for genetic modification of any of the involved proteins. Although its longevity was not obvious in 2001 the PROTAC concept had arrived and was here to stay.

At this point, we must also give credit to the work that paved the way to this initial publication. In 1995, Gosink and Vierstra were able to redirect the specificity of ubiquitination of an E2 enzyme and subsequent adenosine triphosphate (ATP)-dependent degradation *in vitro* by creating a chimera of the E2 ligase and a POI targeting peptide via genetic modification [19]. Similarly, in 2000, Zhou et al.

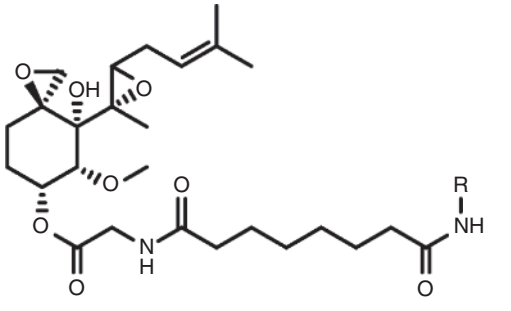


Figure 1.4 Structure of the first PROTAC reported in 2011. The amino acids of the β-TRCP targeting peptide as well as the poly glycine linker are depicted in a single-letter code.

reported the degradation of a target protein after genetically fusing its peptide ligand to a subunit of an E3 ligase complex [20].

After the initial PoC study with MetAP2 in 2001, the next milestone achieved by Crews and coworkers was the degradation of the ER and the AR using two non-covalent PROTACs based on their previously used bisphosphorylated β-TRCP (F-box/WD repeat-containing protein 1A) engaging decapeptide [21]. While already espousing catalytic potential, these PROTACs were still not cell permeable and therefore needed to be microinjected into cells. The first cell-permeable PROTACs were reported in 2004 [22]. Thus, Crews and Deshaies abandoned the bisphosphorylated β-TRCP decapeptide for a heptameric hydroxyproline containing a HIF1α-derived peptide sequence engaging the E3 ligase VHL, which was fused to an octa D-Arg sequence to obtain cell permeability. These tool PROTACs achieved proteasome-dependent degradation of FK506 binding protein (FKBP)-fused constructs as well as AR degradation at high μM concentration and were the first PROTACs engaging VHL instead of β-TRCP, thereby demonstrating that additional E3 ligases are amenable to induced TPD.

Another key step was the transition from cell-based experiments to *in vivo* efficacy, which was partly achieved in 2013 by a less celebrated, albeit fascinating story from the Crews lab reporting conditional PROTACs [23]. A "bait" sequence comprising the tyrosine phosphorylation sequence of either TrkA or ErbB3 was coupled with the peptide ligand for VHL and octa D-Arg. Inside the cell, the "bait" tyrosine phosphorylation sequence is activated by phosphorylation, and the phosphorylated tyrosine sequence is subsequently bound by the actual target protein FRS2α or PI3K. After binding to the "bait" sequence, the target is finally ubiquitinated by VHL and then degraded by the proteasome. Despite limited statistical significance, the conditional PI3K PROTAC showed hints of *in vivo* efficacy after repeated intraperitoneal dosing in a xenograft model. However, all of these early peptide-based PROTACs suffered from poor bioavailability and limited potency, which restricted them from achieving their full potential and generating excitement within the budding TPD community.

The first "all-small-molecule" PROTAC was reported by the Crews lab as early as 2008 (Figure 1.2) [24]. Comprising the MDM2 inhibitor nutlin and a non-steroidal AR ligand connected via a PEG linker, this PROTAC was able to induce the degradation AR at 10 μM in HeLa cells. The choice of MDM2 as the E3 ligase was somewhat surprising; however, in 2008, the nutlins were among the only well-characterized small-molecule E3 ligase binders available. However, MDM2 is a relatively poor E3 ligase and indeed nutlins themselves can induce AR degradation [25, 26], and so the explosion of interest in PROTACs was again unduly postponed.

1.2.3 Milestone 3: Improving Drug-Like Properties – SNIPERs and Peptidomimetic VHL Binders

The endeavor toward more drug-like PROTACs continued in 2010 with a publication from Itoh et al. recruiting the ubiquitin ligase cellular inhibitor of apoptosis protein 1 (cIAP1) to degrade the cellular retinoic acid binding proteins (CRABP-I and -II) via their respective binders methyl bestatin (MeBS) (cIAP1) and *all-trans*

