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A beginner's guide to PROTACs and targeted protein degradation

Alessio Ciulli and **Nicole Trainor** (University of Dundee, UK)

Those with a keen interest in targeting proteins, from chemical biologists to drug hunters alike, cannot help but take notice that a new type of molecule is making waves across this research space. Proteolysis Targeting Chimeras (or PROTACs) are protein degraders, which utilize the cell's own waste disposal machinery to eliminate instead of inhibit a target protein. The key to PROTACs is their bifunctionality: they simultaneously bind a target protein and an E3 ligase protein, which then ubiquitylates the target, marking it for proteasomal degradation. This concept originated in the late 1990s and the first PROTAC was reported in 2001 by the laboratories of Craig Crews and Raymond Deshaies. However, interest in PROTACs did not pick up until 2015 when improved molecules were developed by the laboratories of Jay Bradner, Alessio Ciulli and Craig Crews. Ever since, PROTACs and the wider field of targeted protein degradation have expanded exponentially, with many groups around the world developing degraders as chemical tools to study proteins and as drug candidates for the treatment of diseases.

Why do we need protein degraders?

Before delving into how degraders work, let us first consider the benefits these molecules bring to the fields of chemical biology and drug discovery. A common technique to study a protein and its functions is to remove or deactivate it in cells using genetic knockout or knockdown, for example, using techniques such as CRISPR-Cas9 or RNA interference. Protein degradation by bifunctional molecules complements this approach without the need to manipulate the genome. Treatment of cells with a PROTAC allows for reversible control of protein levels. The effect is time and concentration dependent. To reverse the degradation effect, the PROTAC can be washed out from the cell culture (or stopped being dosed *in vivo*), allowing protein levels to bounce back.

In general, PROTAC-mediated degradation has been demonstrated to closely recapitulate protein depletion via genetic engineering. This is in contrast to inhibition of proteins by small molecules, because blocking a single activity or interaction of a target does not always have the same effect as removal of the protein altogether. This is because most proteins have multiple functions and interactions, and often work together with other molecules as part of large complexes. Some proteins are highly challenging to block with inhibitors and the choice of binding site is crucial to achieve a functional

response. In contrast, PROTACs can recruit proteins via any binding site. This has significant impact on drug discovery: proteins previously described as *undruggable* can now be investigated as to whether they are *degradable*, thus offering new opportunities to develop treatments for diseases with currently unmet medical need.

In addition to bringing new proteins into the druggable space, PROTACs also bring the possibility of improvements to the selectivity of protein targeting. For an increasing number of examples, the selectivity of degradation has been shown to be superior to that of inhibition, even when a promiscuous ligand is used as the warhead. A related advantage, which will be expanded upon in the next section, stems from the catalytic mechanism of action that PROTACs can exhibit. The ability of a single degrader molecule to remove many molecules of a target protein underpins its potent activity at very low concentrations, often well below the level required to saturate the target protein, which in turn reduces the incidence of off-target and potentially toxic effects.

How do PROTACs work?

It is the mode of action of PROTACs that makes them so unique and interesting to study. This mechanism is depicted in [Figure 1](#). The first hurdle, as with any exogenous molecule, is to cross the cell membrane. In fact,

SUMMARY

- PROTACs are molecules which cause protein degradation.
- Protein degradation is akin to gene knockout: it can provide insight into the cellular functions of proteins.
- Simultaneous binding of a target protein and an E3 ligase by a PROTAC within a key ternary complex facilitates ubiquitylation of the target protein.
- The ubiquitylated target protein is degraded by the proteasome.
- This mechanism of action can be catalytic.
- The selectivity of PROTAC-mediated degradation can be superior to inhibition.
- PROTACs may recruit targets via any binding site – functional and sustained inhibition is not required.
- Targeted protein degradation offers new opportunities to tackle previously undruggable proteins.

PROTACs tend to feature properties which can pose a challenge to permeability. For example, molecular weight and number of hydrogen bond donors and acceptors tend to be higher for PROTACs than for inhibitors. When a PROTAC is made of a target inhibitor, that increment comes directly from the addition of the linker and E3 ligase ligand. Uptake into cells occurs in competition with efflux, which is also commonly a problem for large molecules. The molecular properties and cellular uptake of PROTACs are therefore an important area of study. Despite the perceived adversities, many PROTACs are capable of efficiently entering cells and achieve sufficient concentration to allow them to effectively carry out their intended activity.

