REVIEW

Degradation of proteins by PROTACs and other strategies

Yang Wang[a], Xueyang Jiang[d], Feng Feng[c,d], Wenyuan Liu[a,*], Haopeng Sun[b,*]

[a]Department of Pharmaceutical Analysis, Key Laboratory of Drug Quality Control and Pharmacovigilance, Ministry of Education, China Pharmaceutical University, Nanjing 210009, China
[b]Department of Medicinal Chemistry, China Pharmaceutical University, Nanjing 211198, China
[c]Jiangsu Food and Pharmaceutical Science College, Huaian 223003, China
[d]Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing 211198, China

Abstract Abnormal protein expression or activities are associated with many diseases, especially cancer. Therefore, down-regulating the proteins involved in cancer cell survival proved to be an effective strategy for cancer treatment—a number of drugs based on proteolysis-targeting chimaera (PROTAC) mechanism have demonstrated clinical efficacy. Recent progress in the PROTAC strategy includes identification of the structure of the first ternary eutectic complex, extra-terminal domain-4-PROTAC-Von-Hippel-Lindau (BRD4-PROTAC-VHL), and PROTAC ARV-110 has entered clinical trials for the treatment of prostate cancer in 2019. These discoveries strongly proved the value of the PROTAC strategy. In this review, we summarize recent meaningful research of PROTACs, including the molecular design and optimization strategy as well as clinical application of candidate molecules. We hope to provide useful insights for rational design of PROTACs.

*Corresponding authors. Tel.: + 86 18012955795 (Wenyuan Liu), Tel.: + 86 13951934235 (Haopeng Sun).
E-mail addresses: liuwenyuan@163.com (Wenyuan Liu), sunhaopeng@163.com (Haopeng Sun).

1. Introduction

Proteins play critical roles in maintaining the life of organisms[1-3]. Correct protein folding controls cell health and survival[4-6]. However, most proteins are inherently prone to aggregation in their misfolded or partially misfolded state[7]. In addition, misfolding or misregulation of proteins leads to the development of many diseases, including neurodegenerative diseases, cancers and type 2 diabetes mellitus (T2DM)[8-10]. Therefore, cells must constantly adjust their protein composition to maintain normal proteomes[11]. Misfolded proteins are refolded or degraded by quality control systems[12,13], and elimination of misfolded proteins is critical for maintaining protein homeostasis and cell viability[14].

Under physiological conditions a complex network that includes folding enzymes, chaperones, lectins and ATP-driven motors controls the elimination of misfolded proteins. The ubiquitin-proteasome system (UPS) and autophagy are the two major intracellular pathways for protein degradation[15-18]. The UPS and autophagy have long been considered as independent degradation pathways with little or no interaction points. In spite of growing evidence of close coordination and complementarity between the two systems[19], they are actually different mechanisms: UPS is responsible for the degradation of short-lived proteins and soluble misfolded proteins, whereas autophagy eliminates long-lived proteins, insoluble protein aggregates and even whole organelles (such as mitochondria, peroxisomes), macromolecular compounds, and intracellular parasites (e.g., certain bacteria)[20,21].

In addition, small interfering RNA (siRNA)[22] and clustered regularly interspaced short palindromic repeats/associated protein 9 nuclease (CRISPR-Cas9)[23] technologies can also down-regulate or eliminate proteins. However, these 2 technologies also have limitations: for example, CRISPR-Cas9 technology has undesired off-target effects and low efficiency, which limit its application in vivo[24]. Inefficient delivery to target cells in vivo and nonspecific immune responses following systemic or local administration are barriers for the clinical application of siRNA. Researchers are still developing various technology platforms to improve in vivo delivery of therapeutic siRNA[25].

In addition, heat shock proteins (HSPs) also play important roles in protein kinase degradation[26]. For example, the level of many oncogenic kinases, such as ERBB2, BRAF-V600E, FGFR-G719S and BCR-ABL, are reported to be tightly coupled to heat shock protein 90 (HSP90)[27].

The methods mentioned above for controlling protein degradation are mostly achieved via biomacromolecules. In order to target a broader range of proteins with sufficiently high efficiency for clinical application, in recent years pharmaceutical researchers have developed a series of new strategies for protein degradation using small molecules. One representative strategy is proteolysis-targeted chimera (PROTAC) that degrades proteins by hijacking the UPS[28-32]. PROTAC is a bifunctional-hybrid molecule that binds both E3 ubiquitin (U) ligase and target proteins, thereby leading to the exposed lysine on the target protein being ubiquitinated by the E3 ubiquitin ligase complex, followed by UPS-mediated protein degradation[33]. Theoretically, PROTACs not only provide binding activity, but also have great potential to eliminate protein targets that are "undruggables" by traditional inhibitors or are non-enzymatic proteins[34,35], e.g., transcription factors[36,37]. In addition, the PROTAC technique is "event-driven", which does not require direct inhibition of the functional activity of the target protein. These characteristics make PROTAC technology an attractive strategy for targeting protein degradation (TPD).

In this review we summarize the unique advantages and the core design philosophy of PROTAC through representative examples of PROTAC use in recent years. We also highlight the discovery of E3 ubiquitin ligase, the development and optimization of corresponding ligands, and its application to PROTAC technology. We also introduce rapid synthesis of PROTAC based on "click chemistry" reactions. Finally, we present the opportunities and risks of this emerging PROTAC technology in clinical applications. In summary, this work will provide insights for discovering new E3 ubiquitin ligases and designing PROTACs.

2. Degradation of protein by misfolded protein simulator

2.1. Hydrophobic tagging

The hydrophobic tagging (HyT) technology extends the concept of inducing protein instability to a broader range of protein targets by mimicking protein misfolding[38]. The HyT consists of a hydrophobic fragment and a ligand fragment of the protein of interest (POI), which is capable of causing degradation of the POI (Fig. 1A)[22,39,40]. One mechanism is that the HyT destabilizes the POI, thereby recruiting an endogenous chaperone protein to the misfolded protein and then degrading the protein by the proteasome; another mechanism is the direct recognition of the HyT by chaperones, mediating the proteasomal degradation of the tagged protein. The hydrophobic marker then is released and the POI can be destroyed in successive rounds[22]. However, the current literature does not prove the hydrophobic marker is completely released in the protein degradation process induced by HyT, and remains to be further studied.

Protein ubiquitination and degradation can be achieved by recruiting chaperones using lipophilic small molecule tags. For example, HSP70 family members recognize the exposed hydrophobic cores of misfolded proteins to hijack misfolded protein reactions[41]. HSP70 is highly conserved and ubiquitous in microorganisms, plants and animals[42], and is involved in many cellular processes, including protein folding, transmembrane protein translocation and protein degradation regulation[43]. Proteins with mild or partial misfolding are ubiquitinated by HSP40 and HSP70 and then degraded by HSP70 and 26S proteasomes[44] (Fig. 1B).

The HyT technology was further extended to degrade endogenous proteins such as human epidermal growth factor receptor 3 (HER3), a kinase playing important roles in cancer[45]. The effective ligand of HER3[46] is coupled to the adamantane moiety via a short linker to form a HyT degrader known as TX2-121-1 (1) (Fig. 1C). Covalent binding of 1 to HER3 resulted in HER3 degradation at 500 nmol/L and induced HER3-dependent cell death at an EC_{50} of 0.8−1.4 µmol/L[45]. However, the degradation of HER3 using HyT technology still relies on covalent interactions, which are stoichiometric rather than substoichiometric.

The breast cancer drug fulvestrant was originally designed as a selective estrogen receptor modulator (SERM), but was later found to induce degradation of the estrogen receptor alpha (ERα) receptor[47]. By inducing a conformational change to the receptor, fulvestrant causes ERα to expose a hydrophobic side chain mimicking the misfolded portion of the ERα protein recognized by the cell housekeeper, resulting in degradation of the ERα protein[48]. In 2002, fulvestrant was approved by the FDA for treating ER-positive metastatic breast cancer[49].
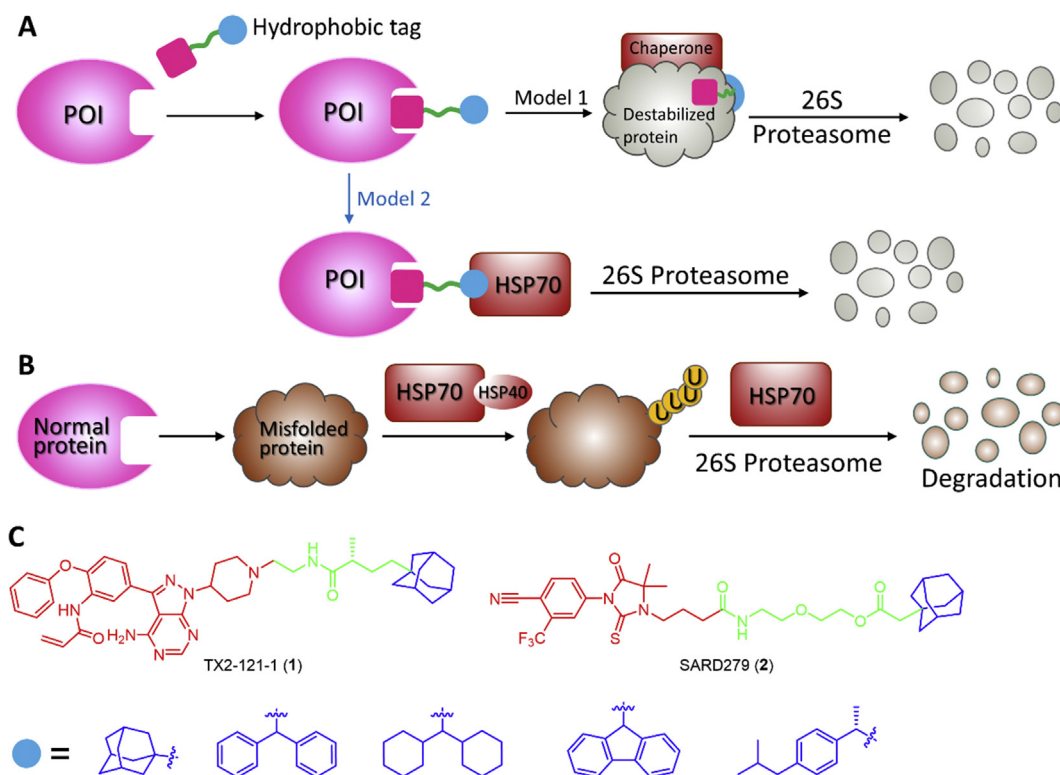
Figure 1 The hydrophobic tagging (HyT) technology. (A) The strategy of protein degradation through induced protein misfolding (or mimicking misfolding) with HyT using bifunctional adamantly-taggd molecules. (B) Degradation of misfolded proteins through HSP70/40 chaperones under normal physiological conditions. (C) The chemical structures of representative HyTs.

Inspired by the clinical success of fulvestrant, a series of selective androgen receptor degraders (SARD) were designed for high affinity to the androgen receptor (AR) agonist, with a polyethylene glycol (PEG) linker to a hydrophobic degron (an adamantyl group)[50]. As the first small molecule SARD[51] (Fig. 1C), SARD279 (**2**) has a 50% degradation concentration (DC_{50}) of 2 μmol/L. Researchers believe that HSPs may be involved in the mechanism of SARD-mediated AR degradation. After incubation with the potent HSP90 inhibitor geldanamycin, the level of HSP70 increased in a geldanamycin-dependent manner, which was consistent with the discovery that HSP90 inhibition resulted in the activation of heat shock factor 1 (HSF1) and its target genes (including HSP70)[52]. This suggests that HSP70 mediated the AR degradation and elevated HSP70 levels were the basis for the increased activities of SARD279 (**2**) in the context of HSP90 inhibition[53].