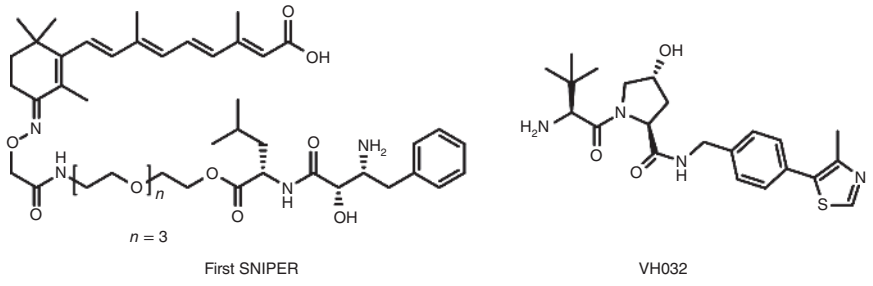


Figure 1.5 Structure of the first SNIPER targeting the E3 ligases cIAP1 and CRABP-I and II and the VHL peptidomimetic VH032.

retinoic acid (ATRA) (cellular retinoic acid-binding protein [CRAPB]-I,II, Figure 1.5) [26]. The target scope of these MeBS degraders was expanded to retinoic acid receptor (RAR) as well as ER and AR, and the acronym SNIPER (specific and non-genetic IAPs-dependent protein erasers) was coined in 2011 [27]. However, while these MeBS SNIPERs or PROTACs showed cellular activity without recourse to microinjection or cell penetrating peptides, unfortunately these compounds induce autoubiquitination and degradation of the IAP E3 ligase itself, thereby hampering the potency of the resultant degraders (DC_{50} values in the μM range).

While SNIPERs were making their debut, Ciulli and Crews were focused on VHL and committing significant effort to convert the heptameric hydroxyproline containing HIF1α peptide into a smaller, more drug-like peptidomimetic using fragment-based drug discovery (FBDD) [28, 29]. They solved numerous crystal structures and tested many substituents around the hydroxyproline core pharmacophore to develop the predecessors of today's well-known VHL ligand VH032 (Figure 1.5). Despite having no direct connection to TPD, this groundbreaking work paved the way for future success in the field.

1.2.4 Milestone 4: PROTACs on the Rise – Small-Molecule Degraders Achieve *in vivo* PoC

Despite the PROTAC concept having been established as early as 2001, the TPD field as we know it today was spurred primarily by two key publications from the Bradner and Crews labs in the spring of 2015 [30, 31]. Winter et al. used the IMiD thalidomide to engage the E3 ligase CRBN and induced the selective degradation of BRD4 *in vitro* and *in vivo*, showing efficacy in a leukemia xenograft model (Figure 1.6) [30].

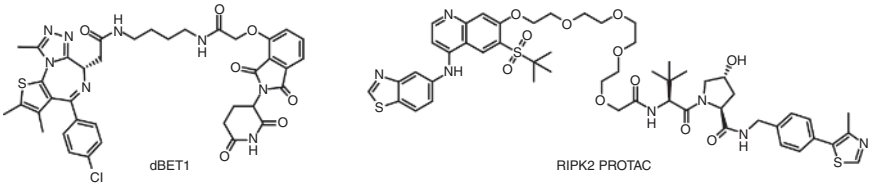


Figure 1.6 Chemical structures of the *in vivo* PROTACs dBET1, engaging CRBN, and the RIPK2 degrader PROTAC engaging VHL.

In another study, Bondeson et al. made use of the previously identified VHL peptidomimetics to degrade the estrogen-related receptor alpha (ERRα) as well as the serine-threonine kinase RIPK2 in various mice tissues and showed that substoichiometric amounts of PROTACs can be sufficient to reach full ubiquitination (Figure 1.6) [31]. Together, these two publications were able to fulfill three important criteria that had previously held the excitement around PROTACs in check: (i) PROTACs are able to induce the ubiquitination of superstoichiometric quantities of their target protein, (ii) PROTACs display very high degradation selectivity across the whole proteome, and (iii) PROTACs can be drug-like and efficacious *in vivo*, showing broad tissue distribution as well as delayed leukemia progression in mice.

That same year, a third publication from Arvinas sparked further excitement around PROTACs and the prospect of achieving clinical use [32]. Lu et al. reported data on how a BRD4-based CRBN PROTAC outperforms its parent small-molecule BRD4 inhibitor in various Burkitt's lymphoma cell lines *in vitro*. These three reports revived the field of small-molecule-induced protein degradation and provided momentum as the pharma industry began to recognize the therapeutic potential of TPD versus traditional small-molecule drugs. The interest continued to grow as Arvinas provided the first data for an orally available AR degrader outperforming the marketed anti-androgen enzalutamide in a prostate cancer xenograft model.

