PROTACs: An Emerging Targeting Technique for Protein Degradation in Drug Discovery

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Proteolysis-targeting chimeric molecules (PROTACs) represent an emerging technique that is receiving much attention for therapeutic intervention. The mechanism is based on the inhibition of protein function by hijacking a ubiquitin E3 ligase for protein degradation. The hetero-bifunctional PROTACs contain a ligand for recruiting an E3 ligase, a linker, and another ligand to bind with the protein targeted for degradation. Thus, PROTACs have profound potential to eliminate “undruggable” protein targets, such as transcription factors and non-enzymatic proteins, which are not limited to physiological substrates of the ubiquitin-proteasome system. These findings indicate great prospects for PROTACs in the development of therapeutics. However, there are several limitations related to poor stability, biodistribution, and penetrability in vivo. This review provides an overview of the main PROTAC-based approaches that have been developed and discusses the promising opportunities and considerations for the application of this technology in therapies and drug discovery.

1. Introduction

The pathogenesis of malignant tumors, a great threat to human health, is a biomedical research target for the development of anti-cancer therapies. Although a variety of therapeutic targets have been identified in the human proteome, a majority of these targets, such as transcription factors, scaffold proteins, and non-enzymatic proteins, cannot be targeted for the development of viable small molecules that inhibit the functions of these proteins by interfering with their interactions with other proteins or nucleic acids. Thus, these proteins are considered “undruggable” targets.[1,2] Furthermore, high drug dosages and continuous exposure to small molecule inhibitors that target proteins with binding sites, such as enzymes and receptors, are usually required for better therapeutic efficacy, which may inadvertently cause off-target effects.[3] As an alternative, current techniques, such as the use of antisense oligonucleotides, RNAi and the emerging CRISPR–Cas9 technology,[4] hold great potential in the elimination of disease-causing proteins. However, the clinical application of these approaches is limited due to a lack of temporal control and undesirable off-target effects.[3] To target a broader range of proteins at the post-translational level, Raymond J. Deshaies’ group developed the first proteolysis-targeting chimeric molecule (PROTAC), which hijacks the ubiquitin-proteasome system (UPS) for protein destruction.[6] The UPS is one of the major pathways responsible for protein degradation for the maintenance of cellular homeostasis. This system comprises ubiquitin, proteasome, three enzymes and intracellular target proteins, designated substrates, and plays key roles in various important biological processes, such as cell cycle progression, signal transduction, maintenance of genome integrity, and tumorigenesis.[7] Protein ubiquitination is an ATP-dependent enzymatic reaction mediated by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (Figure 1). The process involves three steps: first, an E1 activates a ubiquitin molecule by forming an acyl amide acid mediator at the C-terminus of the ubiquitin in an ATP-dependent reaction. Second, the activated ubiquitin is then transferred to an E2. Finally, an E3 ligase, which recognizes and recruits the protein substrate, catalyzes the transfer of the ubiquitin from the E2 to a lysine residue on the substrate via the formation of a covalent bond. Ubiquitin itself contains seven lysine residues and an N-terminal methionine residue, on which polyubiquitin chains are formed after multiple cycles of this reaction. K48- or K11-linked polyubiquitinated proteins are predominantly recognized and destroyed by the proteasome.[8] For the first time, by using engineered E3 ligases (CDC4 and βTrCP), Zhou et al.[9] successfully directed the degradation of stable cellular proteins, which were not physiological substrates of CDC4 or βTrCP, in both yeast and mammalian cells, suggesting that engineered E3 ligases can knock out any cellular protein of interest, which opened the field of PROTAC development.
2. PROTAC Technology: Hetero-Bifunctional Molecules Targeting Proteins for Degradation

PROTACs are hetero-bifunctional molecules that contain a ligand for recruiting an E3 ligase, a linker, and another ligand to bind to the target protein for degradation (Figure 2a).[2,3,10–17] Thus, PROTACs are capable of specifically binding with both the target protein and the E3 ligase simultaneously and hijacking them together, leading to the formation of a ternary complex. As a result, by recruiting an E3 ubiquitin ligase, ubiquitin can be transferred from an E2 to the target protein, which is eventually degraded by the proteasome (Figure 2b).[2,1,10–17]

