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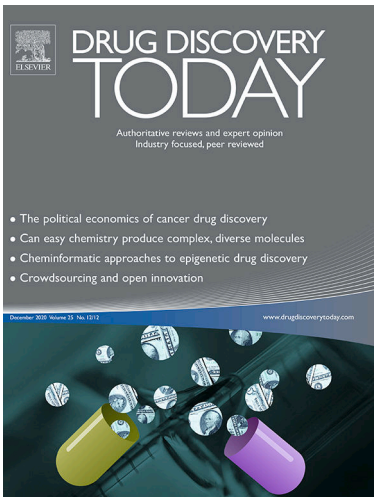

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Frontiers in PROTACs

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Frontiers in PROTACs

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Targeting protein–protein interactions (PPI) is a key focus in the development of new and emerging small-molecule therapeutics. Shallow interacting surfaces can render PPI targeting notoriously difficult. This leaves many therapeutically captivating targets 'undruggable'. Despite these challenges, there has been extraordinary progress circumventing this issue by hijacking the ubiquitin proteasome system (UPS) to target selected substrates for destruction using target-based degradation (TBD) strategies, including bifunctional molecules known as proteolysis-targeting chimeras (PROTACs). In this review, we discuss some of the most recent innovative concepts emerging from PROTAC research and related technologies.

Introduction

The past 25 years have seen an expanding effort into targeting the ubiquitin proteasome system (UPS) for therapeutic purposes. Proteasome inhibitors have achieved US Food and Drug Administration (FDA) approval,[1] proving UPS druggability and research has now extended to manipulating the pathway. Instead of traditional small-molecule inhibition, the UPS can be artificially hijacked to select and degrade specific target proteins of interest via target-based degradation (TBD) strategies. There are a multitude of TBD approaches that have been devised, including proteolysis-targeting chimeras (PROTACs), affinity-directed proteolysis missile systems (AdPROMs),[2] bioPROTACs,[3] molecular glues,[4,5] and selective estrogen receptor downregulators (SERDs).[6] In this review, we highlight novel concepts emerging within the PROTAC field.

PROTACS are bifunctional molecules comprising two ligands joined by a flexible linker. One ligand is the E3 ligase binder, and the other is the target protein binder. Since the first publication on this strategy almost 20 years ago,[7] there has been a huge resurgence of interest within the past 5 years, with Arvinas bringing the first PROTAC molecule into Phase I/II clinical trials.[8] Here, we cover some newly evolving ideas within the PROTAC field (Figure 1), including peptide-based PROTACs (pPROTACs), macrocyclic PROTACs, light-activated PROTACs, and homo-PROTACs. Furthermore, we highlight developing strategies based on the same underlying PROTAC principle yet using cellular phosphorylation-dependent systems.

Advances in peptide-based PROTACs

pPROTACs were an attractive strategy during the early stages of PROTAC development, with studies able to exploit known PPIs for target recruitment. In fact, the first successful PROTAC-mediated degradation of a protein was achieved using a pPROTAC.[7] Despite initial success, the pharmacological limitations of pPROTACs, including peptide instability and poor cell permeability, led to the research field refocusing attention toward small-molecule PROTACs.[8–10] More recently, a resurgence into pPROTACs has become apparent, spurred by research interests into 'undruggable' targets.[11] Chu *et al.* developed a pPROTAC to knockdown specifically endogenous levels of the non-enzymatic intrinsically disordered protein Tau.[12] The protein aggregate α-synuclein is another intrinsically disordered protein of interest, linked to plaque formation in a variety of neurodegenerative disorders, including Parkinson's disease.[13] Typically, α-synuclein plaques are cleared via the lysosomal system.[14] However, because this pathway is also disrupted by α-synuclein aggregation, its therapeutic potential is debatable.[15] Qu *et al.* opted to target α-synuclein by promoting its degradation via the ubiquitin system, successfully downregulating α-synuclein in SK-N-SH neuroblast cell models.[16] This group used a pPROTAC comprising three components, α-synuclein binding, E3 binding, and a c-terminal cell-permeability component encompassing the HIV-1 TAT (YGRKKRRQRRR) sequence.