Shortly after VHL- and CRBN-based degraders had proven their ability to induce *in vivo* target degradation, IAP engaging degraders (SNIPERs) followed suit [33]. In 2018, Ohoka et al. identified an IAP-based ER degrader and demonstrated *in vivo* degradation and efficacy in an ER-positive breast cancer xenograft model. In these studies, the authors moved away from MeBS (used for the first IAP PoC degrader) and focused instead on the peptidomimetic IAP antagonist LCL161. Further variation of the IAP ligand identified ER SNIPERs with superior degradation activity and reduced autoubiquitination and degradation of the IAP E3 ligases [34]. Nevertheless, for some time, IAP PROTACs remained inferior to the VHL- and CRBN-based PROTACs reported since 2015. Like VHL, the IAP ligands represent peptidomimetics comprising an essential N-terminal *N*-methyl alanine, a feature that hampers their potential to achieve oral bioavailability. However, in a recent groundbreaking effort, researchers at GSK identified an extremely long-lasting IAP-based RIPK2 degrader that maintained RIPK2 protein ablation for more than 96 hours after a single subcutaneous dose [35]. The effect could be prolonged to more than 60 days using a slow release formulation [36].

In addition to the two "workhorse" E3 ligases VHL and CRBN (and to some extent IAP), many groups have continued to work on making additional E3 ligases accessible for TPD. A lot of exciting work has come from the labs of Benjamin Cravatt and Daniel Nomura utilizing the power of chemoproteomics and covalent ligand screening to identify novel ligands for E3 ligases [37, 38]. Various members of the CUL4-associated factor (DDB1- and CUL4-associated factor homolog protein [DCAF]) and RING finger protein (RNF) families have been successfully recruited for TPD using covalent binders and fragments. Further details on covalent degraders and the identification of E3 binders using chemoproteomics are reported in Chapter 8 of this book authored by Xiaoyu Zhang.

1.2.5 Milestone 5: Sticking It to the Man! Molecular Glues

In addition to SERDs and PROTACs, molecular glues represent another family of degraders currently generating excitement in both academia and the pharmaceutical industry. At a time when the PROTAC concept was still gathering momentum, research into the MoA of the small-molecule drug thalidomide opened the door to the fascinating world of small-molecule PPI inducers today termed molecular glues (Figure 1.2) [39–42]. The concept of inducing neoprotein–protein interactions has been known since the elucidation of the MoA of cyclosporin A and FK506 in 2021 [43, 44]. However, the realization that the small and simple molecules of the immunomodulatory imide drug (IMiD) family (e.g. thalidomide, pomalidomide, and lenalidomide, Figure 1.7) exert their effect via degradation of the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) came as a great surprise [45, 46]. All the more significant is that such protein targets were previously thought to be "undruggable." The IMiD drugs reshaped the protein surface of the E3 ligase CRBN to selectively recruit neosubstrates for ubiquitination and subsequent proteasomal degradation. Lenalidomide (Revlimid®) has since emerged as a best-selling drug, earning more than US$ 12 billion in revenue in 2020 alone. A detailed understanding of the IMiD MoA informed by multiple ternary complex X-ray structures has enabled the field to further flourish, with multiple IMiDs now in clinical trials (Table 1.3) [47, 48]. By tuning their structures and molecular properties, these compounds display distinct degradation selectivity to various members of the Ikaros family of zinc finger proteins and beyond.

Molecular glues are particularly attractive for drug discovery because of their lower molecular weight and improved drug-like properties versus bifunctional modulators such as PROTACs. However, until recently, the discovery of molecular glue degraders was driven primarily by serendipity. In addition to the IMiD drugs, this was also true of the discovery of the MoA of indisulam, which was revealed to be a degrader of RBM39 via recruiting the E3 ligase DCAF15 [49]. Perhaps, the first "rational" discovery of a molecular glue was the identification of compounds restoring the degradation of mutated β-catenin via its cognate E3 ligase β-TRCP [50]. Recently, work from three independent groups allowed the identification of molecules degrading cyclin K in an E3 agnostic manner [51–53]. The recent progress in the field of molecular glues has driven the foundation of multiple small Biotech companies focused on identifying the next IMiD/CRBN system, as well as expanding the target space and selectivity profiles based on the distinct degron motif identified for all IMiD targets. For further details on molecular glues, we refer the reader to Chapters 4 and 6 authored by Morgan Gadd and Cristina Mayor-Ruiz, respectively.

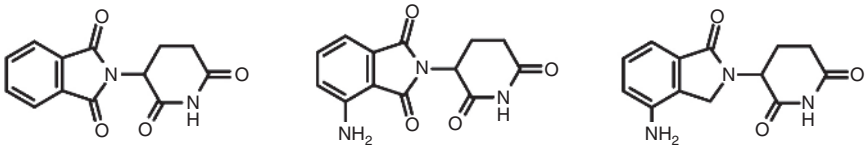


Figure 1.7 Chemical structures of the IMiDs thalidomide, pomalidomide, and lenalidomide.