Once inside the cell, the next step is recruitment of the E3 ligase and the protein of interest. The PROTAC can form a binary (i.e., 1:1) interaction (reversible or irreversible) with either of these proteins first. Once bound, this binary complex will hunt down the other protein, which binds to the other end of the PROTAC, resulting in formation of a ternary (1:1:1) complex. The consequence of this ternary complex is the induced proximity of the target and E3 ligase, enabling formation of novel protein–protein interactions between target and ligase, which would not otherwise occur in the absence of the PROTAC. This complex can enable the E3 ligase to transfer a protein called ubiquitin to the target protein – in fact, multiple molecules of ubiquitin are transferred, both to multiple sites and sequentially to build chains. It is important to note that another class of enzymes called deubiquitinases can counteract this process by removing ubiquitin from target proteins. Nonetheless, this has not

been observed to hamper targeted protein degradation by PROTACs.

Polyubiquitylation is a vital cellular process, which contributes to the regulation of protein homeostasis. Ubiquitylation triggers the delivery of proteins to the proteasome, where they are degraded. At this point the PROTAC's journey is complete: it may dissociate from its bound protein, ready to repeat the cycle again. We have already mentioned how this catalytic mode of action via a ternary complex allows for the dosing of PROTACs at sub-stoichiometric concentrations relative to both target protein and E3 ligase. An example of a PROTAC which demonstrates this mode of action is the SMARCA degrader ACBI1, which binds the bromodomain of SMARCA2 with a K_D of 1.8 μM , but degrades SMARCA2 in cells almost completely at concentrations approximately 200-fold lower than its K_D . Another example that shows this, at the E3 ligase end, is the MZ1 PROTAC bearing a fluoro-hydroxyproline, which despite binding VHL with a K_D of 3 μM , degrades Brd4 with a DC_{50} of 10–30 nM, i.e., at 100-fold lower concentration than its K_D . In both these examples, the PROTACs exhibited high positive co-operativity. It is worth reflecting that this mechanism mitigates the difficulties PROTACs may face towards entering cells or binding to their individual targets. A small number of PROTAC molecules can go a long way.

Challenges in PROTAC development

Bifunctional molecules bear the task of binding two proteins simultaneously, so it is no surprise that they are significantly larger and more complex to work with than conventional small molecules that are meant to bind a single target. Let us begin with discussion of how this impacts the design and synthesis of PROTACs. A modular approach can be applied to PROTACs, whereby they can be broken down into their three constituent parts: the target ligand, the linker and the E3 ligase ligand. If we then imagine variations of each of these three components, the assembly of a library of PROTACs can take the form of a matrix, featuring all the possible combinations. The more variants of ligands and linkers there are, the more laborious this becomes for PROTAC synthesis. While the efficiency of this task can be improved in principle by high-throughput combinatorial chemistry techniques, it is often nonetheless an enormous undertaking to implement in practice.

To help focus the search for effective PROTACs, biological assays have been developed to interrogate each step in the PROTAC journey. Arguably, the study of PROTAC activity requires more assays and a greater diversity of techniques than inhibitor development. Degradation of a target protein can be monitored using