The first PROTAC, developed by Sakamoto et al.,[6] consisted of a phosphopeptide (DRHDpSGLDpSM) derived from NF-κB inhibitor-α (IκBα) to recruit SCFβ-TrCP E3 ligase; ovalacin (OVA), which can covalently bind to the active site (His-231) of methionine aminopeptidase-2 (MetAP-2), targeting MetAP-2 for ubiquitination; and a linker that connects the phosphopeptide and OVA (Figure 2c and d). The phosphopeptide is sufficient and necessary for binding of the F-box protein β-TrCP with SCFβ-TrCP.

Figure 1. The ubiquitin-proteasome system. Ubiquitin is activated by a ubiquitin-activating enzyme (E1) via an ATP-dependent reaction and then transferred to the cysteine of a ubiquitin-conjugating enzyme (E2). A ubiquitin ligase (E3) finally catalyzes the transfer of the ubiquitin from the E2 to a substrate. The polyubiquitinated substrate is finally recognized and degraded by the proteasome.

E3 ligase, the substrate recognition component of the E3 ligase complex. The constitutive activation and F-box-protein-mediated substrate selection of SCF complexes make it possible to eliminate specific proteins. Indeed, there is substantial evidence that this chimeric PROTAC serves as a bridge to recruit MetAP-2 to SCFβ-TrCP, resulting in the ubiquitination and degradation of MetAP-2, which is not known to be a physiological substrate of the SCF complex. Specifically, MetAP-2, which specifically binds to the OVA moiety in a dose-dependent manner, can be recruited to SCFβ-TrCP in Xenopus egg extracts. Moreover, endogenous SCFβ-TrCP degrades MetAP-2-PROTAC, but not free MetAP-2, in a time-dependent manner. SCFβ-TrCP triggers polyubiquitination of MetAP-2, whereas the proteasome inhibitor L1nL or epoxomicin inhibits the degradation of the MetAP-2-PROTAC complex. Therefore, the PROTAC hijacks SCFβ-TrCP to target the ubiquitination and degradation of MetAP-2. Thereafter, two additional PROTACs were developed, targeting the estrogen receptor (ER) and androgen receptor (AR), important receptors in the progression of breast and prostate cancers, respectively.

3. Cell-Permeable PROTACs: The First Ones Entering Cells

The first in vivo PROTAC was developed by Craig M. Crew’s group in 2004. In this PROTAC, a seven-amino-acid sequence (ALAPYIP), replacing the IκBα-phosphopeptide component, is conjugated to an artificial ligand (AP21998) to target (F36V)FKBP12 proteins (Figure 3).[19] ALAPYIP is the minimal amino acid sequence of the hypoxia inducible factor 1α (HIF1α) and is recognized by the von Hippel-Lindau tumor suppressor protein (VHL), the substrate recognition component of CRL2VHL E3 ubiquitin ligase.[20] Under normoxic conditions, HIF1α is hydroxylated by a proline hydroxylase at P564, leading to the constant recognition and polyubiquitination of HIF1α by CRL2VHL E3 ubiquitin ligase, which ensures low levels of HIF1α.[21,22] The artificial ligand AP21998 binds to the mutant FKBP12 protein, instead of the wild-type protein, specifically and orthogonally[23,24] to facilitate the degradation of (F36V)FKBP12 by CRL2VHL E3 ubiquitin ligase (Figure 3a). More importantly,
an eight-poly-D-arginine tag, which is located at the carboxyl terminus of ALAPYIP, improves cell permeability and prevents nonspecific proteolysis (Figure 3b).\textsuperscript{[25,26]}

This cell-permeable PROTAC has been shown to destroy its target proteins in a cellular context. After treatment with the PROTAC targeting (F36V)FKBP12, a loss of green fluorescence was observed in HeLa cells stably expressing EGFP-(F36V)FKBP12, but the fluorescence was retained in 786-O cells, which cannot produce the VHL protein. The degradation of (F36V)FKBP12 mediated by the PROTAC was also confirmed by