1.2.6 Milestone 6: Human After All – TPD in Clinical Trials

Possibly, the first example of intentional TPD in human clinical trials was fulvestrant, an ER ligand later found to induce degradation of the target protein (a class of compounds later termed SERDs). Similarly, thalidomide and related molecular glues were identified through serendipitous discovery. In contrast, the current generation of clinical degraders has been purposefully designed to induce degradation, often with an underlying rationale for why TPD might be advantageous compared to traditional occupancy-based inhibition. In the case of bifunctional degraders such as PROTACs, their structural features typically engender "beyond rule-of-5" chemical properties, which brings significant challenges for drug delivery and oral bioavailability [54, 55]. Overcoming this challenge for such molecules and achieving bioavailability is a success story that has allowed these compounds to recently enter human clinical trials (Figure 1.2).

Fulvestrant was the first SERD drug to be approved in the United States in 2002 (Figures 1.2 and 1.3). Although fulvestrant has proven clinically effective, its oral bioavailability is severely limited because of its high lipophilicity, and therefore, the drug must be administered by intramuscular injection. To overcome this limitation, a new generation of SERD drugs is now under development to improve upon the DMPK profile of fulvestrant (Table 1.1) [56]. Several next-generation SERDs are now under clinical evaluation for the treatment of breast cancer, including four

Table 1.1 SERD and SARD degraders in clinical trials.

Drug name	Company	Target protein(s)	Indication(s)	Clinical phase
Camizestrant (AZD9833)	AstraZeneca	ER	Breast cancer	Phase III
Elacestrant (RAD1901)	Radius Health	ER	Breast cancer	Phase III
Rintodestrant (G1T48)	G1 Therapeutics	ER	Breast cancer	Phase I
Giredestrant (GDC-9545)	Roche Genentech	ER	Breast cancer	Phase III
Amcenestrant (SAR439859)	Sanofi	ER	Breast cancer	Phase III
LY3484356	Loxo oncology	ER	Breast cancer	Phase I
Zn-c5	Zentalis	ER	Breast cancer	Phase I
D-0502	InventisBio	ER	Breast cancer	Phase I
Galeterone (TOK-001)	University of Maryland	AR	Prostate Cancer	Phase II
ASC-J9	AndroScience	AR	Acne vulgaris	Phase IIb (discontinued)
AZD3514	AstraZeneca	AR	Prostate cancer	Phase I (discontinued)

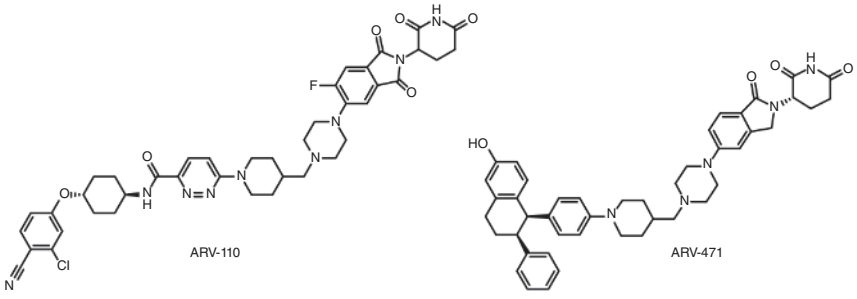


Figure 1.8 Chemical structures of the first two PROTACs to enter clinical trials; the AR degrader ARV-110 and the ER degrader ARV-471.

compounds currently in phase III trials. In contrast with SERDs, there has been less progress in the clinical development of SARD drugs targeting the AR. AZD3514 was under development as a first-in-class, orally bioavailable SARD for the treatment of prostate cancer and entered phase I trials in 2010, where it showed moderate antitumor activity but caused significant nausea and vomiting precluding further investigation [57]. ASC-J9 is another SARD that was under clinical evaluation for the topical treatment of acne vulgaris. Although the compound showed improved efficacy compared with placebo, its clinical development has not progressed beyond phase IIb (Table 1.1) [58].

In 2019, the first bifunctional degraders entered human clinical trials. Like SARD drugs, ARV-110 targets the AR for the treatment of prostate cancer, while ARV-471 is an ER degrader for the treatment of breast cancer (Figure 1.8). Both drugs have since demonstrated the proof of mechanism, providing useful levels of pharmacokinetic exposure and the modulation of hormone-related biomarkers in clinical volunteers, allowing these drugs to progress to phase II trials [59, 60]. These pioneering studies aimed to tackle their respective cancer indications via clinically validated protein targets, a strategy that helped to minimize the risk of failure by ensuring that their novel mode of action was counterbalanced by proven disease biology. Since 2019, more than 10 additional bifunctional degraders have entered the clinic, including several compounds that target novel biological pathways (Table 1.2) [61]. Although many of these degraders focus on oncology indications, both KT-474 from Kymera® and NX-5948 (Nurix®) target autoimmune diseases, thus demonstrating the potential for TPD in therapeutic areas other than cancer.