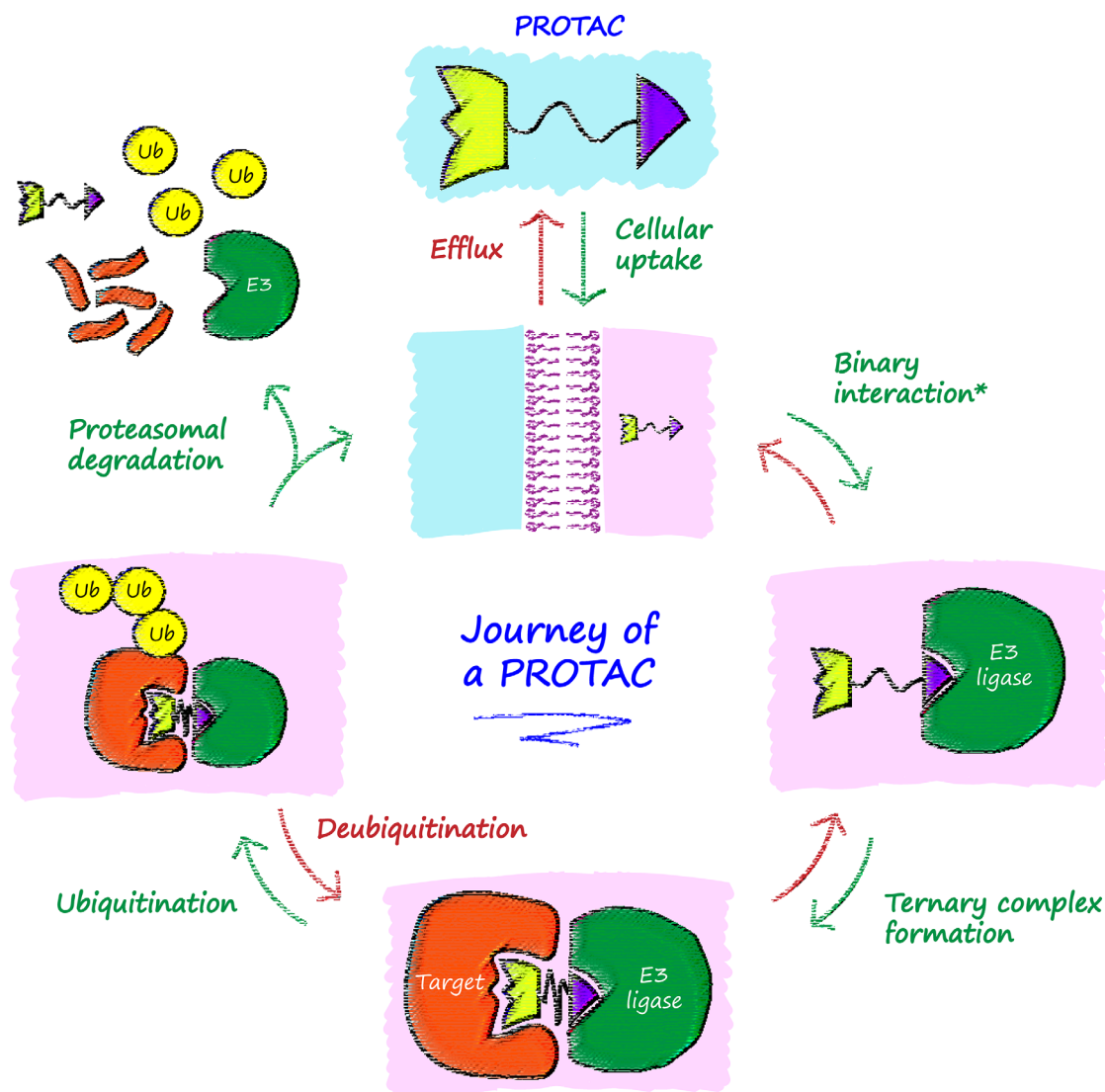


Figure 1. The Journey of a PROTAC. Cartoon depiction of the journey of a PROTAC molecule, starting with entry into the cell and resulting in degradation of a protein target. Once complete, the PROTAC is free to repeat the cycle. *The binary interaction step can occur with either E3 ligase or protein target.

quantification of the protein levels in PROTAC-treated versus DMSO-treated cells. Degradation can even be detected in live cells using microscopy to detect the target protein, provided it is tagged with a fluorescent or bioluminescent label. To complement the measurement of PROTAC activity in cells, several assays enable the study of PROTAC–protein interactions in purified as well as cellular environments. The affinity of a PROTAC for the E3 ligase or target protein may be measured using the same range of biochemical or biophysical assays commonly employed to study inhibitors, using both direct binding assays, e.g. surface plasmon resonance or isothermal titration calorimetry, and competition assays, e.g. fluorescence polarization. Such assays have been repurposed to interrogate ternary complexes:

pre-formed PROTAC–protein binary complexes can be titrated against the second protein species (or vice versa). Specifically to the PROTAC mode of action via the ternary complex, proximity-based assays are also invaluable to monitor PROTAC-mediated formation of ternary complex, e.g. using time-resolved fluorescence resonance energy transfer (TR-FRET) or the amplified luminescent proximity homogenous assay (Alpha-LISA). Ultimately, illuminating the structure of such ternary complexes at atomic level can provide invaluable information to their mode of action. A growing number of ternary complex structures have been reported in the literature since 2017, when the first one solved using X-ray crystallography was published by our laboratory; see Figure 2 for an example. These complexes provide a