The addition of a cell-permeable sequence has become a standard practice to improve pPROTAC modality. Ma *et al.* also chose to use this cell-permeable pPROTAC strategy to target the oncoprotein cell cycle-related and expression-elevated protein in tumor (CREPT) as a treatment for pancreatic cancer.[17] A lack of reported inhibitors led to the group adopting a *de novo* approach, designing an alpha helix ligand to mimic the leucine-zipper-like motif (lys266-val286) found in CREPT. This helix formed a homodimer with the CREPT coiled coil terminus (CCT) domain and was linked to a Von Hippel-Lindau tumour suppressor (VHL) ligand to form a functional pPROTAC, before being further optimized with the addition of a C-terminal transport pentapeptide (RRRRK) to improve its cell permeability. Although promising, the pPROTAC modality still contains many other limitations requiring consideration, such as peptide instability. Stabilizing pPROTAC via conformational constrictions could provide a potential solution, as demonstrated by Jiang *et al.*[18] However, this and other strategies to improve the pPROTAC modality are discussed in more detail elsewhere.[11]

Structure-based PROTAC design of a macrocyclic PROTAC

The Ciulli group investigated the structural basis of the complex formation between E3 ligase, target substrate, and PROTAC. They solved the X-ray structure of pVHL, a BET bromodomain and a selected PROTAC (MZ1) at 2.7 Å resolution.[19] The crystal structure revealed the architecture of the ternary structure. Based on this, they used a structure-based design strategy to enhance the selectivity to target the BET family member Brd4. Importantly, this study demonstrated that evaluating the ternary structure of a PROTAC complex can inform rational design of the PROTAC molecule to improve its specificity. Building on this work, they pursued

macrocyclization of the PROTAC.[20] Macrocyclization of small-molecule ligands is an attractive endeavor because it is favorable thermodynamically to adopt a bound state because of reduced conformational flexibility.[21,22] The macrocyclization of MZ1 was achieved by adding an additional linker between the two ligand moieties (Figure 2, compound **1**). Testing of the binding affinity and thermodynamics of MacroPROTAC 1 to VHL found K_D and ΔH values comparable to those of MZ1 (47 nM and –6.7 kcal mol^{-1}, compared with 66 nM and –7.7 kcal mol^{-1} from MZ1). However, when testing the binding affinities to $Brd2^{BD1}$ and $Brd4^{BD2}$, weaker affinities were detected. Interestingly there was a stronger affinity to $Brd4^{BD2}$ compared with $Brd2^{BD1}$, indicating that the macrocycle PROTAC can discriminate between highly homologous BET bromodomains.

Light-activated PROTACs

Akin to traditional small molecules, PROTACs still have the undesired property of having off-target systemic effects. The growing field of photopharmacology aims to circumvent this by using photoswitchable ligands to provide optical control over the desired target.[23] The application of photopharmacology to PROTACs was first published by the Trauner group.[24] They incorporated the photoswitchable azobenzene group into CRBN ligands for the light-controlled degradation of BET proteins and FKBP12. Continued irradiation of the photo-PROTAC ('PHOTAC') with 390-nm light resulted in the knockdown of the desired proteins via the active *cis* conformation of the PHOTAC. In a complementary approach, Pfaff *et al.* considered how a 3 Å difference in linker length can crucially affect the activity of a PROTAC.[25] The authors intuitively noted that the 3 Å difference is similar to the 3-4 Å topological distance difference between *cis* and *trans* azobenzenes. It was theorized that a PROTAC with a *cis* azobenzene linker would be inactive because it would be too short to reach the binding pocket of the second protein, whereas the *trans* isomer would be at the optimal length to form the ternary complex. Taking this into account, the authors designed a biologically stable *cis* azobenzene linked PROTAC that could reversibly photo-isomerize. The resulting PROTAC comprised a stable *cis o*-F4-azobenzene flanked by the VHL and the JQ1 ligands (Figure 2, compound **2**). The *cis*/ *trans* switch occurred at 415 nm with a 95% yield of the *trans* isomer. Irradiation at 530 nm retrieved 68% of the *cis* isomer in the photo-stationary state. Upon cellular testing, the photoPROTAC was stable and spatiotemporal control of its activity was achieved.[26] Shortly afterwards, work on another light-controlled azo PROTAC was published.[26] The CRBN ubiquitin ligase was used to degrade the VCR-ABL complex selectively in a similar photochemical manner, but with the *cis* isomer being the active form.

Another alluring strategy to incorporate optical control over PROTACs is to include a photocaged group (pc-PROTAC). Xue *et al.* incorporated the 365-nm photocleavable dimethoxy-2-nitrobenzyl (DMNB) group into the thalidomide and JQ1 moieties to form two separate photocaged (pc)-PROTACs. The photocaged JQ1 pc-PROTAC (Figure 2, compound **3**) was the more active and stable variety. The pc-PROTAC demonstrated controlled and potent Brd4 degradation in live cells and zebrafish.[27] Liu *et al.* subsequently harvested optical control over protein degradation by adding a photolabile caging group to pomalidomide. The addition of a nitroveratryloxycarbonyl (NVOC) on the glutarimide nitrogen of pomalidomide blocked a key hydrogen bond interaction with the backbone carbonyl group of His380 in CRBN. This opto-pomalidomide was ineffective at binding to CRBN. The NVOC group underwent photolysis at 365 nm and released the active pomalidomide. This photocaged strategy provided optical control over the degradation of Ikaros zinc finger transcription factors.[28] A photocaging group has been used on the VHL ligand to control PROTACs optically.[29] The crucial hydroxyl group on the VHL ligand was functionalized with a photocleavable DNMB group. The caged degrader was attached via the flexible linker to the BRD4 degrader JQ1. Intracellular activation of this photocaged PROTAC using 365 nm light resulted in VHL-mediated proteasome-induced removal of BRD4.