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**Figure 2.** Schematic diagram of PROTAC. a) A PROTAC molecule consists of a ligand for recruiting an E3 ubiquitin ligase, a linker, and a ligand binding to the target protein. b) The PROTAC binds with both the target protein and the E3 ligase simultaneously to induce the formation of a ternary complex. The target protein is then polyubiquitinated and undergoes proteolysis. A schematic diagram (c) and chemical structure (d) of the first PROTAC. This PROTAC contains a phosphopeptide derived from IκBα to recruit the E3 ubiquitin ligase SCF\textsuperscript{β-TrCP}, a linker and ovalicin to bind with MetAP-2, which can trigger MetAP-2 ubiquitination. The ligand to bind to target protein is shown in brown box, and the ligand to recruit E3 ubiquitin ligase is shown in blue box.
Western blotting.\textsuperscript{[19]} In addition, the AR-targeting PROTAC with DHT conjugated to the same HIF1\(\alpha\)-polyarginine peptide sequence causes a significant decrease in GFP-AR levels in HEK293 cells.\textsuperscript{[19]} Interestingly, another peptide-based PROTAC was developed to antagonize and destroy the cancer-forming X-protein of the hepatitis B virus (HBV). This PROTAC, consisting of the oligomerization domain of X-protein fused to the oxygen-dependent degradation (ODD) domain of HIF1\(\alpha\) and a polyarginine cell-penetrating peptide (CPP) at the N-terminus, induces the degradation of the total X-protein via a proteasome-dependent mechanism.\textsuperscript{[27]}

In summary, the development of cell-permeable PROTACs is a crucial breakthrough in the development of PROTAC technology and provides the possibility of targeting disease-causing proteins in vivo, leading to promising applications in cell biology and especially in drug development.

4. PhosphoPROTACs: A Conditional Knockdown Strategy

4.1. The Principle of PhosphoPROTACs

Phosphorylation is an important post-translational protein modification that regulates the activity of proteins, especially kinases. For instance, when receptor tyrosine kinase (RTK) pathways are activated, several tyrosine (Tyr) residues of RTKs are phosphorylated, leading to the recruitment and phosphorylation of downstream targets with phosphotyrosine-binding (PTB) andSrc homology 2 (SH2) domains to further activate the cascade.\textsuperscript{[28]} Epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER2), two proteins from RTK pathways, are frequently found to be overactivated in lung and breast cancers, respectively. Therefore, selectively
targeting recruited proteins with PTB or SH2 domains for degradation can inactivate tyrosine kinase signaling to suppress tumor cell proliferation. Based on this idea, a phospho-dependent proteolysis-targeting chimera (phosphoPROTAC) was developed (Figure 4). It is conceivable that this phosphoPROTAC has low toxicity to normal cells, since it only suppresses activated RTK signaling, which usually occurs in cancer cells. In addition, this phosphoPROTAC would not induce mutations in the RTK leading to drug resistance and would bypass mutant RTKs found in cancer cells, since this phosphoPROTAC inactivates RTK signaling pathways by targeting downstream effector proteins for degradation.

PhosphoPROTACs contain an RTK phosphorylation sequence, a peptidic VHL-binding fragment, a poly-D-Arg sequence for increasing cell permeability, and a linker. On one hand, the Tyr residue of the RTK phosphorylation sequence is phosphorylated by activated RTK, which is followed by the recruitment of proteins with PTB and SH2 domains. On the other hand, the peptidic VHL-binding fragment, upon hydroxylation of the proline residue, recruits the E3 ubiquitin ligase CRL2VHL to induce polyubiquitination and subsequent degradation of proteins with PTB and SH2 domains, eventually resulting in the inactivation of tyrosine kinase signaling (Figure 4a). Thus, although the use of phosphoPROTACs is a conditional knockdown strategy that depends on kinase activity, phosphoPROTACs could interfere with protein functions rapidly and directly.