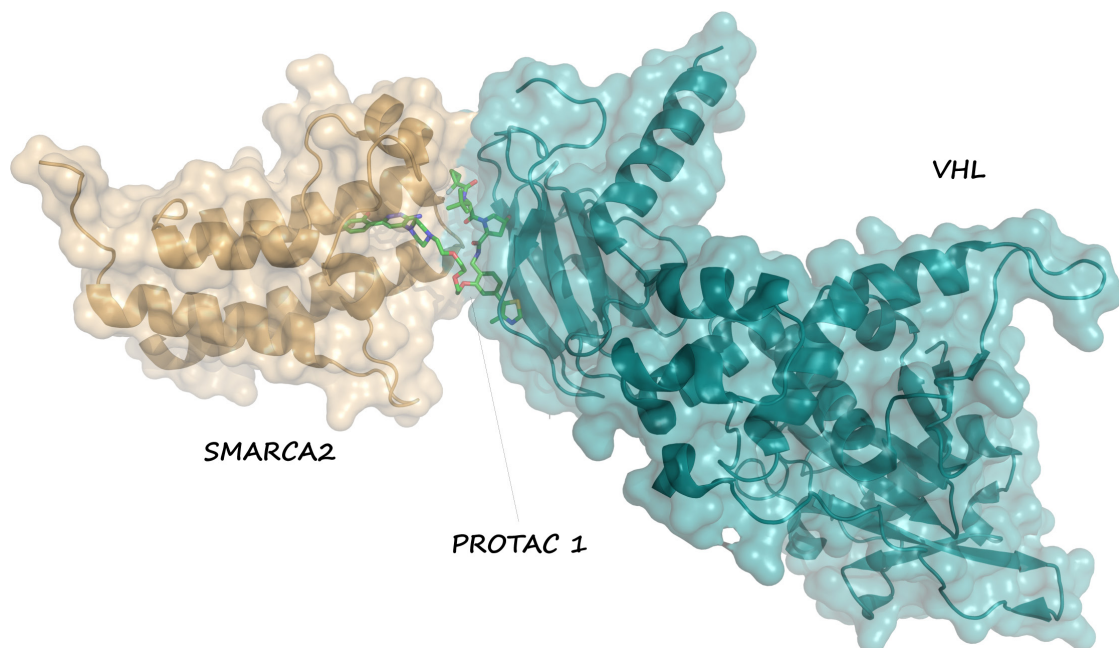


Figure 2. Example of a ternary complex. X-ray crystal structure of PROTAC 1 (green) bound in a ternary complex with the bromodomain of its protein target SMARCA2 (pale orange) and the E3 ligase VHL (teal).

means of prioritizing design ideas via structure-based design and lead the compound optimization process into areas of chemical space typically underexplored when screening combination of ligands and linkers without the guidance of such structure.

Another challenge in PROTAC development is choosing which E3 ligase to hijack. There are approximately 600 known E3 ligases, so which one is the best? There appears to be no “one size fits all” for every target protein. In fact, for some targets it has been observed that the choice of which E3 ligase to recruit is critical as to whether protein degradation will be observed

or not with an initial set of PROTAC compounds. Thankfully, in most cases it has been found that multiple E3 ligases are capable of effecting degradation of a given target when hijacked by PROTACs. Figure 3 shows the chemical structures of three PROTAC degraders for the same target protein (BRD4), designed from three different E3 ligases: von Hippel-Lindau protein (VHL), cereblon (CRBN) and inhibitor of apoptosis protein (IAP). To date, only a handful of E3 ligases have been co-opted for use with PROTACs, which is largely owing to the paucity of good-quality ligands for other ligases. An increase in the breadth of our chemical targeting