E3 versus E3

E3 ligases themselves are targets for a multitude of therapeutic areas, including cancers,[30] neurological disorders,[31] and antimalarials.[32] VHL and Cereblon are two popular E3 ligase degraders and have significant biological interest. The transcription factor hypoxia inducible factor 1α (HIF-1α) is the main substrate for VHL. HIF-1α is continually expressed and degraded by the proteasome in normal oxygen conditions. Prolyl hydroxylase domain (PHD) enzymes hydroxylate HIF-1α, targeting it for ubiquitin-mediated proteasomal destruction by VHL. Small-molecule inhibition of the pathway reproduces low oxygen conditions by upregulating genes involved in the hypoxic response.[33] This pathway has been targeted by the development of PHD inhibitors for chronic anemia associated with chronic kidney disease.[34] However, issues with selectively led to interest in targeting downstream of the PHD enzymes to VHL.[35] An iterative medicinal chemistry process led to potent VHL inhibitors,[36–38] which have subsequently served PROTAC degraders. This spawned the interesting concepted 'Homo-PROTACs' devised by the Ciulli group.[39] Homo-PROTACs are PROTACS designed to dimerize E3 ligase and pit themselves against each other for degradation. Using crystallographic evidence of bound VHL inhibitors VH032 and VH298, solvent-exposed regions of the ligands could be identified where derivatization would not result in a strong change of affinity. Three designs of homo-PROTACs were synthesized: symmetric via a connection on the left-hand acetyl groups (Figure 3a), symmetric via a connection on the right-hand phenyl group (Figure 3b), and asymmetric via the acetyl on one and phenyl on the other (Figure 3c). Each design comprised either three, four, or five polyethylene glycol (PEG) chains as the linker unit. Upon biological testing, the most potent degrader was found via connecting the acetyl groups. Steinebach *et al.* used the same homodimerizing concept to self-direct the ubiquitination and degradation of CRBN.[40] Building upon this idea, the Ciulli group designed, synthesized, and evaluated 'heterodimerizing' PROTACS. These are PROTACs that highjack two ligases against each other,[41] in this case CRBN versus VHL. When tested at 1 μM, compound **4** (Figure 2) was the most potent heterodimerizing PROTAC in degrading CRBN. When testing the compound at the lower concentration of 10 nM, diminished levels of VHL were observed. This suggests that the specificity of each ligase in heterodimerizing PROTACs is determined by concentration.

Beyond PROTACs: targeting other cellular pathways

The idea that PROTACs modulate post-translational modifications has sparked interest in using bifunctional molecules to take control over other cellular processes. Yamazoe *et al*. described a chemical strategy to induce dephosphorylation of kinases. This was carried out using bifunctional molecules, which promoted the interaction between protein phosphatase 1 (PP1) and oncogenic kinases.[42] To

test the concept, a HaloTag-PP1-FLAG vector was transfected into LnCAP cells. An AKT inhibitor was modified with a chloroalkyl group that would react with the HaloTag.[43] When these cells were treated with the chloroalkyl-modified AKT inhibitor, reduced AKT levels were observed. This was assumed to be the result of the HaloTag-PP1-FLAG fusion protein binding to the chloroalkyl AKT inhibitor, promoting PP1-induced dephosphorylation of AKT. To develop the bifunctional molecule, the covalent AKT inhibitor was connected to a PP1-binding synthetic peptide via a polyethylene bridge.[44] The initial bifunctional compounds containing the 20-amino acid peptide showed dephosphorylation in a recombinant *in vitro* biochemical system. However, when applied to LnCAP cells, insufficient cell permeability and/or proteolytic instability were observed. After shortening the 20-amino acid peptide to the minimal RVSF PP1 recognition motif (Figure 2, compound **5**), a decrease in phosphorylated AKT at Thr308 and Ser473 was found in LnCAP cells. The concept was also tested with epidermal growth factor receptor (EGFR). Using HaloTag-PP1-FLAG transfected HCC827 cells dosed with a chloroalkyl-modified EGFR inhibitor (AZD-9291), a significant reduction in EGFR phosphorylation at Tyr1068 occurred.