4.2. Two PhosphoPROTACs: TrkAPPFRS2α and ErbB2PPPI3K

Hines et al.[29] synthesized two phosphoPROTACs, which utilize tyrosine phosphorylation sequences derived from either the

![Figure 4. PhosphoPROTACs. Schematic diagram (a) and chemical structures (b) of phosphoPROTACs. The PhosphoPROTAC is composed of an RTK phosphorylation sequence, VHL-binding sequence and a poly-D-Arg sequence connected via a linker, illustrated in the circle with broken line. Once RTK is activated, the Tyr of the RTK phosphorylation sequence is phosphorylated and then recruits and activates proteins with PTB and SH2 domains. Meanwhile, the peptidic VHL-binding fragment, upon hydroxylation of the proline residue, recruits the E3 ubiquitin ligase CRL2VHL to induce polyubiquitination and subsequent degradation of proteins with PTB and SH2 domain, thus inactivating tyrosine kinase signaling. The ligands to bind to target proteins are shown in brown boxes, and the ligand to recruit E3 ubiquitin ligase is shown in blue box.](image-url)
nerve growth factor receptor TrkA or the neuregulin receptor ErbB3, to recruit specific target proteins (Figure 4b). Specifically, TrkAP3FRS2α comprises a TrkA-derived decapeptide sequence (IENPQYFSDA) with a TrkA autophosphorylation site on the central tyrosine to recruit the neurotrophic signaling effector fibroblast growth factor receptor substrate 2α (FRS2α) and a seven-amino-acid sequence derived from HIF-1α for binding to the E3 ligase CRIL2/VHL. Similar to previous PROTACs, the knockdown of FRS2α at the protein level by the PROTAC TrkAP3FRS2α occurs in a dose- and time-dependent manner. Furthermore, FRS2α protein levels in PC12 cells were reduced by ≈90% after treatment with NGF and TrkAP3FRS2α for 60 min, suggesting that this PROTAC has very high efficiency.[29] ErbB2PPt3K is another phosphoPROTAC targeting the ErbB2/Erβ3-PI3K-AKT pathway, which is frequently activated in many human cancers and plays a key role in mitogenesis and apoptosis inhibition.[30] The activation mechanism of the pathway is as follows: ErbB2 and ErbB3 form heterodimers induced by neuregulin (NRG), which is followed by ErbB3 phosphorylation on Tyr residues; this further activates PI3K (phosphatidylinositool-3-kinase) via the SH2 domain of the p85 regulatory subunit of PI3K. As a result, AKT is phosphorylated and activated,[31,32] which consequently promotes tumor cell survival and proliferation.[33] In addition, ErbB2, when overexpressed in breast and ovarian cancers, functions as a co-receptor with ErbB3 and is phosphorylated in the absence of NRG, thus eventually activating AKT. Thus, the ErbB2/ErbB3-PI3K-AKT pathway is critical for tumor cell growth and much effort is being made to discover methods of targeting this pathway. Fortunately, ErbB2PPt3K was developed, which can eliminate the p85 subunit of PI3K in a dose-dependent manner in MCF7 cells, leading to the inactivation of AKT with no effect on total AKT, as expected. Furthermore, a mouse xenograft model shows that tumor size is significantly reduced, to ≈40%, upon daily treatment with ErbB2PPt3K, strongly suggesting promising therapeutic effects of this phosphoPROTAC in vivo.[29]

Although phosphoPROTACs show good therapeutic efficacy for cancer treatment in mouse xenograft models, these molecules are not desirable anticancer drugs because of the poor cell permeability and high molecular weight of these molecules as well as their metabolic instability in cells. Encouragingly, significant progress has been recently made in designing nonpeptidic PROTACs by replacing peptide ligands with small molecules.