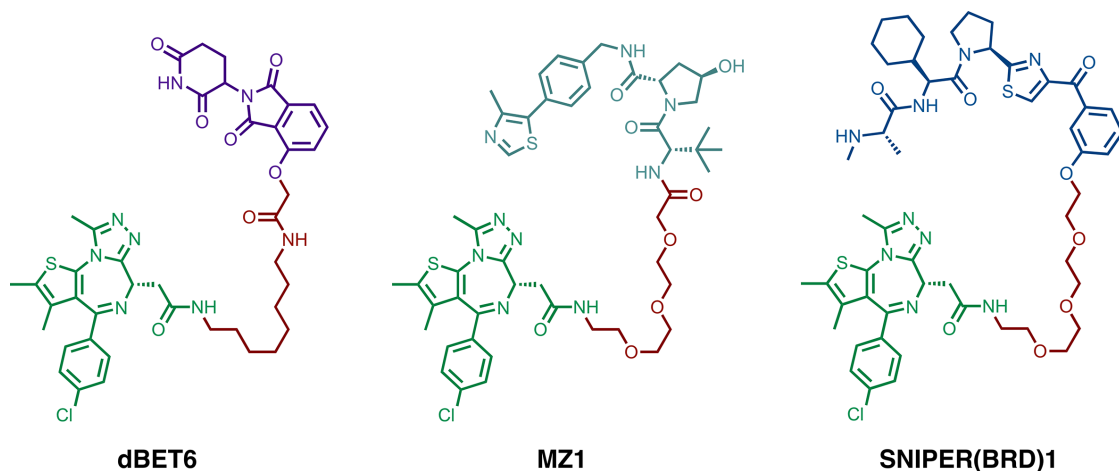


Figure 3. Examples of PROTAC chemical structures. Chemical structures of three BRD4 degraders: (left to right) CRBN-based dBET6, VHL-based MZ1 and IAP-based SNIPER(BRD)1.

of E3 ligases is expected to greatly expand the scope of targeted protein degradation.

An extra level of complexity is encountered when aiming to develop PROTACs as drugs. Most PROTACs fall outside what are traditionally considered drug-like space in terms of their physicochemical properties. It is worth re-iterating that the catalytic mode of action of degraders can compensate for hurdles such as cell permeability and this contributes towards achieving a suitable therapeutic dose. Indeed, some PROTACs have been shown to be orally bioavailable despite breaking conventional medicinal chemistry rules such as Lipinski's Rule of Five. Greater understanding of how PROTACs can be purposefully designed to be orally bioavailable is currently needed, as the discovery of orally bioavailable degraders has thus far been largely serendipitous.

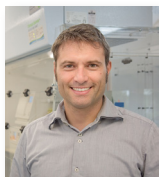
Where PROTACs are already making a difference

Over the past few years, the number of new PROTACs being reported in the literature has been rising exponentially. For chemical biology applications, many

have now been qualified and made available as chemical probes, allowing for the study of the importance and function of their target proteins within the cell. PROTACs are also advancing in drug discovery pipelines: although there are none so far which have been approved for use in patients, at least five have progressed to clinical trials. Arvinas is developing an androgen receptor degrader, ARV-110 (in phase II) for prostate cancer, as well as an estrogen receptor degrader, ARV-471 (phase I) for breast cancer. Additionally, Bristol-Myers Squibb also has an androgen receptor degrader, Kymera therapeutics an IRAK4 degrader for autoimmune disorders and Dialectic Therapeutics a BCL-xL PROTAC for various solid and liquid cancer indications – all compounds currently in phase I. These trials give hope that patients may have access to new therapies within the next few years. Early signs of tolerance and efficacy in these trials also reassure the many scientists worldwide who are currently striving to develop PROTACs to combat myriad diseases with unmet medical need. In summary, PROTACs are already transforming the way we study proteins and treat diseases, and the future of targeted protein degradation just keeps getting brighter. ■

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Nicole Trainor is a project lead scientist at the University of Dundee, working within the PROTAC drug discovery collaboration team established between Professor Alessio Ciulli and Boehringer Ingelheim (AC-BI). She joined the team in 2017 shortly after obtaining her PhD at Imperial College London, where she investigated the chemical biology of histone lysine methyltransferases under the supervision of Professor Matthew Fuchter. To enable these studies, she synthesized chemical probes and characterized their activity using mass spectrometry proteomics. Since joining AC-BI, she has contributed to the design and synthesis of PROTACs as well as evaluation of their selectivity through proteomics assays. Email: n.z.trainor@dundee.ac.uk.