Molecular glues have also played a significant role in the transition of TPD to the clinic. Inspired by the earlier IMID drugs such as thalidomide, researchers have designed new compounds that specifically degrade protein targets linked to lymphoma, leukemia, and solid tumors (Table 1.3) [61]. In contrast to bifunctional degraders, the physicochemical properties of molecular glue degraders comply more closely with Lipinski's "rule of 5," [55], providing an advantage for achieving a pharmacokinetic profile with efficacious clinical exposure. Although the rational design of molecular glue degraders remains a challenge, recent advances may facilitate their discovery and development in the future [51, 62].

Table 1.2 Bifunctional degraders in clinical trials.

Drug name	Company	Target protein(s)	Indication(s)	Clinical phase
ARV-110	Arvinas	AR	Prostate cancer	Phase II
ARV-766	Arvinas	AR	Prostate cancer	Phase I
ARV-471	Arvinas	ER	Breast cancer	Phase II
AC682	Accutar	ER	Breast cancer	Phase I
CC-94676	Bristol Myers Squibb	AR	Prostate cancer	Phase I
AC0176	Accutar	AR	Prostate cancer	Phase I
HP-518	Hinova Pharmaceuticals	AR	Prostate cancer	Phase I
GT20029	Suzhou Kintor Pharmaceutical	AR	Androgenic alopecia, Acne	Phase I
DT2216	Dialectic	BCL-XL (B-cell lymphoma-extra large protein)	Liquid and solid tumors	Phase I
KT-474	Kymera	IRAK4	Autoimmune diseases	Phase I
KT-413	Kymera	IRAK4	DLBCL (diffuse large B-cell lymphoma)	Phase I
KT-333	Kymera	STAT3	Solid and liquid tumors	Phase I
NX-2127	Nurix	BTK	B-cell cancers	Phase I
NX-5948	Nurix	BTK	B-cell cancers, autoimmune diseases	Phase I
FHD-609	Foghorn	BRD9	Synovial sarcoma	Phase I

1.2.7 Milestone 7: Beyond the Proteasome – Inducing Proximity to Modulate Protein Levels

In the wake of the excitement generated by TPD and the realization that functional neo-PPIs can be induced via bifunctional molecules, additional strategies to rationally modulate protein levels have been discovered [63, 64]. In 2018, the Disney lab reported the recruitment of an RNA nuclease to degrade the microRNA-96 (miR-96) resulting in increased levels of the pro-apoptotic transcription factor FOXO1 and coined the term ribonuclease targeting chimeras (RIBOTAC) for ribonuclease targeting chimeras [65]. Additional PoC work followed; however, at this point of time, RIBOTACs might still be considered as chemical biology tools rather than upcoming therapeutics [66, 67].

Moving beyond the power of the UPS to degrade and remove disease-causing proteins, other groups have focused on the second major mammalian degradation machine; the lysosomal pathway [68, 69]. In 2020, Banik et al. reported that by engaging the cell surface mannose-6-phosphate receptor (M6PR), the internalization of

Table 1.3 Molecular glue degraders in clinical trials.

Drug name	Company	Target protein(s)	Indication(s)	Clinical phase
Avadomide CC-122	Bristol Myers Squibb	IKZF1, IKZF3, DNA-PK	Lymphoma	Phase II
DKY709	Novartis	IKZF2	Solid tumors	Phase I
CC-90009	Bristol Myers Squibb	GSPT1	Acute myeloid leukemia	Phase I
Mezigdomide CC-92480	Bristol Myers Squibb	IKZF1, IKZF3	Multiple myeloma	Phase I
Iberdomide CC-220	Bristol Myers Squibb	IKZF1, IKZF3	CML (chronic myeloid leukemia), NHL (non-Hodgkin's lymphoma)	Phase I
CC-99282	Bristol Myers Squibb	IKZF1, IKZF3	Lymphoma	Phase I
CFT7455	C4 Therapeutics	IKZF1, IKZF3	Multiple myeloma and Lymphoma	Phase I
BTX-1188	BioTheryX	IKZF1, IKZF3, GSPT1	Acute myeloid leukemia, NHL	Phase I

protein cargo and its subsequent degradation via the lysosome can be triggered [70]. They named their system lysosome targeting chimeras (LYTACs, Figure 1.9). LYTACs are capable of degrading extracellular as well as membrane proteins and thereby address a niche that PROTACs and molecular glues are unable to reach, as the latter modalities are restricted to intracellular targets. LYTACs were further expanded to include the asialoglycoprotein receptor (ASGPR), which is exclusively expressed on hepatocytes via *N*-acetylgalactosamine (GalNAc). The Biotech company LYCIA Therapeutics® was later founded based on LYTAC technology [71]. Besides LYTACs, sweeping antibodies or Seldegs have also been reported to degrade extracellular protein constructs [68]. For membrane-bound POIs, antibody-based PROTACs (AbTACs) have been employed by the Wells lab, which comprise bispecific antibodies of which one arm is designed to engage the POI while the other arm recruits a membrane-bound E3 ligase to induce ubiquitination on the cytosolic side [72].