5. All-Small-Molecule PROTACs: A Potent and Promising Tool to Efficiently Target Undruggable Pathogenic Proteins

All-small-molecule PROTACs have achieved rapid target degradation and better cell permeability by using small-molecule ligands to recruit both target proteins and E3 ligases. Notably, hetero-bifunctional all-small-molecule PROTACs exhibit better stability and biodistribution in cells as well as passive cellular entry. A variety of small molecules that have been shown to bind with several E3 ligases or substrate receptor proteins of the CRL E3 ligase complex, such as MDM2, cIAP1, CRBN, and VHL, have been utilized in PROTAC development (Figure 5).

5.1. MDM2-Based PROTACs

The first synthetic all-small-molecule PROTAC is a hetero-bifunctional compound consisting of a non-steroidal androgen receptor ligand (SARM) and a Nutlin, linked via a PEG-based linker (Figure 5a).[34] Androgen receptor (AR), which is a member of the steroid nuclear receptor family and is widely expressed in prostate cancer tissues, directs the assembly and stabilization of the basal transcription apparatus, thereby transactivating the expression of the target.[35] SARM binds with AR at a Kd of 4 nM.[36] Nutlins are a class of potent and selective small-molecule antagonists of MDM2, the major E3 ligase targeting tumor suppressor p53 for polyubiquitination and degradation. The Nutlin binds to the p53-binding pocket of MDM2 to regulate the stability and transcriptional activity of p53 by disrupting the interaction of MDM2 with p53 but not affecting the E3 ligase activity of MDM2.[37] Thus, Nutlin can fulfill the function of hijacking MDM2 for PROTACs. This MDM2-based PROTAC targets AR for proteasomal degradation at a concentration of 10 μM in HeLa cells. Although the ability of this PROTAC to degrade AR is not as strong as that of previously developed PROTACs, it provides proof-of-concept that PROTACs can be developed as small-molecule drugs.[34]

5.2. cIAP1-Based PROTACs

In 2010, Yuichi Hashimoto’s group successfully designed a hybrid molecular compound in which methyl bestatin (MeBS) and all-trans retinoic acid (ATRA) were connected by spacers of different lengths (Figure 5b).[38] ATRA, an endogenous ligand of retinoic acid receptors (RARs), can recruit intracellular retinoic acid-binding proteins (CRABP-I and CRABP-II). MeBSs are a class of small molecules that directly and selectively bind to the BIR3 domain of cIAP1, the RING domain of which promotes auto-ubiquitination.[39,40] Thus, the cIAP1-based PROTAC can successfully induce the degradation of the intracellular CRABP-I and CRABP-II proteins via the ubiquitin-proteasome pathway. Biologically, due to its better permeability and stability in cells, this PROTAC has been proven to inhibit the migration of neuroblastoma IMR-32.[39,40] Therefore, this PROTAC was thought to be a promising therapeutic strategy for various diseases. However, MeBS has various off-target side effects owing to the inhibition of both arginyl aminopeptidases and leukotriene A4 hydrolase by MeBS.[3] Hashimoto’s group improved this cIAP1-based PROTAC by replacing the MeBS moiety with an MV1 moiety, a cIAP1/cIAP2/XIAP pan-ligand. The advanced PROTAC can achieve double protein knockdown of cIAP1 and CRABP-II, thereby exhibiting higher potency in the inhibition of the proliferation of IMR32 cells.[41]