The early HyT technology was based on the adamantane HyT strategy and has been applied to a broad range of objectives. In addition to adamantyl, *tert*-butyl carbamate-protected arginine (Boc₃-Arg) can also be used as a HyT to induce protein degradation[38]. Although Boc₃-Arg has a higher molecular weight than adamantane, the capability of Boc₃-Arg to induce protein degradation was confirmed in 2012[54]. The non-covalent inhibitor trimethoprim (TMP) binds Boc₃-Arg to form TMP-B₃A (**3**) (Fig. 2B), which degrades dihydrofolate reductase at a micromolar concentration. Similarly, the covalent inhibitor ethacrynic acid (EA) was ligated with Boc₃-Arg to form EA-B₃A (**4**) (Fig. 2B), which degraded Glutathione-*S*-transferase (GST). In addition, the protein degradation occurred in U- and ATP-independent manners

by the proteasome[54]. Taking the GST protein degradation agent EA as an example, as a recognition fragment of the GST protein (Fig. 2B, the red part of the structure), the double bond of EA can alkylate the Cys residue in the active site of GST. Moreover, Boc₃-Arg may bind to the 20S proteasome, which makes the GST protein passively captured and degraded by the 20S proteasome.

The 20S proteasome is a 700 kDa barrel-shaped protein consisting of four loops with two stacked β loops sandwiched between two α loops[55]. The 20S proteasome is widely distributed throughout the cell and degrades most of the oxidized proteins in U and ATP-independent processes[54,56−58]. The U pathway of the 20S proteasome is required for the degradation of oxidatively damaged proteins[59]. In addition, protein cofactors such as HSP90 can synergize with the 20S proteasome to promote protein degradation[60]. The 20S proteasome can also induce POI degradation in combination with HyT (Fig. 2A).

There are three possible mechanisms of Boc₃-Arg-mediated degradation: First, the Boc₃-Arg portion can enter the proteasome and "drag" the rest of the protein into the proteolytic chamber. Second, the Boc₃-Arg group can be embedded in the target protein to expose its hydrophobic surface to interact with the 20S proteasome. Third, Boc₃-Arg may interact with other protein factors such as HSP90.

However, how the Boc₃-Arg portion targets the protein remains to be elucidated. A direct non-covalent interaction between Boc₃-Arg and the 20S proteasome was discovered: Boc₃-Arg activated the purified 20S proteasome, indicating that the tag binded directly to the 20S proteasome, and Boc₃-Arg targeted the target protein to 20S proteasome[61]. In addition, the proteasome subunits $\alpha 7$ and $\beta 7$ were
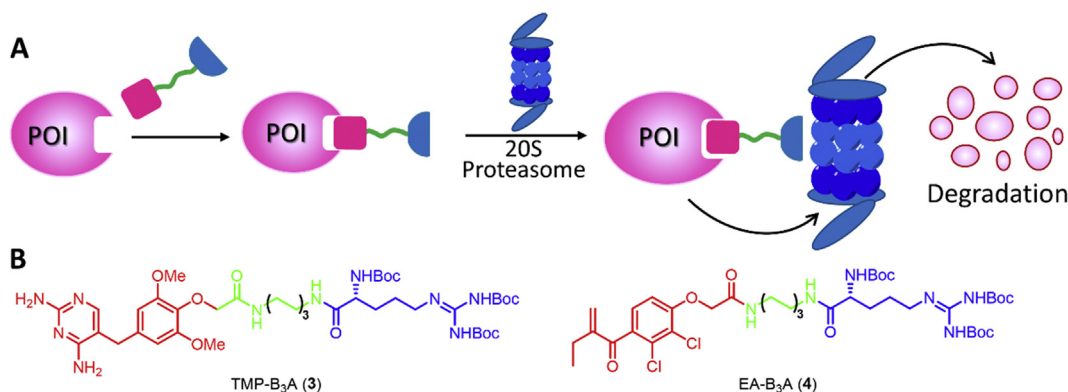
Figure 2 The 20S proteasome. (A) Degradation of POI through 20S proteasome. (B) The representative chemical structures of Boc3-Arg.

strongly enriched in a resin-binding protein pool linked to Boc3-Arg. The purified 20S proteasome was sufficient to degrade the target protein in a Boc3-Arg-dependent manner in the presence of its respective Boc3-Arg. This was the first example using bifunctional small molecules to target protein directly to the 20S proteasome and degrade protein. The Boc3-Arg portion inhibits the translational machinery mediated by the mammalian target of rapamycin complex 1 (mTORC1) pathway, and the off-target effect may potentially limit the clinical application of Boc3-Arg as a HyT[62].

The HyT method has used covalent and non-covalent ligands to initiate protein degradation. However, its physicochemical and pharmacokinetic (PK) properties may be the major challenge for clinical development.

2.2. Fusion-based degron (HaloTag)

Some protein fusion tags have been extensively studied: *e.g.*, fluorescent proteins[63], His tag[64], FLAG tag[65], etc. HaloTag is a modified bacterial dehalogenase enzyme that covalently binds with a hexyl chloride label. HaloTag fusion protein has been widely used as a biological orthogonal marker[66,67], *e.g.*, *in vivo* molecular imaging, protein purification/transport, high-throughput detection, etc. HaloTag forms stable covalent bonds with compounds containing alkyl chlorides[68] *via* a very simple binding moiety with low molecular weight and reasonable cell permeability[69]. More importantly, HaloTag has high selectivity and sensitivity[70].

The HaloTag-based bifunctional molecule contains an alkyl chain HaloTag and a ligand that binds to the target protein. This bifunctional molecule transfers the fusion domain onto the POI, binds the bacterial HaloTag protein and generates a hydrophobic group on its surface, which is mediated by a chaperone. The protein then becomes unstable and is subsequently degraded by the proteasome (Fig. 3). More importantly, PK studies of adamantane-based HyT in mice indicated that 75% of HyT is still present after 24 h, and that HyT compounds also inhibited 80% of tumor growth in xenograft HaloTag-HRAS-G12V-driven mice[70]. Immunoprecipitation showed that the addition of the adamantane-labeled HaloTag fusion protein was associated with HSP70, suggesting that the adamantyl-mediated degradation is related to HSP70[71].

3. PROTAC technology

Traditional small molecule inhibitors can inhibit the activity of some enzymes and block the function of certain disease-related proteins, but they still have some limitations in applications[72,73]. TPD is a new direction in the field of drug discovery. Traditional small molecule inhibitors only block part of the protein's function, while TPD eliminates all the functions of the protein.

As a protein degradation system, UPS is a key post-translational modification process[74] and is involved in protein quality control, antigen processing, signal transduction, cell cycle control, cell differentiation and apoptosis. UPS plays a key role in regulating protein homeostasis[75,76]. E3 ubiquitin ligase is a specific substrate member of UPS and represents an attractive protein target for drug discovery. The molecular mechanism of proteasome protein degradation is driven by the sequential actions of three enzymes (E1-activation, E2-conjugation and E3-binding)[77,78] (Fig. 4A). First, with ATP as the energy source, the carboxyl group at the end of U glycine is linked to the thiol group of the U-activating enzyme E1 to form a thioester bond between U and E1. Second, E1 transfers the activated U to E2 through a lactide process. Third, E3 binds E2 U to the target protein and releases E2 to leave a specific ubiquitinated protein[79]. Finally, ubiquitinated proteins are recognized by specific proteasomes and degraded into short peptides or amino acids by proteases[80].

In physiological conditions E3 ubiquitin ligase requires a special recognition signal to recruit and ubiquitinate its target protein[81]. Generally, ubiquitination occurs on the lysine residues of a target protein. PROTAC is a bifunctional-hybrid compound consisting of three parts: one side is the ligand to the target protein, the opposite side is the ligand to the E3 ubiquitin ligase, and the middle is the linker connecting two ligands[82]. Small molecule PROTAC can bind to both E3 ubiquitin ligase and the target protein and induce the formation of a ternary complex which leads to polyubiquitination and subsequent degradation of the target protein (Fig. 4B). Meanwhile, PROTAC can be recycled for subsequent rounds of degradation[83].

3.1. The first generation of peptide PROTAC

In 2001 the first bifunctional molecule compound **5** was reported (Fig. 5B), which induced the degradation of the target protein methionine aminopeptidase-2 (MetAP-2) by recruiting the ubiquitinated protein β-TRCP (F-box protein) in a compound **5**-dependent manner[84] (Fig. 5A). To overcome the impermeability of SCFα-TRCP binding to the IB phosphopeptide moiety, injection of β-TRCP-based PROTAC into HEK293 cells by microinjection was found to induce AR degradation[85]. It was the first time that the concept of PROTAC was clearly proposed with a comprehensive
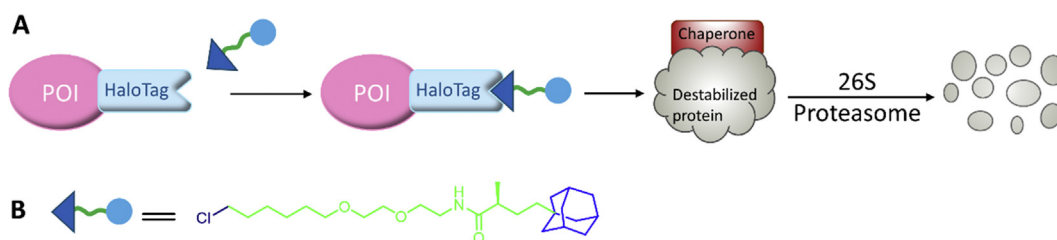
Figure 3   HyT strategy developed based on HaloTag fusion protein system for protein degradation.
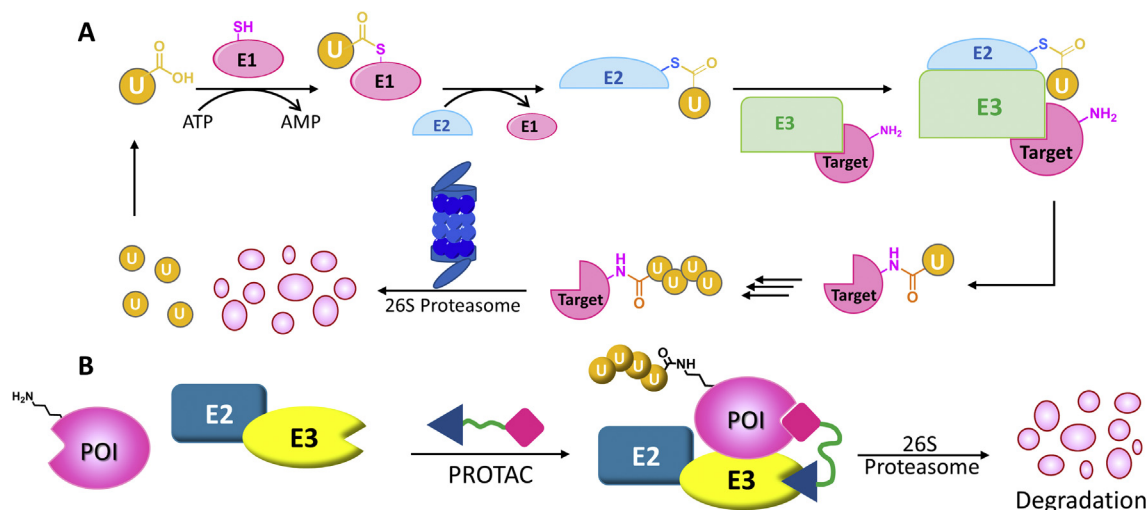

Figure 4   The ubiquitin−proteasome system and PROTAC. (A) Ubiquitin (U) is activated by ubiquitin-activating enzyme (E1). Activated ubiquitin is transferred in thioester linkage from E1 to ubiquitin-conjugating enzyme (E2). Ubiquitin ligase (E3) catalyzes the transfer of ubiquitin from E2 to substrate *via* lysine residues. (B) The PROTAC bind 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 PROTAC molecule consists of a ligand for recruiting E3, a linker, and a ligand binding to POI.

validation *in vitro*. Other small molecule protein-targeting chimeras were also developed to specifically degrade target proteins by recruiting E3 ubiquitin ligase[86]. However, the first generation of PROTAC were polypeptides that did not have favorable physiochemical properties for therapeutics. Besides a high molecular weight and low activity, the major problem of the peptide-based PROTAC is the poor permeability to intracellular targets[87].