Controlling phosphorylation is just one example of how new ideas for cellular pathway manipulation to dispose of proteins are arising. Other examples include autophagy-targeting chimeras (AUTACs).[45] AUTACs are bifunctional chimeras that target proteins and dysfunctional mitochondria for autophagy. Extracellular and membrane proteins have been targeted via nonproteasomal destruction pathways using lysosome-targeting chimera (LYTACs).[46,47] Boyd *et al.* provide a more extensive perspective on the use of bifunctional molecules in biology.[48]

Concluding remarks and outlook

The concept of targeted protein degradation using PROTACs has sparked great interest within academia and industry. As research progresses, so does our mechanistic understanding and recent studies further highlight exciting new options for finely tuned control over these bifunctional molecules. Furthermore, the application of PROTACs to other scientific areas is ever-expanding, and PROTAC technology is being exploited as a chem-proteomic tool in mapping the degradable kinome.[49] However, there are topics that warrant further research. For example, the HECT ligases are yet to be trialed in PROTAC-based strategies. HECT ligases intrinsically attach ubiquitin to the protein of interest (POI), unlike the RING ligases, which act as a mediator in bringing E2 and the POI together. Therefore, it would be interesting to establish whether these HECT E3 ligases would still function as PROTAC degraders.

Hijacking the UPS to dispose of proteins in a catalytic manner, including 'undruggable' proteins, is a powerful strategy that is now being validated in the treatment of human disease. Arvinas currently has two PROTACs in clinical trials: (i) the androgen receptor-targeting ARV-110 is in Phase II for the treatment of metastatic castration-resistant prostate cancer; and (ii) the estrogen receptor (ER) targeting ARV-471 is in Phase I for the treatment of $ER^+/HER2^-$ breast cancer. The results of these trials are eagerly anticipated, and will pave the way for the wider clinical application of PROTACs.

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Figure 1. Schematic summary of new and emerging proteolysis-targeting chimera (PROTAC) strategies. A PROTAC is a bifunctional molecule with an E3 ligase-binding region (red triangle) linked to a substrate-targeting moiety (a blue box). Top left to right: PROTAC activity can be controlled by incorporation of a photoswitchable linker; cell-permeable PROTACs containing peptides as the substrate-targeting modality have been developed; degraders can target themselves using homo-PROTACs. Bottom left to right: Photolabile groups incorporated into the PROTAC adds a layer of control to targeted degradation; macrocyclization of a PROTAC via the linker.

Figure 2. Structures of selected proteolysis-targeting chimeras (PROTACs). The E3-binding ligand moiety is represented in blue, the substrate ligand in red, and the linker in black. Compound **1:** Macrocyclic PROTAC MZ1. Macrocyclization of the MZ1 PROTAC had minimal effects on VHL binding, but resulted in decreased $Brd2^{BD1}$ and $Brd4^{BD2}$-binding affinities. However, selectivity to $Brd4^{BD2}$ over $Brd2^{BD1}$ was observed. Compound **2:** diazobenzene-functionalized light-activated PROTAC. The diazobenzene switch is incorporated into the linker (orange). Upon irradiation of the inactive *cis* form with 415-nm light, the PROTAC photoisomerizes to the active *trans* form. Compound **3:** photocaged PROTAC (pc-PROTAC). The dimethoxy-2-nitrobenzyl group (orange) is incorporated into the JQ1 moiety. Gain of function is achieved upon cleavage at 365-nm light. Compound **4:** Cereblon versus Von Hippel-Lindau (VHL) PROTAC. Differential degradation of the ligases was observed at different concentrations. Compound **5:** bifunctional compound inducing dephosphorylation of AKT. The protein phosphatase 1 (PP1)-targeting ligand is shown in blue and the AKT inhibitor in red.

Figure 3. Chemical structures of the three strategies for homo-proteolysis-targeting chimera (homo-PROTAC) design with VH032. **(a)** Linker attached via the terminal acetyl groups. **(b)** Linker attached via the phenyl groups. **(c)** Linker attached via the acetyl and phenyl groups. Linking the acetyl groups resulted in the most=potent degrader.

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Highlights

- PROTACs hijack the ubiquitin proteasome system to eliminate select disease proteins.
- Cell permeable peptide PROTACs have been developed to target oncoproteins.
- Macrocyclic and light controlled PROTACs offer specificity and spatiotemporal control.
- PROTACs have been developed to act as homo-dimerising compounds to pit E3 ligases against themselves
  The PROTAC principle can now be applied to cellular phosphorylation systems.

Short teaser: PROTACs as targeted protein degradation tools represent an attractive method for disposing of unwanted proteins that cause human disease. In this mini review we present some intriguing emerging concepts in PROTAC design geared towards improving their effectiveness and versatility.