Further applications of lysosomal degradation include the so-called autophagy targeting chimeras (AUTACs) and autophagy tethering compounds (ATTECs, Figure 1.9) [69, 73, 74]. While AUTACs are in principle capable of degrading everything within the cell, including whole-cell organelles (e.g. mitochondria), their full MoA remains unknown. ATTECs on the other hand simultaneously engage the LC3 as well as their protein target and have been successfully applied to induce the degradation of mutant Huntington (mHTT) protein via autophagy.

Modulating POI levels has emerged as an exciting playground for novel chemical biology tools, and their use has consequently helped to better understand the

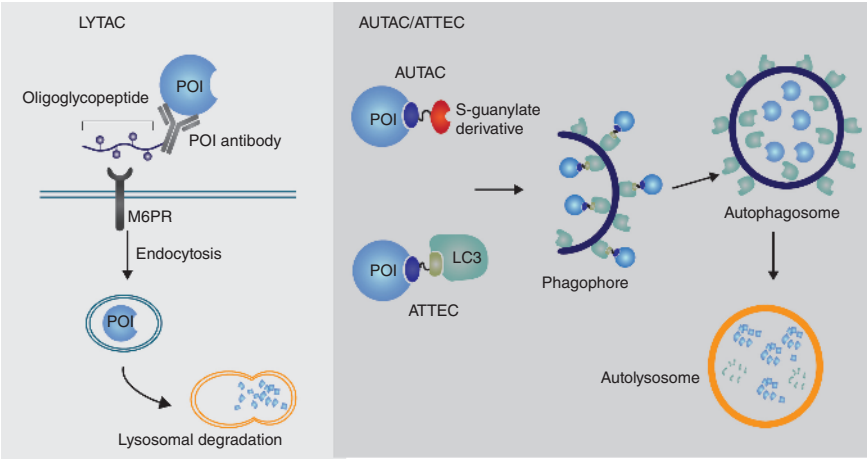


Figure 1.9 Schematic representation of LYTAC and AUTAC/ATTEC mode of action.

biological processes behind protein degradation. However, in their current state, many of these techniques do not yet provide the necessary features to advance to the preclinical stage.

1.2.7.1 Is TPD Just the Beginning? Bifunctional Modulators of Post-transcriptional Modification

Alongside the development of bifunctional degraders that induce ubiquitination (e.g. PROTACs), there have been significant advances in the design of bifunctional compounds that induce other types of protein modification. This includes phosphorylation, dephosphorylation, deubiquitylation, and acetylation, all of which have been achieved via bifunctional modulators analogous to PROTACs. In contrast to polyubiquitination, which results in degradation, these modulators can drive a variety of cellular phenotypes. While PROTACs that have made the transition to clinical development, the therapeutic potential of these agents remains in its infancy – no such bifunctional modulators have yet reached the clinic, despite their potential to modify disease states in cell-based models. As such, the field continues to develop rapidly.

In 2020, a collaboration between Genentech® (USA) and Pharmaron® (China) reported the development of bifunctional compounds that engage the ubiquitously expressed phosphatase PP1 using a peptidic warhead linked to a POI ligand binding to either protein kinase B (AKT) or epidermal growth factor receptor (EGFR), both of which are kinases of therapeutic interest [75]. In this case, the phosphatase warhead plays a similar role to the E3 ligase binding component found in PROTACs. The authors termed these compounds PhoRCs (phosphatase recruiting chimeras, Figure 1.10). In the first instance, they demonstrated a concentration-dependent effect upon the phosphorylation of AKT at threonine 303 and serine 473, both of which are linked to AKT activity. A second PhoRC compound using a targeting