3.2.   KEAP1-dependent peptide PROTAC

Kelch-like ECH-associated protein 1 (KEAP1) is a Cullin3−RBX1 U ligase substrate adaptor protein[88] containing a BTB domain at its N-terminus. The BTB domain mediates the interaction with Cullin3 (Cul3)[89]. KEAP1 acts as a sensor for reactive oxygen species (ROS) and protects cells from oxidative damage[90].

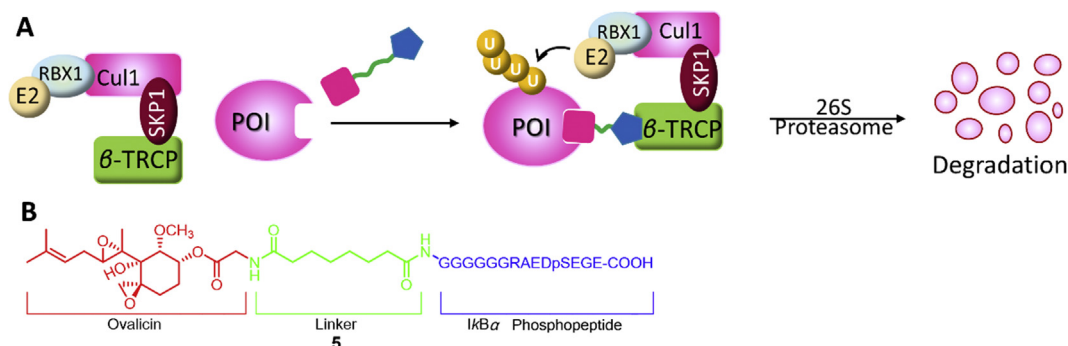**Figure 5**   The first PROTAC. (A) A schematic diagram of the first PROTAC. This PROTAC contains a phosphopeptide derived from IκBα to recruit the E3 ubiquitin ligase SCFβ-TRCP, a linker and ovalicin to bind with MetAP-2, which triggers MetAP-2 ubiquitination. (B) Structure of first PROTAC, compound 5. Blue: ligand for target proteins is shown; Red: ligand for recruiting E3 ubiquitin ligase.

One substrate for the KEAP1−Cul3 complex is nuclear factor erythroid 2-related factor 2 (NRF2). NRF2 is an important transcription factor for regulating oxidative/xenobiotic stress response and anti-inflammation[91].

Ubiquitination is the most important pathway to regulate the activity of NRF2[92]. Three E3 ligases have been discovered to control the ubiquitination of NRF2, namely KEAP1−Cullins3−RING-box protein 1 (KEAP1−Cul3−RBX1)[93], β-TRCP-S-phase kinase-associated protein 1−Cul1−RBX1 (SKP−Cul1−RBX1)[94], and HMG-COA reductase degradation 1 homologue (HRD1)[95]. Under normal conditions KEAP1 inhibits NRF2 signaling through UPS[96]. After exposure to electrophiles, ROS, and reactive nitrosated species (RNS) or heavy metals, the cysteine residue modification in KEAP1 results in a conformational change in the complex to impede the proteasome-mediated degradation of NRF2. In this case NRF2 remains stable and accumulates in the nucleus[97].

The development of PROTAC with these potent small molecule KEAP1−NRF2 inhibitors may be a better strategy for inhibiting the protein−protein interaction (PPI) of KEAP1−NRF2 (Fig. 6). The KEAP1−NRF2 signaling pathway might be a valuable therapeutic target for the treatment of neurodegenerative diseases[98]. A peptide-PROTAC which recruits KEAP1−Cul3 U E3 ligase is reported to induce Tau degradation in cells[34]. The peptide 1 strongly binds to KEAP1 and Tau *in vitro* with K_d values of 22.8 and 763 nmol/L, respectively. Peptide 1 induces the degradation of Tau in a time- and concentration-dependent manner, suggesting the usage of PROTAC to recruit KEAP1 to induce Tau degradation to treat neurodegenerative diseases.

Subsequent research into PROTAC has focused on the development of small molecule PROTAC technology with *in vivo* stability.

4. Small molecule PROTAC

There has been great progress in the discovery of E3 ubiquitin ligases: more than 600 human genome-encoded E3 ubiquitin ligases have been identified[99]. Von-Hippel-Lindau (VHL), murine double minute 2 (MDM2), cell inhibitor of apoptosis protein (cIAP) and CRBN (cereblon), have been utilized successfully for small molecule PROTAC, and these molecules are likely to become therapeutic candidates[100]. However, which other E3 ligases can be recruited to PROTAC needs further study.

4.1. MDM2-based PROTAC

MDM2 is a key oncogenic protein that contributes to cell growth, survival, invasion and chemotherapy resistance in cancer[101]. As a negative regulator of the tumor suppressor P53, MDM2 directly binds to the transactivation domain (TAD) of P53 protein and blocks P53 transcriptional activity[102,103]. The P53 protein upregulates *MDM2* gene expression levels, while MDM2 promotes P53 export from the nucleus and promotes its proteasome-mediated degradation[104]. In addition, MDM2 also acts as an E3 ubiquitin ligase to reduce P53 levels[105,106]. Since P53 regulates many important processes in cells, including DNA repair, cell cycle arrest and apoptosis, and tumor therapy[107,108], it is important to maintain an appropriate amount of P53 in the nucleus. Therefore, inhibiting the interaction between P53 and MDM2 to restore normal P53 levels may be a good strategy[109,110].

In recent years, several small molecule inhibitors of the P53−MDM2 interaction have been developed[111]. One class are *cis*-imidazoline derivatives called nutlins[112]. In this class, compound **6** (nutlin-3) had the best inhibitory activity (Fig. 7A). In

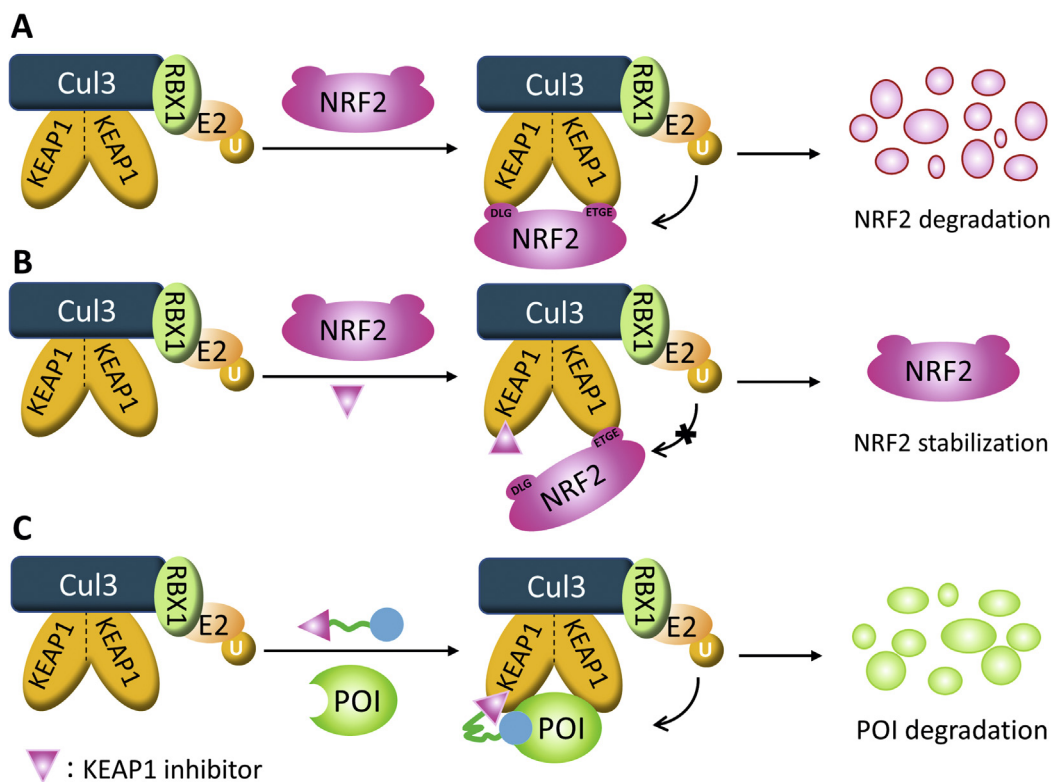**Figure 6**   Development of PROTAC based on KEAP1 as E3 ubiquitin ligase. (A) Under physiological conditions, endogenous substance NRF2 is recognized by KEAP1, ubiquitinated, and degraded by the proteasome. (B) KEAP1 inhibitors block the interaction of KEAP1−NRF2, leading to stabilization and activation of NRF2. (C) PROTAC developed based on KEAP1 inhibitors is used for the degradation of POI.

addition, compound **6** also inhibits cancer cell growth, cell migration and induces apoptosis[113,114]. Compound **6** act as a ligand for MDM2 to recruit E3 ubiquitin ligase[115]. On this basis, a new generation of molecules were developed, including RG7112 (**7**)[116] and RG7388 (**8**)[117] (Fig. 7A). Their inhibitory efficiency is significantly higher than that of nutlin-3. The development of PROTACs based on MDM2 as E3 ubiquitin ligase is shown in Fig. 7B.

In 2008 researchers reported the first all-small molecule PROTAC, compound **9**[115], which targeted the AR in HeLa cells. Compound **9** was a combination of a non-steroidal AR ligand and an imidazoline derivative known to bind to MDM2 *via* a PEG linker. The soluble PEG linker provides compound **9** with an acceptable level of cell permeability. After incubation of HeLa cells with compound **9** significant degradation of AR can be achieved at micromolar concentrations, with a DC_{50} value of 10 μmol/L.

Poly(ADP-ribose) polymerases (PARPs) are post-translational modification enzymes that play an important role in DNA repair[118,119]. Among them, PARP1 is a valid target for cancer treatment[120]. Recently, an alkyne analogue of niraparib and the compound **6** derivative were linked by "click chemistry" to develop the PARP1 degradation inducer compound **10**[121]. Compound **10** selectively induced significant PARP1 degradation in MDA-MB-231 cell line with a potency 5-fold higher than that of niraparib, olaparib and veliparib. The maximal level of degradation (D_{max}) of **10** was 70% with a $DC_{50} = 4-6$ μmol/L. This indicates that this PARP1-targeting PROTAC has great potential for treating the MDA-MB-231 cell-like subtype of triple negative breast cancer (TNBC).

Recently a PROTAC, A1874 (**11**)[122] targeting BRD4 was developed by linking a bromodomain and an extra-terminal (BET)

inhibitor and a MDM2 inhibitor with a 13-atom long PEG linker. Different concentrations of **11** were applied to the colon cancer cell line HCT116, and the $D_{max} = 98\%$ at 100 nmol/L, which yielded a dose-dependent degradation of BRD4. In addition, the P53 level in HCT116 cells also showed dose-dependent stabilization. In particular, its potency ($DC_{50} = 32$ nmol/L) is significantly higher than that of **9** ($DC_{50} = 10$ μmol/L). The MDM2-based PROTAC can lead to oncoprotein degradation, indicating that these PROTACs have great potential as cancer therapeutics.