5.3. CRBN-Based PROTACs

Recently, thalidomide and its derivatives were found to bind to CRBN, the substrate recognition component of the ubiquitin ligase CRL4CRBN, leading to lower protein levels of the anti-apoptotic protein Bcl2 and interferon regulatory factor 4.
(IRF4) as well as to the degradation of IKZF1 and IKZF2 in multiple myeloma (myelodysplastic syndrome), suggesting that thalidomide and its derivatives can hijack the ubiquitin ligase CRL4CRBN for protein degradation. CRL4CRBN, an E3 ubiquitin ligase complex consisting of cereblon (CRBN), damaged DNA-binding protein 1 (DDB1), cullin-4A/B, and regulator of cullins 1 (RBX1), is involved in the degradation of various substrates in several biological processes. It is well established that CRBN contains a conserved CUL1 domain in which all crucial residues of the thalidomide-binding site are located. Given that pomalidomide is able to specifically bind to CRBN, Craig M. Crews's group generated the PROTAC ARV-825 by coupling a BRD4-binding moiety (OTX015) with pomalidomide via a linker, inducing efficient ubiquitination and proteasomal degradation of the transcriptional coactivator BRD4 (Figure 5c). BRD4, a member of the bromodomain and extraterminal domain (BET) family, links acetylated histones and transcription factors to the locally assembled transcriptional apparatus at specific cis-regulatory elements. Thus, BRD4 plays a key role in the proliferation of cancer cells by regulating the expression of many important oncogenes, such as c-myc, bcl-xL, and bcl-6. Indeed, independent groups have shown that BRD4 knockdown triggers terminal differentiation and apoptosis in acute myeloid leukemia (AML) cells, and small-molecule inhibitors of the BET family induce early cell cycle arrest and apoptosis in MLL-fusion leukemic cell lines. This novel bifunctional PROTAC (ARV-825) mediates rapid degradation of BRD4, leading to suppression of proliferation and induction of apoptosis in Burkitt's lymphoma (BL) cell lines. Moreover, ARV-825 almost completely degrades BRD4 at 10 nM within 6 h, and sustains BRD4 degradation and downstream effects for up to 24 h, indicating the profound efficiency and endurance of this molecule. Notably, compared with small-molecule inhibitors such as JQ1 and OTX015, ARV-825 treatment caused more significant and lasting suppression of c-MYC, PIM1, CDK4/6, JAK2, and Bcl-xL and more pronounced proliferation inhibition and apoptosis induction in all the tested BL cell lines. Therefore, ARV-825 was the first example of a potent and promising PROTAC tool that efficiently targeted undruggable pathogenic proteins, indicating the great potential of PROTAC technology for the pursuit of effective therapeutics and for significant improvement of drug development strategies. Similarly, another small-molecule BRD4 inhibitor, JQ1, was conjugated to a thalidomide derivative to construct another bifunctional PROTAC, named dBET1 (Figure 5c).
completely degraded after treatment with dBET1 at 100 nM for 2 h in AML cells. Moreover, the protein levels of PIM1, MYC, BRD2, and BRD3 were also significantly decreased. Likewise, dBET1 exhibited greater and more potent apoptosis induction in AML cells than JQ1 in cell-based assays and in a xenograft mouse model of AML. Significant attenuation of tumor progression was achieved in a 14-day treatment with administration of dBET1 to tumor-bearing mice.\[^{[56]}\] Taken together, these data indicate that dBET1 exhibited greater and more potent apoptosis induction in AML cells than JQ1 in cell-based assays and in a xenograft mouse model of AML. Significant attenuation of tumor progression was achieved in a 14-day treatment with administration of dBET1 to tumor-bearing mice.\[^{[56]}\]

In addition to the E3 ubiquitin ligase CRL4\(^{CRBN}\), CRL4\(^{DCAF15}\) was recently demonstrated to be a good choice for the molecular design of PROTACs. The groups of Drs. Nijhawan and Owa found that the anticancer sulfonamides indisulam, E7820, and chloroquinoxaline sulfonamide can recruit CRL4\(^{DCAF15}\) to selectively degrade RBM39 or CAPERa, which suggests that sulfonamides are promising ligands for the development of PROTACs\[^{[57,58]}\].

### 5.4. VHL-Based PROTACs

Although peptidic VHL PROTACs were developed a long time ago, the development of these PROTACs as therapeutic drugs has been limited by the lack of high-affinity small-molecule ligands to hijack the CRL2\(^{VHL}\) complex. During this time, much emphasis has been placed on the discovery of small molecules that specifically bind to the HIF1a-binding site of VHL. Inspiringly, a high-affinity, high-specificity, small-molecule ligand with a hydroxyproline moiety for VHL binding was developed to generate novel non-peptidic VHL-based PROTACs targeting estrogen-related receptor alpha (ERR\(α\)) for degradation (Figure 5d).\[^{[59]}\] ERR\(α\), an orphan nuclear receptor, plays central roles in the regulation of cellular energy homeostasis by controlling the transcription of genes involved in glucose and fatty acid metabolism and mitochondrial biogenesis.\[^{[60,61]}\] A dose-dependent decrease of ERR\(α\) protein levels in a VHL-dependent manner was observed in MCF7 breast cancer cells treated with PROTAC_ERR\(α\). In addition, at a concentration of 100 nM, PROTAC_ERR\(α\) downregulates ERR\(α\) levels by 50%. Moreover, PROTAC_ERR\(α\) has broad tissue distribution and is efficacious in mice; reduced ERR\(α\) protein levels in tumor xenografts and multiple tissues, such as heart and kidney tissues, are readily observed.\[^{[59]}\]