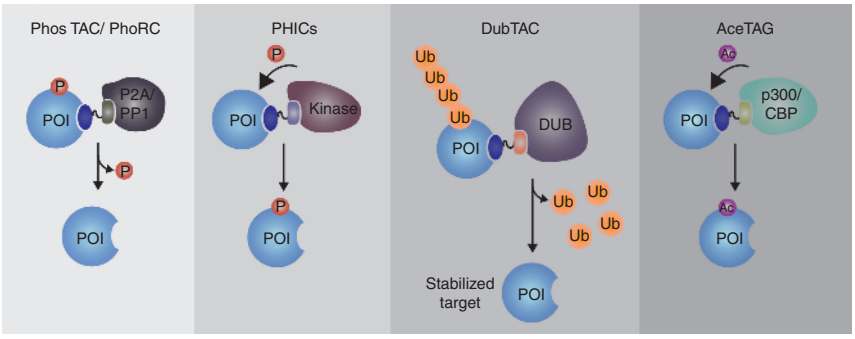


Figure 1.10 Schematic representation of post-translational modification modulators.

ligand derived from the EGFR inhibitor AZD-9291 (Tagrisso) similarly showed decreased levels of EGFR phosphorylation at the tyrosine 1068 residue. Although modest cell activity was demonstrated in this report, the peptidic warhead represents a potential drawback for the PhoRC modality as this feature limits their permeability. Phosphatase ligands with improved physicochemical properties may prove more optimal in the design of future PhoRC compounds.

A related platform recruiting phosphatases for the targeted dephosphorylation of neosubstrates was reported by Crews and coworkers [76]. Termed PhosTACs (phosphorylation targeting chimeras), these compounds rely on the use of tagged proteins – namely, a target phosphatase bearing an FKBP12 tag and a substrate bearing a HaloTag. In this way, a bifunctional compound linking a high-affinity FKBP12 ligand to a chloroalkane binding to the HaloTag can artificially induce ternary complex formation to drive dephosphorylation. The authors exemplified their platform with the fusion proteins for the phosphatase PP2A and either of two substrates: PDCD4 and FOX03a. In both cases, successful dephosphorylation was demonstrated in engineered HeLa cells by Western blot.

Around the same time as the publication of PhoRCs and PhosTACs, a team from the Broad Institute (USA) reported the inverse protein modification, in this case, co-opting kinase proteins for the phosphorylation of a neosubstrate [77]. These compounds were titled phosphorylation-inducing chimeric small molecules (PHICs), phosphorylation-inducing chimeric small molecules (Figure 1.10). In initial studies, the target protein for phosphorylation was the bromodomain-containing protein BRD4 for which a high-affinity ligand (S)-JQ1 and an inactive tool compound (R)-JQ1 were already available. To illustrate the generality of the PHIC modality, phosphorylation was demonstrated in cell-free experiments using two different kinases, AMP-activated protein kinase (AMPK) or protein kinase C (PKC), neither of which are known to interact with BRD4 under normal conditions. By linking a kinase ligand with (S)-JQ1, the resulting PHICs induced phosphorylation as demonstrated via immunoblotting for phospho-Ser484/488 of BRD4. In contrast, PHICs using the inactive isomer (R)-JQ1 showed no phosphorylation. Unfortunately, in cell-based experiments, these initial PHICs proved inactive, possibly owing to

differences in the subcellular localization of the BRD4 and the target kinases. The authors then generated PHICs that recruited AMPK kinase for the phosphorylation of the neosubstrate Bruton's tyrosine kinase (BTK) to successfully achieve intracellular phosphorylation. This report expands upon the toolbox of bifunctional modulators but also highlights the potential limitations of such approaches.

As with phosphorylation and dephosphorylation, recent advances have extended upon PROTAC-induced ubiquitination to enable the reverse transformation: deubiquitylation. Termed deubiquitinase-targeting chimeras (DUBTACs, Figure 1.10), these molecules enable targeted protein stabilization (TPS), the inverse of TPD [78]. Using a chemoproteomic approach pioneered in their labs, Nomura and coworkers first discovered a covalent ligand that binds to an allosteric site on OTUB1, a highly expressed deubiquitinating enzyme (DUB) that cleaves K48-linked ubiquitin chains. The OTUB1 recruiting ligand was then linked to a ligand derived from lumacaftor, a cystic fibrosis drug that engages the protein cystic fibrosis transmembrane conductance regulator (CFTR). The resulting DUBTAC compounds therefore induce ternary complex formation between OTUB1 and CFTR, driving deubiquitylation and stabilizing the CFTR protein in a disease-relevant bronchial epithelial cell line.

Acetylation is another post-transcriptional modification that has yielded bifunctional modulators termed AceTAGs (acetylation tagging system, Figure 1.10) [79]. In this case, the authors recruited the highly homologous lysine acetyltransferase (KAT) proteins p300/cyclic adenosine monophosphate response element CREB-binding protein (CBP) for which a high-affinity targeting ligand was already available. Echoing the earlier PhosTAC approach, the acetyltransferase recruiting warhead was linked to an FKBP12 ligand, which bound to an FKBP12-tag modified target protein. Using this platform, several POI targets could be acetylated in a dose-dependent manner in HeLa cells, including histone H3.3, p65/relA, and p53.