Structures and protein degradation activities of MDM2-based PROTACs **9**−**11** are shown in Fig. 8 and Table 1.

4.2. cIAP1-based PROTAC

"Inhibitor of apoptosis" proteins (IAPs) were first discovered in baculovirus[123] and play an important role in maintaining cellular homeostasis. They also control a range of biological processes such as the inflammatory response, cell death, cell division, cell proliferation and cell differentiation[124,125]. Among them, XIAP, cIAP1, cIAP2, LIVIN/ML-IAP and IAP-like protein 2 have a conserved RING domain at their C-terminus, and the RING domain binds E2 U-conjugating enzymes (UBCs). This allows the RING-containing protein to become an E2 U-binding enzyme that catalyzes the recruitment of U into the target protein. The RING domain is endowed with E3 ubiquitin ligase activity, which acts as an E3 ubiquitin ligase[126−128].

cIAP1 is overexpressed in many cancers and down-regulating cIAP1 expression may be a promising approach for cancer treatment[129]. Bestatin is an aminopeptidase inhibitor with immunomodulatory activity and is approved for the treatment of acute non-lymphocytic leukemia in Japanese adults[130]. Studies have shown that bestatin-methyl ester MEBS (**12**) selectively down-regulate cIAP1 (Fig. 9A). **12** directly interacts with the cIAP1-BIR3 domain, promoting self-ubiquitination depending on its RING domain and subsequent proteasomal degradation of cIAP1[131]. In 2007, researchers reported that a new class of small molecule IAP antagonists binds to the BIR domain of IAP proteins, leading to the rapid ubiquitination and proteasomal degradation of cIAP proteins[132]. To elucidate the mechanism of IAP antagonism, a structure-based design was used to develop a novel monovalent MV1 (**13**) targeting the IAP protein, yielding a pan-antagonist of all XIAP, cIAP1 and cIAP2[133] (Fig. 9A).

Both **12** and **13** can induce self-ubiquitination and proteasomal degradation of cIAP1 E3 ligase[131,133], which may limit protein knockdown efficacy. Based on this, a number of IAP antagonists have been developed, and some have been evaluated as anti-tumor drugs in clinical phase studies[134,135]. These IAP antagonists have higher affinity for IAP than **12**, and they have been used to develop a compound with potent protein knockdown activity. This LCL 161 derivative (**14**) (Fig. 9A) has been used as an IAP ligand[136].

Coupling **12** and all-trans retinoic acid (ATRA) with a spacer yielded "specific and non-genetic IAP-dependent protein eraser 4" (SNIPER (4)) (**15**)[137], which selectively induced degradation of cellular retinoic acid-binding proteins-I and II (CRABP-I and −II). At the concentrations of 10 and 30 μmol/L **15** significantly reduced cell migration by about 75% and 95%, respectively. However, the ester type **15** had poor selectivity to its target protein, and the ester group could be easily hydrolyzed in cells. Therefore, an amide-type SNIPER (6) (**16**) capable of overcoming these problems was developed: **16** selectively induced degradation of CRABP-II but did not induce IAP[138]. Later, researchers
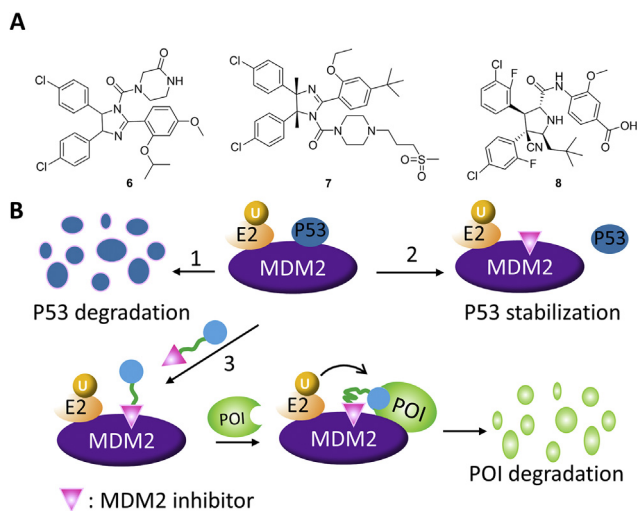
Figure 7 Structures of MDM2 ligands and development of PROTAC based on MDM2. (A) Structures of MDM2 ligands (nutlin-3, **6**) and a new generation of nutlin-like molecules (**7** and **8**). (B) Development of PROTAC based on MDM2 as E3 ubiquitin ligase. Under normal physiological conditions, endogenous substance P53 is recognized by MDM2, ubiquitinated and degraded by the proteasome. MDM2 inhibitors block the interaction of MDM2−P53, leading to stabilization and activation of P53. PROTAC developed based on MDM2 inhibitors is used for the degradation of POI.
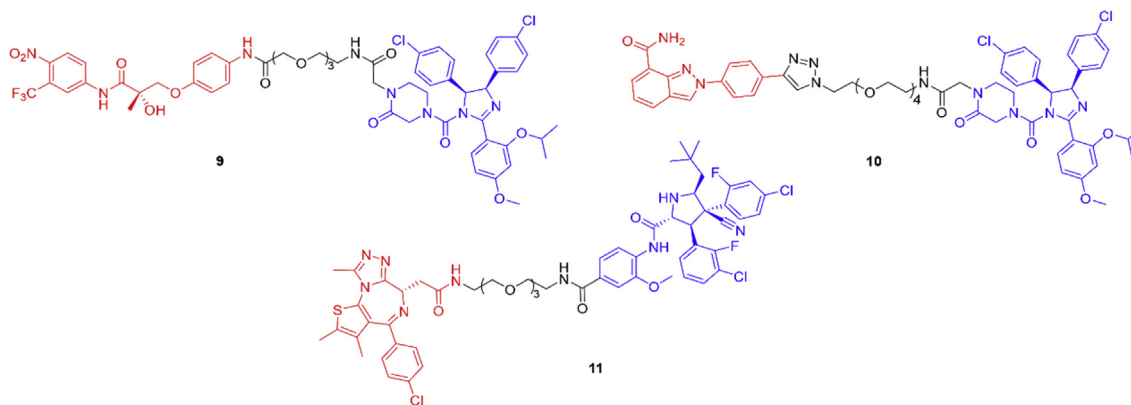
Figure 8 Structures of PROTACs **9**−**11** based on MDM2 as E3.

developed compound **17** by combining ATRA and **12** through a linker moiety, which could induce the degradation of cIAP1 and CRABP-II in a proteasome-dependent manner at a concentration of 1 μmol/L. It also effectively inhibited the proliferation of IMR32 cells[139].

In 2013 the Naito[140] team used **12** and 4-hydroxy tamoxifen (4-OHT) to synthesize compound **18**, which significantly reduced ERα and cIAP1 at 30 μmol/L. In order to increase the target protein degradation efficiency, they replaced **12** with the IAP antagonist **13**, which had a higher affinity for IAP than bestatin. A series of SNIPER(ER) compounds containing different ER ligands or different linker lengths were developed with improved activities as compared to **18**. Compound **19** was found to be more effective in reducing ER levels than **13**. ER concentration was reduced by 50% at concentrations as low as 3 nmol/L, and maximum activity was observed at about 100 nmol/L, with a D_{max} value of 70%[136]. In 2018 the Ohoka team[141] reported that the derivatization of the IAP ligand module produced SNIPER(ER) with excellent ERα activity, preferentially recruiting XIAP instead of cIAP1. This improved SNIPER(ER) showed higher binding affinity to IAP and more efficient degradation of ERα than compound **19**, with a maximum degradation of 70%. Of these SNIPER(ER)s, compound **20** ($DC_{50}=1$−10 nmol/L, $D_{max}>80\%$) was more effective in inhibiting the growth of MCF-7 tumor xenografts in mice when compared to the previously characterized compound **19**.

Transforming acidic-coiled coil 3 (TACC3) is a spindle regulatory protein that is overexpressed in many cancers, including ovarian cancer, breast cancer, squamous cell carcinoma, cell carcinoma and lymphoma[142−144], indicating that TACC3 is a molecular target for anticancer drugs. Compound **21** significantly reduced TACC3 levels in human fibrosarcoma HT1080 cells ($DC_{50}=10$−30 μmol/L, $D_{max}>50\%$)[145].

In 2017 PROTAC bound to the allosteric site of the oncoprotein BCR-ABL was first reported[146]. SNIPER(ABL)-062 (**22**) had a relatively good binding affinity for ABL1, cIAP1/2 and XIAP. BCR-ABL protein could be efficiently and selectively degraded at 30 nmol/L ($DC_{50}=30$−100 nmol/L, $D_{max}>70\%$) of **22**. The method of degrading target proteins by binding ligands to the allosteric sites of target proteins provides new approaches to future protein degradation[146].

The Naito[147] team developed a novel SNIPER that induces proteasome degradation of the AR, which recruits IAP U ligase to degrade target proteins. Hybrid optimization strategies include: using multiple AR antagonists and E3 ligase ligands, and altering the linker and junction sites.

First, the bestatin moiety is replaced by 2 specific IAP antagonists, and a series of compounds are further derivatized by linking different linkers, such as amides, ethers, acetylenes or alkyl groups. Among them, SNIPER(AR)-51 (**23**) was developed by linking **14** and an AR antagonist with a PEG linker to effectively degrade AR. Based on compound **23**, the effect of different attachment sites of the IAP ligand on the activity of SNIPER was further explored. The *m*-analogue showed the same ability to reduce the level of AR protein as the *p*-analogue, but the *o*-analogue showed no activity against AR protein at a concentration of 1 or 3 μmol/L. In evaluating the effect of the type of the linker on the activity of the SNIPER-degrading protein, the optimal protein degradation activity was found in the SNIPER containing the flexible PEG-linked chain. In summary, **23** presented the best performance in reducing AR protein compared with other compounds.

Structures and protein degradation activities of cIAP-based PROTACs **15**−**23** are shown in Fig. 9B and Table 2.

4.3. VHL-based PROTAC

The *VHL* gene has been referred as a tumor suppressor[148]. Its germline mutations are associated with the inherited VHL cancer syndrome[149]. VHL is a component of the multi-subunit E3 ligase containing ELONGINB (ELOB), ELONGINC (ELOC), Cul2 and RBX1 (VBCCR complex)[150]. The major substrate for VHL is hypoxia inducible factor-1α (HIF-1α), a transcription factor that upregulates many proteins[151], *e.g.*, pro-angiogenic factors, vascular endothelial growth factor (VEGF), glucose transporters, GLUT1, etc. VHL recognizes and labels the prolyl-hydroxylated transcription factor HIF-1α and marks it to be degraded by the

Table 1 PROTACs **9**−**11** with the ability to degrade target proteins developed based on MDM2 as E3[a].