Similarly, PROTAC_RIPK2 is another VHL-based PROTAC, with the same small-molecule ligand for VHL binding, which targets the serine/threonine kinase RIPK2 for degradation (Figure 5d). RIPK2 activates the NF-κB and MAPK signaling pathways and plays a critical role in the innate immune response by interacting with the bacterial sensors NOD1 and NOD2.\[^{[62]}\] PROTAC_RIPK2 utilizes the tyrosine kinase inhibitor vandetanib, an anti-cancer drug for the treatment of medullary thyroid cancer, to recruit the protein target RIPK2.\[^{[63]}\] In addition, PROTAC_RIPK2 links vandetanib to the VHL ligand via a 12-atom linker, leading to highly selective downregulation of RIPK2. PROTAC-mediated RIPK2 degradation is 1) potent, with a \(D_{\text{max}}\) of 95% at a concentration of 10 nM; 2) fast, as protein reduction by nearly 50% was observed as early as 1 h post-treatment, and almost complete degradation was achieved after 4 h of treatment; and 3) highly specific, since only RIPK2 and an unrelated protein out of nearly 7000 proteins were degraded in THP-1 cells treated with 30 nM PROTAC_RIPK2 for 18 h. Moreover, no obvious toxicity was observed at any concentration of PROTAC_RIPK2, and the downregulation of RIPK2 protein levels mediated by PROTAC_RIPK2 is reversible after withdrawal of PROTAC_RIPK2.\[^{[59]}\]

MZ1 and MZ2 are another two VHL-based PROTACs targeting BRD4 for degradation with different lengths of linkers to JQ1 for BRD4 binding and modulation of the VHL ligand (Figure 5d).\[^{[64]}\] Notably, the efficiency of BRD4 degradation by MZ1, which has a shorter linker, was higher than that by MZ2, with a longer linker, suggesting that the length of the linker is important for the degradative activity of PROTACs. Unlike the CRBN-based dBET1, both MZ1 and MZ2 preferentially promote the degradation of BRD4 over BRD2 and BRD3, indicating that specific E3 ligases selectively degrade target proteins.

Compound 3i was recently developed as VHL-based PROTAC targeting another serine/threonine kinase, TANK-binding kinase 1 (TBK1), which has been implicated in innate immune response, tumorigenesis, and development. Compound 3i can completely knockdown TBK1 in several cancer cell lines at a concentration of 100 nM, indicating the excellent efficiency of this compound (Figure 5d).\[^{[65]}\] In addition, VHL-based PROC-TACs have been shown to target transmembrane receptor tyrosine kinases (RTKs) as well as mutants of RTKs.\[^{[66]}\]