These studies illustrate the growing arsenal of methods to harness post-transcriptional modification for chemical biology. In principle, ternary complex formation between any two proteins could be utilized to induce a wide variety of phenotypes. Although such techniques have not yet made the transition from the laboratory to clinical development, there is clear potential for their future development in the pursuit of new therapeutic agents.

1.3 Conclusion and Outlook

The field of TPD and related technologies is undergoing a period of rapid development. Many such compounds are currently under evaluation in clinical trials or are expected to soon reach the clinic (Tables 1.2 and 1.3). Despite the many new molecules, modalities, and technologies already reported to date, we expect that the current wave of discovery represents just the "tip of the iceberg" with significant progress still to come.

However, despite the present explosion of research, unresolved problems and the need for caution remains. As the first PROTAC drugs proceed through clinical trials, we will come to understand the unique safety concerns associated with this

modality. It is still unclear whether recruiting E3 ligases might sequester these proteins from their natural roles within the cell (at the time of writing, primarily VHL and cereblon) to any clinically significant extent. If so, there may be a duality to the safety profile of bifunctional molecules, which incur on-target safety risks for both the protein target and the E3 ligase. There is also a double concern regarding the emergence of resistance, where mutations could emerge in either protein (POI or E3 ligase) to overcome engagement. Initial reports on preclinical resistance have primarily identified mutations of the E3 ligase complexes and other proteins involved in the degradation cascade [80–82]. However, at the current stage of investigation, these reports are inconclusive. The final picture of resistance mutations will be painted only after market approval and long-term investigation in real-world patient populations. For oncology, this could be of particular concern as resistance mutations are often a limiting factor for clinical efficacy and durability of response.

On the topic of safety, there is also a persistent concern in the use of these novel modalities for benign diseases. A quick glance at Tables 1.1–1.3 that describe agents in current clinical trials shows an overwhelming bias toward oncology drugs; this is partly driven by the different safety requirements in indications other than cancer. In addition, cereblon-derived PROTACs and molecular glues continue to bear close chemical similarity to thalidomide, which was withdrawn because of the tragic discovery of its teratogenic effects. The drug discovery community has a moral imperative to ensure that future clinical therapies are safe for patients beyond reasonable doubt.

There has been significant progress in overcoming the oral bioavailability concerns of PROTACs, both for cereblon-derived compounds (e.g. clinical candidates ARV-110 and ARV-471, Figure 1.8) and very recently for PROTACs that engage VHL [83, 84]. Despite these few successful examples, it remains difficult to rationally design compounds that achieve bioavailability. In contrast to traditional drug-like small molecules, the chemical features of PROTACs make this task much more challenging, and the "rules" for future drug design are still under active investigation [54].

Despite these concerns, there is still a great deal of optimism surrounding the field. The rapid evolution of new modes of bifunctional modulation (LYTACs, DUBTACs, etc.) has provided unprecedented access to new biological tools that will likely find applications in target validation, fundamental biology, and ultimately as clinical therapeutic agents. These bifunctional modulators might also follow in the footsteps of small-molecule drugs that eventually gave rise to protein and antibody drugs; thus, researchers have been experimenting with bifunctional proteins that engage targets in a similar manner to PROTACs to enable targeted degradation. The so-called "predator" system reported in 2020 is one such example, in which the authors link the E3 ligase Trim21 to a target protein, enabling proteasomal degradation [85]. Although these technologies are in their infancy, we expect significant progress in the development of protein-based bifunctional degraders in the future.

Despite being a relatively young field of research, TPD has already proven tremendously successful. A multitude of small biotech companies have emerged which specialize in all facets of TPD and beyond, including monovalent molecular glues, bivalent protein degraders, lysosome recruiting LYTACs, and autophagy-inducing molecules.

Many of these companies have already advanced toward clinical investigation of their first assets and have secured multibillion USD funding and/or built successful partnerships with major clinical-stage pharmaceutical corporations. Furthermore, most (if not all) large- or medium-sized pharmaceutical companies are conducting their own work in the field of TPD. The realization that the functional interaction of two protein targets can be successfully induced using synthetic small molecules has revolutionized the current paradigm in pharmaceutical research and sparked a "gold rush" in TPD drug discovery [86]. Although it is impossible to predict the clinical outcome and ultimate success of the all degrader molecules currently under investigation, we believe that the research landscape has already changed for the better, leading researchers to explore new technologies that seemed impossible only a few years ago. These are exciting times to work in TPD and we are keen to see which barriers will be broken and which new possibilities will emerge in the coming years.

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