Compd.	Target	Degradation in cell lines		Ref. No. (Year)
		DC_{50}	D_{max} (%)	
9	AR	10 μmol/L	NA	115 (2008)
10	PARP1	4−6 μmol/L	70	121 (2019)
11	BRD4	32 nmol/L	NA	122 (2019)

[a]DC_{50}: the concentration at which 50% degradation was observed. D_{max}: the maximal level of degradation. NA: not available.
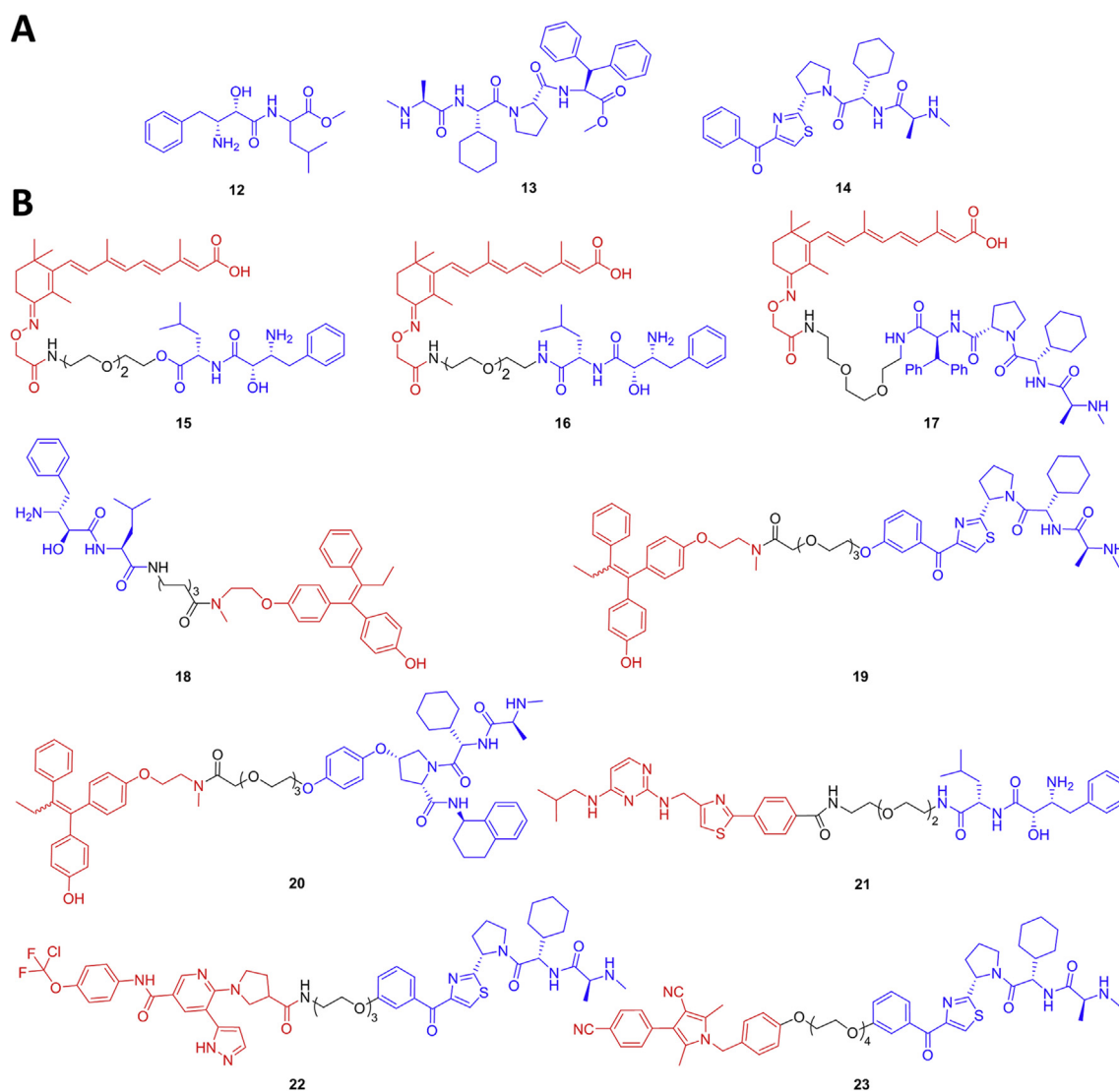
Figure 9 cIAP ligands and PROTACs. (A) Development of cIAP ligands **12**−**14**. (B) Structures of PROTACs **15**−**23** based on cIAP as E3.

proteasome[152,153]. HIF-1α is recruited to the CRL2^VHL complex *via* the β domain of pVHL, which recognizes post-translational modifications of hydroxyproline. Once bound to CRL2^VHL, HIF-1α is ubiquitinated and then undergoes proteasome-mediated targeted degradation[154].

Fragment screening is widely used to find an attractive starting point in drug design. Ciulli et al.[155] used it to study the interaction of pVHL: HIF1α, which was roughly divided into three sub-regions: the left side (LHS), the central core, and the right side (RHS). Compound **24** (hydroxyproline diamide 13) (Fig. 10A) had a fairly modest 0.01 nmol/L affinity, but it provided the greatest free energy for initial inhibitory binding to be a useful starting point for constructing promising small molecules. Later, they found (3*R*,4*S*)−*F*-Hyp (**25**) (Fig. 10B) acts as the best VHL ligand fragment to target protein degradation[156].

Based on these studies, researchers used the HIF-1α peptide structure as a starting point to design VHL ligands, and the first small molecule VHL ligand compound **26** was developed. However, its binding affinity to pVHL is not high ($K_d = 5.3$ μmol/L), and its lipophilicity is poor[157,158]. Later, a second VHL small molecule ligand **27** was developed with an affinity to VHL of

$K_d = 1.5$ μmol/L, and lipophilicity (LE=0.25), which are improved compared to the first VHL ligand[159]. Based on the structural design, the second-generation VHL small molecule ligand VHL-1 (**28**) was obtained ($K_d = 185$ nmol/L and clog$P$=1.71). This ligand inhibitor is more active than the 10-mer HIF-1α peptide model, verifying that VHL was an excellent starting point as a drug target[160].

The small molecule inhibitor compound **28** has limited activity and cannot induce intracellular HIF accumulation. One optimization strategy is to retain the carbonyl group on the LHS to maintain the hydrogen bond between the bag and the structured water, and replace the three hydrogens on the LHS with electron-withdrawing groups such as cyano and cyclopropyl, yielding VH298 (**29**). VH298 is the primary target for VHL with $K_d = 185$ nmol/L and clog$P$=1.71, indicating that **29** can permeate the cell membrane more efficiently[161,162]. The graphical representation of the discovery, development, and optimization of the VHL ligands discussed above is shown in Fig. 10C. VHL is widely used as a PROTAC-conjugated E3 ligase ligand for TPD, and the development of PROTACs based on VHL as an E3 ubiquitin ligase is shown in Fig. 11.

Table 2 PROTACs **15–23** with the ability to degrade target proteins developed based on cIAP as E3[a].

Compd.	Target	Degradation in cell lines		Ref. No.
		DC_{50}	D_{max} (%)	(Year)
15	CRABP-I, −II	NA	NA	137 (2010)
16	CRABP-II	NA	NA	138 (2011)
17	cIAPI, CRABP-II	NA	NA	139 (2012)
18	ER	NA	NA	140 (2013)
19	ER	1–3 nmol/L	>70	136 (2017)
20	ER	1–0 nmol/L	>80	141 (2018)
21	TACC3	10–30 μmol/L	>50	145 (2014)
22	BCR-ABL	30–100 nmol/L	>70	146 (2017)
23	AR	1–3 μmol/L	>80	147 (2018)

[a]DC_{50}: the concentration at which 50% degradation was observed. D_{max}: the maximal level of degradation. NA: not available.

In 2015, PROTAC_ERRα (**30**) and PROTAC_RIPK2 (**31**) which almost completely degrade estrogen-related receptor-α (ERRα) and RIPK2 with nanomolar activity were developed[163]. The ERRα levels were dose-dependently reduced in MCF-7 breast cancer cells after incubation with **30** ($D_{max}=86\%$, DC_{50} ∼100 nmol/L). After concentration escalations of **31**, human THP-1 monocytes showed a stable and dose-dependent RIPK2 degradation ($D_{max}>95\%$ at a concentration of 10 nmol/L and $DC_{50}=1.4$ nmol/L). Furthermore, **30** reduced ERRα in mouse hearts, kidneys, and MDA-MB-231 tumors by approximately 44%, 44% and 39%, respectively. This was the first evidence that

the small molecule VHL-based PROTACs have substoichiometric activity in a mouse model.

BET protein families, including BRD2, BRD3, BRD4 and testicular-specific BRDT, play an important role in cancers[164]. A VHL-based PROTAC ARV-771 (**32**) was developed[165], which showed <5 nmol/L potency of BRD2/3/4 degradation in several prostate cancer cell lines ($DC_{50}<1$ nmol/L, $D_{max}>90\%$). In addition, unlike BET inhibitors, **32** leads to inhibition of AR signaling and AR levels, and such an anti-proliferative effective is 500 times more active than other BET inhibitors in these cell lines. **32** also leads to tumor regression in castration-resistant prostate cancer (CRPC) mouse xenograft models. It not only validates BET protein degradation as a promising clinical strategy for anti-metastatic CRPC, but also demonstrates the feasibility of using PROTACs to treat small tumor-mediated protein-degrading solid tumor malignancies.

The dysregulation of BET protein, especially BRD4, is closely related to cancer and inflammations, making BET protein an attractive drug target[166]. JQ1 was linked to VHL to form PROTAC MZ1 (**33**)[167], and more than 90% of the BET protein was degraded at a concentration as low as 1 μmol/L. The degradation of BRD4 ($DC_{50}<100$ nmol/L, $D_{max}>90\%$) was stronger than BRD2 and BRD3. Researchers further developed AT1 (**34**) based on the crystal structure of **33**[168]. After treatment with **34**, BRD4 was significantly degraded ($DC_{50}=10–100$ nmol/L, $D_{max}>90\%$), however, **34** had no effect on the levels of BRD2 and BRD3. VZ185 (**35**) induced a rapid and effective degradation of BRD7 and BRD9 within a few hours (BRD9: $DC_{50}=4$ nmol/L; BRD7: $DC_{50}=34$ nmol/L, $D_{max}=95\%$)[169].

The VHL-based PROTAC **36** mediated the degradation of c-ABL[170]. TANK-binding kinase 1 (TBK1) is an atypical member
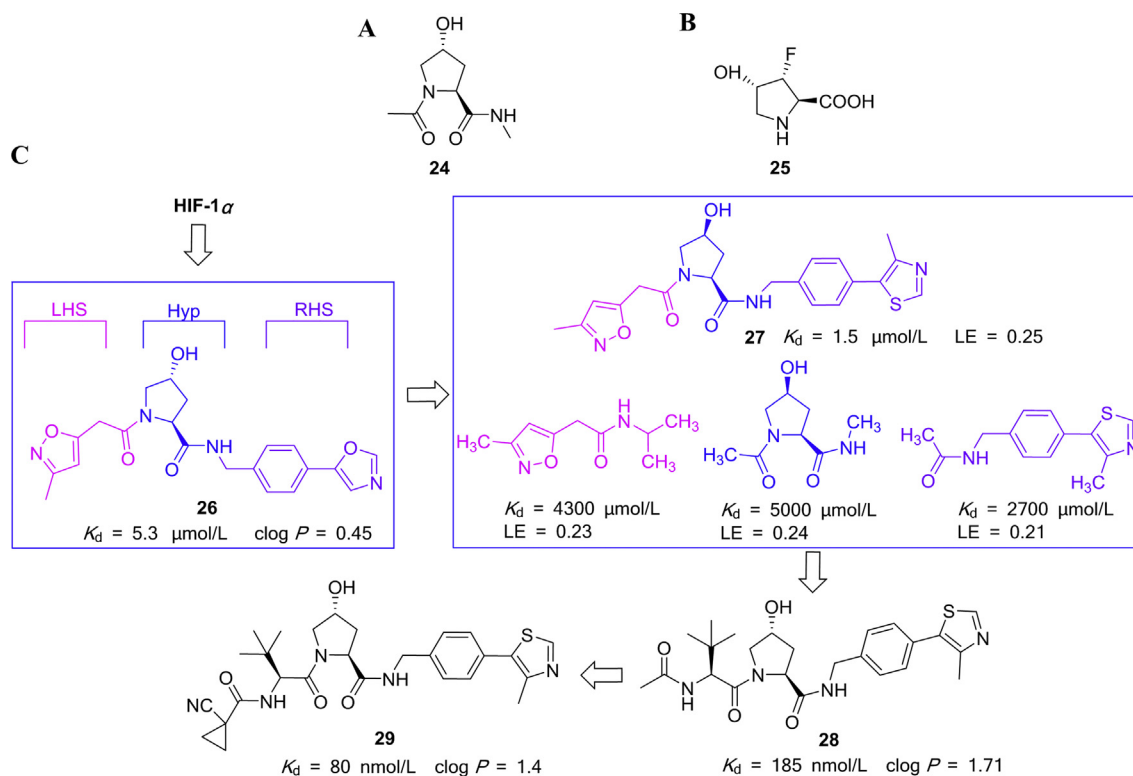