### 6. CLIPTACs: In-Cell Click-Formed Proteolysis-Targeting Chimeras Assembled from Two Small Precursor Molecules

Given that current PROTACs are composed of three parts, namely, a target-protein-binding moiety, a ligand for recruiting the degradation machinery, and a chemical linker, these heterobifunctional molecules have some limitations, such as high molecular weight and poor stability and penetrability in vivo. In addition, only few linkers are functional. Thus, PROTACs need to be further improved. Recently, Tom D. Heightman’s group developed an advanced PROTAC technology named CLIPTAC, in-cell click-formed proteolysis-targeting chimeras, which are actually CRBN-based PROTACs formed by rapid reaction of a tetrazine-tagged thalidomide derivative (Tz-thalidomide) with a trans-cyclo-octene (TCO)-tagged ligand in cells. This CLIPTAC can simultaneously recruit target proteins and the E3 ligase CRBN and promote the proteasomal degradation of the target protein (Figure 6).\[^{[67]}\] Notably, the two individual small precursor molecules, which are able to self-assemble to form a functional PROTAC in cells, have lower molecular weights and better penetrability than previous PROTACs. Indeed, two key oncoproteins, BRD4 and ERK1/2, were successfully degraded by sequential treatment of cells with one precursor followed by the other. Intriguingly, treatment with a pre-clicked CLIPTAC, formed outside the cells, caused no degradation of the two proteins, suggesting that the functional CLIPTAC forms in cells after the two precursors enter the cells, resulting in the degradation of target proteins. This study provides an advanced strategy to design proteolysis-targeting chimeras, which can be extended to the destruction of any protein of interest via in-cell
click-based formation of the PROTAC from a tagged ligand of these proteins and a tagged E3 ligase recruiter.

7. Conclusions and Future Perspectives

In summary, the PROTAC strategy has been continuously and significantly improved: from peptides to all-small-molecules, the stability, cellular permeability, solubility, and tissue distribution of PROTACs has been improved. Several oncoproteins, such as androgen receptor, estrogen receptor, ERRα, and BRD4, have been specifically ubiquitinated and destroyed via PROTACs, which function bridges between the target protein and an E3 ligase. Furthermore, treatment with PROTACs triggers a pronounced phenotype by eliminating target proteins via ubiquitination and degradation rather than by inhibiting the activity of these proteins, as in the case of traditional small-molecule inhibitors, to interfere with only one aspect of protein function. Thus, PROTAC technology provides a promising opportunity to target undruggable disease-causing proteins. In particular, thalidomide-like drugs, to perform antitumor functions, act as "simple" PROTACs to target IKZF1 and IKZF3 for degradation,[44,45] which proves the therapeutic potential of PROTACs. However, a number of fundamental questions that limit the future therapeutic application of PROTACs remain to be addressed. First, the design of PROTACs depends strictly on the development of ligands binding to target proteins and ubiquitin E3 ligases. Thus, the generation of small-molecule ligands with high specificity and affinity will remarkably accelerate the development of PROTACs. Second, since PROTACs with different linker lengths exhibit obviously different efficiencies for target degradation,[65] the principles of linker design need to be elucidated. Third, PROTACs with the same ligand for the target protein and different ligands for E3 ubiquitin ligases have variable selectivity for target proteins and efficiencies of target protein degradation.[64,85] The human genome encodes more than 600 E3 ubiquitin ligases.[86] Thus, future studies should identify suitable E3 ubiquitin ligases for PROTACs to target specific proteins.[68,69] Finally, further reduction of the molecular weight and toxicity and improvement of the pharmacokinetics, bioavailability and tissue distribution of PROTACs deserve extensive future investigation. Overall, by overcoming these limitations, PROTAC molecules could be eventually developed as a novel class of drugs for the treatment of human diseases by specific elimination of disease-causing proteins.

Abbreviations

AR, androgen receptors; ATRA, all-trans retinoic acid; β-TrCP, β-transducin repeat-containing protein; CDC4, cell division control protein 4; CRBN, Cereblon; cIAP1, cellular inhibitor of apoptosis protein 1; CLIPTAC, in-cell click-formed proteolysis-targeting chimeras; CRL, cullin-RING ligase; DHT, dihydroxytestosterone; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; ER, estrogen receptor; HIF1, hypoxia-inducible factor 1; MDM2, mouse double minute two homolog; MeBS, methyl bestatin; PROTAC, proteolysis-targeting chimeric molecule; PTB, phospho-Tyr-binding; RTK, receptor tyrosine kinase; SCF, Skp1-cullin-F-box; SH2, Src homology 2; UPS, ubiquitin-proteasome system; VHL, von Hippel-Lindau.

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Conflict of Interest

The authors declare no conflict of interest.

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