Figure 10 The development of VHL ligands. (A) **24** is a useful starting point for constructing VHL small molecule ligands. (B) **25** acts as the best VHL ligand fragment. (C) Development of VHL ligands. LE: ligand efficiency.
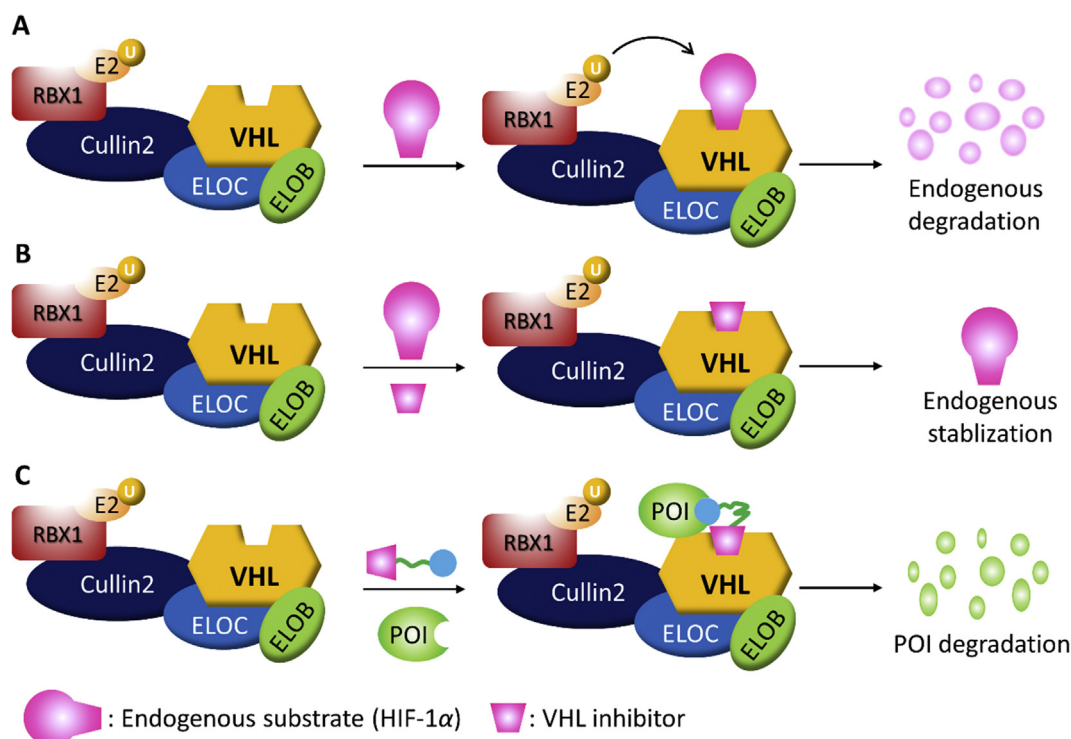
Figure 11   Development of PROTAC based on VHL as E3 ubiquitin ligase. (A) Under normal physiological conditions, endogenous substance HIF-1α is recognized by VHL, ubiquitinated, and degraded by the proteasome. (B) VHL inhibitors block the interaction of VHL−HIF-1α, leading to stabilization and activation of HIF-1α. (C) PROTAC developed based on VHL inhibitors used for the degradation of POI.

of the IKK family of serine/threonine kinases involved in the development of innate immune responses, tumor and various cellular functions[171,172]. Subsequently, researchers reported a proteasome-dependent PROTAC **37**[173]. It was a potent TBK1 degrader (DC$_{50}$=12 nmol/L, D_{max}=96%), completely degraded TBK1 at the concentration of 100 nmol/L in several cancer cell lines, and had excellent selectivity over the related kinase IKK.

Using VHL-based PROCTACs to induce degradation of the active receptor tyrosine kinase (RTK) proved feasible[174]. Compound **38** targeted EGFR (epidermal growth factor receptor) (DC$_{50}$=215.8 nmol/L); compound **39** targeted c-Met, contrary to inhibition of kinase activity, which was a strategy for degrading RTK. Recently, an FMS-like tyrosine kinase 3 (FLT-3) PROTAC **40**[175] was developed by linking the clinical candidate quizartinib[176] (AC220) to the VHL ligand *via* an optimized linker. It effectively induced degradation of FLT-3 ITD protein in MV4-11 cells and MOLM-14 cells at nanomolar concentrations. In addition, **40** inhibited cell growth more effectively than a single warhead.

TRIM24 is a multidomain protein extensively characterized as a transcriptional co-regulator[177]. dTRIM24 (**41**) was synthesized using VHL and TRIM24[178]. Dose- and time-dependent degradation of TRIM24 was observed in 293FT cells (DC$_{50}$=2.5−5 μmol/L and D_{max}=70% at 5 μmol/L). As an emerging cancer-dependent chemical probe, **41** provides a good approach of utilizing selective but ineffective ligands of POI. The anaplastic lymphoma kinase (ALK)-PROTAC TD-004 (**42**) consists of ceritinib and VHL ligands to degrade the ALK fusion protein, such as NMP−ALK or EML4−ALK[179]. Compound **42** effectively inhibited cell proliferation of the SU−DHL-1 cancer

cell line that expresses ALK-fusion protein (IC$_{50}$=60 μmol/L, H3122, IC$_{50}$=180 μmol/L). However, **42** did not inhibit the growth of the A549 cell line, which does not express the ALK-fusion protein (IC$_{50}$>0.01 nmol/L). Focal adhesion kinase (FAK) is a key player in tumor invasion and metastasis[180]. Approved kinase inhibitor drugs have limited inhibition of FAK[181]. In contrast, the degradation of FAK may greatly reduce FAK signaling. Thus, researchers reported a selective and potent FAK-degrader, compound **43**[182] (DC$_{50}$=3.0 nmol/L, D_{max}=99%). It is regarded as a drug candidate for certain tumor diseases.

Recently, Shaomeng Wang group[183] developed a group of potential PROTAC AR degraders using different linkers to link VHL ligands with different AR antagonists. The solubility of PROTAC depends on the linker, thus a pyridyl group directly linking the ethynyl group led to a good solubility of PROTAC. Among these PROTACs, ARD-69 (**44**) appeared to be the most effective AR degrader. The DC$_{50}$ values of **44** in the LNCaP and VCaP cell lines were 0.86 and 0.76 nmol/L respectively, and the D_{max}>95% is at a concentration of 10 nmol/L. In 22Rv1 cells, the DC$_{50}$=10.4 nmol/L, and 1 μmol/L of **44** almost completely degraded AR. **44** effectively inhibited the expression of the prostate specific antigen (PSA), *TMPRSS2* and *FKBP5* genes in LNCaP and prostate vertebral body cancer (VCaP) cell lines in a dose-dependent manner. It also reduced mRNA levels of the *PSA* and *TMPRSS2* genes. In addition, **44** is 100 times more potent than the AR antagonist in inhibiting AR-regulated gene transcription in LNCaP and VCaP cells. AR signaling drives cell growth of AR-positive prostate cancer cells. Compound **44** strongly inhibited the growth of LNCaP, VCaP, and 22Rv1 cell

lines and the IC_{50} values were 0.25, 0.34 and 183 nmol/L respectively, which were >100-fold more potent than the 2 AR antagonists. More importantly, **44** effectively reduced AR and PSA proteins in mouse VCaP xenograft tumor tissues and such an effect was sustained for at least for 48 h. Further optimization of **44** may lead to drug candidates for the treatment of metastatic castration-resistant prostate cancer (mCRPC).

PROTAC can lead to degradation of different target proteins. However, for closely related protein families, controlling the selectivity of degradation is still a challenge. In order to develop isoform-selective PROTACs selectively targeting p38α and p38δ in the p38 MAPK family, researchers used different linkers to link a single warhead (foreinib) with VHL[184], as well as changing the orientation of the VHL recruiting molecule. Researchers found that PROTAC consisting of different attachment sites of VHL ligands yields selectivity to degrade different proteins. SJFα (**45**) (t-butyl position of VHL ligand) degraded the p38α in human breast cancer MDA-MB-231 cells ($DC_{50} = 7.16$ nmol/L, $D_{max} = 97.4\%$), but the efficiency of degrading p38δ was significantly lowered ($DC_{50} = 299$ nmol/L, $D_{max} = 18\%$). In addition, other p38 isoforms were not degraded (β and γ) at concentrations up to 2.5 μmol/L, indicating that **45** can selectively degrade p38α. However, SJFδ (**46**) (phenyl position of VHL ligand) is capable of degrading p38δ ($DC_{50} = 46.17$ nmol/L, $D_{max} = 99.4\%$) but has no effect on the level of p38α, β or γ. This indicated that such PROTACs can selectively target protein members even in closely related protein families.

Interleukin-1 receptor-associated kinase 4 (IRAK4) is a serine/threonine kinase involved in toll-like receptors (TLRs) and white blood cells[185]. The transduction pathway stimulated by the interleukin-1 (IL-1) receptor family further leads to the activation of IRAK4, which is associated with various diseases such as psoriasis, rheumatoid arthritis and cancer[186]. Therefore, it is important to develop drugs that can promote IRAK4 protein degradation. Recently, the protein degradation drug development team of GlaxoSmithKline (GSK) Drug Research Center[187] reported the results of a PROTACs study on IRAK4 protein. Compound **47** was most effective in PBMC cells ($DC_{50} = 151$ nmol/L). Furthermore, compound **47** was able to induce a decrease in IRAK4 protein levels in cells of human skin fibroblasts ($DC_{50} = 36$ nmol/L), but did not inhibit IL-6 secretion. Therefore, PROTAC-targeted degradation of IRAK4 may open up opportunities to develop new therapeutic drugs for the treatment of inflammatory and neoplastic diseases.

PROTACs promise to expand the druggable proteome as degradation is not limited to the protein domain which is functionally responsible for the disease. The ATP-dependent activities of the BAF (SWI/SNF) affect the localization of nucleosomes on DNA, thereby affecting many cellular processes related to chromatin structure, including transcription, DNA repair and decatenation mutant cancers, etc[188]. The BAF complex is mutated in approximately 20% of human cancers, and SMARCA2 and SMARCA4 are 2 mutant subunits of which. These mutants have different roles in different cancers; SMARCA4 inhibits cancer in solid tumors and promotes cancer cell growth in acute myeloid leukemia (AML)[189]. Therefore, targeting the above proteins is crucial for cancer treatment. The Ciulli team[190] showed how structure-based PROTAC design can identify potent degraders of SMARCA2 and SMARCA4 with anticancer activity.

They first designed the PROTAC 1 (**48**) molecule based on SMARCA bromodomain inhibitors. By observing the eutectic structure of the high resolution SMARTA2BD:PROTAC 1:VCB

(VHL−ELONGINC−ELONGINB complex) ternary complex, researchers performed a reasonable structural optimization of PROTAC. This operation is mainly to insert benzene on the linker which can form pi−π stacking with Tyr98 of VCB. The optimized PROTAC 2 (**49**) was found to have better molecular recognition ability and synergism of SMARCA than **48**, as well as improved stability of the ternary complex. Next, the researchers observed the eutectic structure of the second-generation ternary complex SMARTA2BD:PROTAC 2:VCB, and added an oxygen to the linker to increase the length of the linker and the permeability of the molecule to obtain the PROTAC molecule ACBI1. The results showed that PROTAC ACBI1 (**50**) has good cell permeability and can induce the degradation of SMARCA2 ($DC_{50} = 6$ nmol/L) or SMARCA4 ($DC_{50} = 11$ nmol/L) and PBRM1 ($DC_{50} = 32$ nmol/L) rapidly, effectively, and specifically. Further, **50** can induce anti-proliferative and apoptotic effects in acute myeloid leukemia cells, which provides a new possibility for treating cancers that are sensitive to BAF complex loss.

Structures and protein degradation activities of VHL-based PROTAC 30−50 are shown in Fig. 12 and Table 3.

In this section, we discuss how VHL acts as an E3 ligase and its interaction with HIF-1α. We also highlight the optimization of VHL ligands and the development of VHL-based PROTAC. We hope to provide a reference to find more VHL ligands and the development of more VHL-based PROTACs in the future.

4.4. CRBN-based PROTAC

The immunomodulatory drugs (IMiDs) based on thalidomide (**51**) as a lead compound have been successfully repurposed for erythema nodosum leprosum, multiple myeloma (MM) and myelodysplasia. Thalidomide was found to bind to CRBN, leading to the identification of more drug-like E3 ligase binders. CRBN was the primary teratogenic target of thalidomide (**51**)[191]. The C-terminal domain of CRBN, named CULT, has been defined as a binding site of cellular ligands and thalidomide (**51**). The crystal structures of the DDB1−CRBN complex bound to thalidomide (**51**), lenalidomide (**52**) and pomalidomide (**53**) (Fig. 13A) have been established and provided the molecular basis for teratogenicity[192]. Unexpectedly, evidence demonstrated that **51** and its derivatives **52** and **53** exerted immunomodulatory and anti-proliferative activities by reducing protein levels of the anti-apoptotic protein. Selective ubiquitination and degradation of specific targets including transcription factors, Ikaros (IKZF1) and Aiolos (IKZF3), casein kinase 1α through hijacking U ligase CRBN provided a novel mechanism of therapeutic activity for proteins[193−195].

CRBN is ubiquitously expressed in physiologic and pathophysiologic tissues, but CRBN modulators may exert tissue-specific effects. Inspired by the retrieval of CRBN using **51**, a series of bifunctional PROTACs have been rationally designed. Especially, experiments have demonstrated that the aryl ring of **53** can tolerate chemical substitution in PROTAC. The phthalimide (**53**)-based conjugated ligands have been widely used to develop the libraries of CRBN-targeting PROTACs, which can be easily converted into multiple PROTAC precursors[196]. Recently, several CRBN modulators have been reported, which were demonstrated to mediate antitumor effects through the ubiquitination and degradation of the translation termination factor G1 to S phase transition 1 protein (GSPT1)/Ikaros and Aiolos. Studies have revealed that glutarimide mediates substrate binding on the surface of CRBN via a PPI[197−199].
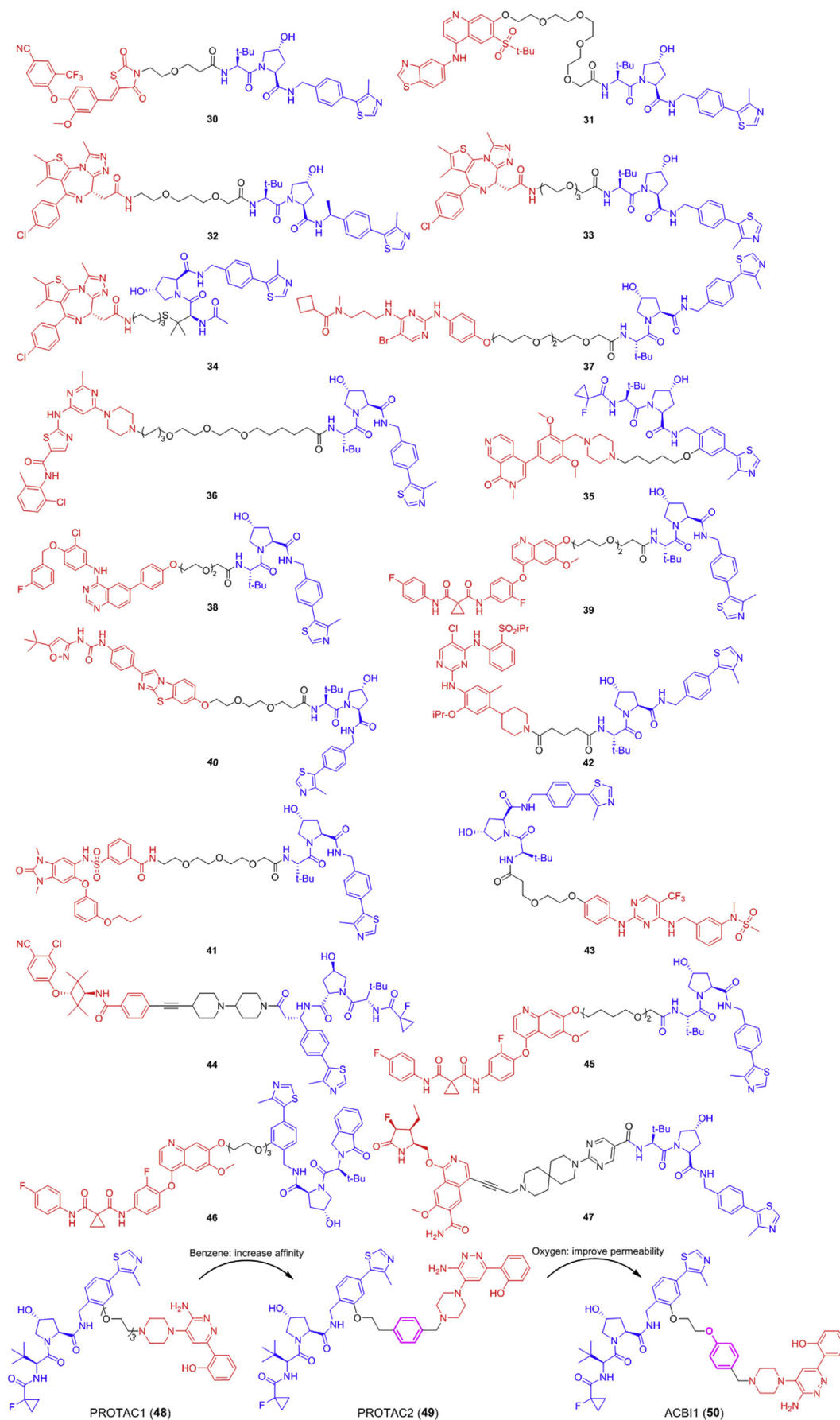
Figure 12 Structures of PROTACs **30–50** based on CRBN as E3.

Table 3 PROTACs **30−47** with the ability to degrade target proteins developed based on VHL as E3[a].

Compd.	Target	Degradation in cell lines		Ref. No. (Year)
		DC_{50}	D_{max} (%)	
30	ERRα	100 nmol/L	86	163 (2015)
31	RIPK2	1.4 nmol/L	>95	163 (2015)
32	BRD2/3/4	<5 nmol/L/<1 nmol/L for BRD2/3/4	>50	165 (2016)
33	BRD4	<100 nmol/L	>90	167 (2015)
34	BRD4	10−100 nmol/L	>90	168 (2017)
35	BRD9/7	4 nmol/L for BRD9 34.5 nmol/L for BRD7	95	169 (2018)
36	C-ABL	NA	NA	170 (2016)
37	TBK1	12 nmol/L	96	173 (2018)
38	EGFR/HER2	215.8 nmol/L	79.1	174 (2018)
39	c-Met	NA	NA	174 (2018)
40	FLT-3	43 nmol/L for FLT-3 ITD 36 nmol/L for FLT-3 WT	NA	175 (2018)
41	TRIM24	2.5−5 μmol/L	70	178 (2018)
42	ALK	NA	NA	179 (2018)
43	FAK	3.0 nmol/L	99	182 (2018)
44	AR	<1 nmol/L	95	183 (2018)
45	p38α	7.16 nmol/L	97.4	184 (2019)
46	p38δ	299 nmol/L	99.4	184 (2019)
47	IRAK4	151 nmol/L	NA	187 (2019)

[a]DC_{50}: the concentration at which 50% degradation was observed. D_{max}: the maximal level of degradation. NA: not available.

ARV-825 (**54**) was the first CRBN based PROTAC generated by Crews group[200], which mediates the degradation of the oncoprotein BRD4 in a substoichiometric but dose-dependent manner. BRD4 plays a pivotal role in regulating essential oncogene expression, including *c-MYC*, *BCL-xL* and *BCL-6*[200,201]. BRD4 is a promising therapeutic target in multiple cancer types, *e.g.,* CRPC[202] and pancreatic cancer[203]. Compound **54** completely degraded BRD4 at 10 nmol/L within 6 h (DC_{50}<1 nmol/L). In a word, **54** can efficiently lead to degradation of pathogenic proteins.

Another small molecule BRD4 degrader, dBET1 (**55**), was developed using JQ1 and thalidomide (**51**) derivatives, resulting in the chemical recruitment of BRD4. Treatment with increasing concentrations of **55** in human AML cell lines (MV4; 11) for 18 h yielded good results (DC_{50}=430 nmol/L, D_{max}>95% for BRD4)[41]. Comparing the 2 PROTACs (**54** and **55**), they have similar binding moieties but different linkers. Interestingly, the degradation activity of **54** was 10-fold greater than that of **55**, suggesting that careful design of the "linker" region may improve PROTAC's selectivity and affinity[204].

CRBN-based PROTAC **56** not only retains the ability to induce c-ABL degradation (>85% degradation in 1 μmol/L), but also induces BCR-ABL degradation (>60% degradation in 1 μmol/L)[170]. Compound dBRD9 (**57**) is a PEG-linked **53** conjugate that promotes rapid degradation of BRD9 over a wide range of concentrations[205].

Cyclin-dependent kinase 9 (CDK9) is a member of the CDK family. CDK9 inhibitors have therapeutic effects on hematological malignancies and HIV infection[206,207]. Compound **58** selectively leads to degradations of CDK9 in HCT116 cells[208], without affecting other CDK family members. In addition, a series of wogonin-based PROTACs were developed by "click chemistry" to link the target protein to CRBN[209]. Among these compounds, a compound having a triazole linker was more effective than a compound having an alkane chain, and a compound **59** having a

linker of 10 atoms length exhibited an optimum CDK9 degrading activity and could selectively down-regulate CDK9 levels in a concentration-dependent manner. CDK9 was inhibited at submicromolar concentrations (IC_{50}=523±12 nmol/L), while the CDK2, CDK4, CDK5, CDK7 and CDK8 levels remained unchanged. Compound **59** inhibited the proliferation of MCF-7 cells at a concentration (IC_{50}=17±1.9 μmol/L) lower than that of wogonin (IC_{50}=30±3.5 μmol/L), indicating that **59** is a potent inducer of apoptosis. In conclusion, this wogonin-based PROTAC is highly selective in CDK9 high expression cell lines, and will be a useful tool for further study of CDK9-dependent effects in cancer cells. THAL-SNS-032 (**60**)[210] was also a selective CDK9 degrader, consisted of a SNS-032 ligand and a thalidomide (**51**) derivative. Compound **60** induced rapid degradation of CDK9 without affecting the levels of other SNS-032 targets (DC_{50}<250 nmol/L). Compound **60** has improved degradation efficiency compared to the previously reported degradation of CDK-producing PROTAC.

In the CDK family, CDK4/6 can regulate G1-S cell cycle transition by phosphorylating retinoblastoma tumor suppressor protein, further triggering the gene expression process that promotes the entry of S phase. Therefore, CDK4/6 is a very important target for cancer therapy. Some ATP-competitive CDK4/6 inhibitors have been reported to show significant clinical activity[211], but they cannot specifically recognize CDK4 and CDK6. On this basis, researchers developed a series of bifunctional small molecules that jointly or selectively target CDK4/6. Among them, a pomalidomide (**53**)-based degrader BSJ-03-123 (BSJ) (**61**)[212] uses the protein interface to selectively degrade CDK6 within the proteome range. In addition, BSJ-02-162 (**62**) induces the degradation of both CDK4 and CDK6, whereas BSJ-01-187 (**63**) selectively targets CDK4, and YKL-06-102 (**64**) targets CDK6[213].

Compound **65** was developed by the cycloaddition reaction which linked a thiramide-derived azide to an alkynylation inhibitor[214]. Compound **65** induced isoform-selective Sirtuin 2 (SIRT2)

degradation (IC$_{50}$=0.25±0.02 μmol/L). Recently, **66** was designed by combining an indazole-based BET inhibitor and a **51** analog[215]. It effectively induced degradation of BRD2-4 at concentrations as low as 0.1−0.3 nmol/L in the RS4-11 acute leukemia cell lines (IC$_{50}$=51 pmol/L). The second-generation BRD inhibitor BETd-246 (**67**)[216] has good selectivity and anti-tumor activity. It induced selective degradation of BET protein in TNBC cells at low nanomolar concentrations, and showed excellent cytotoxicity in most TNBC cell lines. In a word, **66** and **67** have potent therapeutic activity against acute leukemia and TNBC. In 2018, compound **68** was developed by combining a BET inhibitor and CRBN ligands to target the BET protein[36]. After incubation with **68**, the levels of BRD2, BRD3 and BRD4 in leukemia cell lines were effectively reduced at concentrations of 30−100 pmol/L. The 3 proteins were completely degraded in the RS4-11 cell line (DC$_{50}$<0.5 nmol/L, D$_{max}$ ∼100%). Furthermore, **68** was 1000-fold more potent than dBET1 and at least 10-fold more potent than **54** in inducing protein degradation. **68** inhibited cell growth in the MV4-11, RS4-11, and MOLM-13 human leukemia cell lines with IC$_{50}$ values of 8.3, 62 and 32 pmol/L, respectively. This suggested that **68** provided a more powerful strategy for the treatment of acute leukemia.

Researchers have developed some potential multi-kinase degraders by combining highly promiscuous kinase inhibitors with CRBN ligands. Among them, TL13-149 (**69**)[217] and DD-04-015 (**70**)[217] selectively target FLT-3 and Bruton's tyrosine kinase (BTK). A small molecule PROTAC MT-802 (**71**) targets the wild-type and C481S mutant BTK[218]. The experimental results showed that **72** had good BTK degradation activity for wild-type BTK (DC$_{50}$=14.6 nmol/L, D$_{max}$>99%) and C481S mutant BTK (DC$_{50}$=14.9 nmol/L, D$_{max}$>99%), which indicated that **71** is a therapeutic candidate for targeting mutant BTK. In addition, compound **72** also has good activity to reduce BTK levels (DC$_{50}$ ∼10 nmol/L)[219].

Although the above multi-kinase degraders can target many kinases, some pathogenic kinases such as ALK still cannot be effectively degraded. ALK is a tyrosine kinase receptor and involved in the development of a variety of human cancers[220]. Some researchers have reported a novel PROTAC for ALK. Considering the high potency and selectivity of ceritinib for ALK, ceritinib was selected as the ALK binding moiety. The X-ray crystal structure of ALK in the complex with ceritinib (PDB ID:4MKC) indicated that the piperidine group is located in the solvent-exposed zone. Therefore, the researchers accessed different lengths and types of linkers from the piperidine position of ceritinib to the ligand of the CRBN[221]. Among these PROTAC molecules, MS4078 (**73**) showed high affinity for ALK with a K_d value of 19±3 nmol/L in SU-DHL-1 cells. **73** effectively reduce the cellular level of oncogenic activity of ALK fusion protein in SU-DHL-1 lymphoma (DC$_{50}$ ∼11 nmol/L, D$_{max}$=90%) and NCI−H2228 lung cancer cells (DC$_{50}$=59 nmol/L) in a concentration- and time-dependent manner. In addition, **73** effectively inhibited the proliferation of SU-DHL-1 cells. More importantly, **73** showed good plasma exposure in mouse PK studies. Therefore, **73** can be a useful chemical tool for *in vivo* efficacy studies, laying the foundation for the development of the next generation of ALK PROTAC.

P300/CBP-associated factor (PCAF) and general control non-repressed protein 5 (GCN5) are closely related epigenetic proteins[222], and PCAF can produce some inflammatory factors[223]. In THP1 cells, GSK983 (**74**)[224] induced the concentration-dependent degradation of PCAF (DC$_{50}$=1.5 nmol/L, D$_{max}$=80%).

Similarly, it also degraded GCN5 (DC$_{50}$=3 nmol/L, D$_{max}$=80%). Thus, **74** may provide a new anti-inflammation strategy.

Casein kinase 2 (CK2) is a highly pleiotropic active serine/threonine protein kinase overexpressed in many cancers. CK2 inhibitors are linked to porphyrins by a "click chemistry" reaction to form several PROTAC targeting CK2[225]. Among them, compound **75** induced CK2 degradation in a dose- and time-dependent manner, and had the best inhibitory effect at 10 μmol/L, maintaining CK2 at a low basal level through UPS. Recently, compound **76**, a B-cell lymphoma 6 (BCL6) PROTAC was reported[226]. In a range of diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL) cell lines, **76** effectively induced BCL6 degradation at a concentration of 1 μmol/L. MDM2 is a key oncogenic protein that contributes to cell growth, survival, invasion and therapeutic resistance in cancer[101]. Using the potent MDM2 inhibitor and CRBN ligand lenalidomide (**52**), researchers successfully obtained an effective PROTAC MD-224 (**77**)[227]. Even at concentrations<1 nmol/L, **77** still induced rapid degradation of MDM2 protein in human leukemia cells. **77** was 10−50-fold more potent than MDM2 inhibitors in inducing P53 activation and inhibiting cell growth in RS4-11 and MV4. PK data showed that a single dose of **77** continuously degraded MDM2 and up-regulated P53 over 24 h.

Recently, a new method for preparing PROTAC conjugates using solid phase organic synthesis (SPOS) has been reported[228]. A pre-loaded resin, composed of a thalidomide moiety and an ethylene-oxyl linker, can simply and rapidly synthesize PROTAC. Compound **78** prepared by this method induced BTK degradation in a dose-dependent manner, reducing BTK levels to 15% at 2000 μmol/L concentration, IC$_{50}$=0.16±0.04 μmol/L[228]. Of course, this thalidomide-prepackaged resin (TPR) can also be applied to other proteins that can be obtained with suitable inhibitors/regulators/ligands, suggesting this method has useful versatility. In addition, researchers also developed the next generation of BTK degrader, L18I (**79**), a molecule that combined ibrutinib and lenalidomide (**52**) *via* PEG linkers[229]. Compound **79** efficiently degraded C481S BTK in HBL-1 cells (DC$_{50}$=29 nmol/L). More importantly, **79** had significant anti-tumor effects in a mouse xenograft model inoculated with C481S BTK HBL-1 cells. These results indicate that **79** has great potential in the treatment of drug-resistant cancers.

A new generation of multifunctional histone deacetylase 6 (HDAC6) degraders was reported by combining the selective HDAC6 inhibitor Nexturastat A with the CRBN ligand[230]. These new degraders can synergize with HDAC6 degradation for the antiproliferation of MM. By optimizing the linker length and the position of the linker, compound **80** has the best potency and selectivity for degrading HDAC6 (DC$_{50}$=1.6 nmol/L, D$_{max}$=86%). More importantly, due to the multifunctionality of the degrader, **80** also has significantly more potent anti-proliferative effects over HDAC6 inhibitor alone, IMiD alone, or its combination in MM cancer cell lines. These multifunctional HDAC6 degraders may provide a novel strategy to therapy MM.

The small molecule ARV-110, an orally available PROTAC protein degrader, binds specifically to AR and mediates AR degradation[231]. ARV-110 completely degraded AR in all tested cell lines (DC$_{50}$<1 nmol/L). Recently, Arvinas company announced that ARV-110 has been administered to patients in Phase I clinical trials[232].

In preclinical studies, the AR degradation agent ARV-110 showed promising activity[233]. In the enzalutamide-sensitive model, ARV-110 was able to significantly reduce PSA levels at low doses and was superior to enzalutamide. In an *in vivo* model

of acquired and intrinsic resistance to enzalutamide, the ARV-110 pair showed 70% and 100% tumor inhibition rates, respectively. ARV-110 degrades 95%−98% of AR in various cell lines typically used in prostate cancer research. In VCaP, ARV-110 can reduce AR in a time-dependent manner, where AR was degraded almost completely within 4 h after administration. In a VCaP xenograft mouse model, ARV-110 was able to reduce PSA in plasma at lower doses and was superior to enzalutamide. In an *in vivo* model of acquired enzalutamide resistance, daily and orally delivered ARV-110 (3 mpk) inhibited the tumor growth by up to 70%. In a PDX patient model, orally delivered ARV-110 (10 mpk) significantly inhibited the growth of enzalutamide-insensitive tumors. ARV-110 is currently undergoing Phase I clinical trials to evaluate its safety and tolerability in patients with mCRPC who have progressed on standard treatment options.

Structures and protein degradation activities of CRBN-based PROTACs **54−80** are shown in as Figs. 13B,14 and Table 4.

Small molecule PROTACs have been successfully developed to target MDM2, cIAP, VHL, CRBN. More importantly, a series of patent applications were filed reporting the synthesis of these PROTACs, such as PROTAC **33**[234], PROTAC **37**[235], ARV-825 (**54**)[236]. The patent applications also claimed that these PROTACs can degrade different proteins and provide a good strategy for treating diseases such as cancer.

5. The advantages of PROTACs

Traditional small molecule inhibitors have many limitations. The selection pressure of small molecule kinase inhibitors often results in drug resistance in cancer cells. For instance, long-term clinical application of gefitinib leads to EGFR mutations[237]. Moreover, some protein structures do not support high-affinity inhibitors, and low-affinity inhibitors at high concentrations may cause unfavorable off-target effects[238]. The successful PROTAC targeting RTK suggests that the PROTAC degradation strategy may be superior to inhibitors in several key aspects. First, PROTACs may effectively induce protein degradation even at low concentrations. Second, PROTACs result in more effective and durable signal transduction inactivation and growth inhibition, without the concern about rewiring in the kinase group. Finally, PROTACs may induce degradation of mutant proteins to prevent them from "disengaging"[174]. We will thoroughly discuss the advantages of PROTACs in the following paragraphs.

5.1. Targeted degradation of undruggable proteins

The FDA has approved drugs for ∼400 human proteins[248], but there are about 3000 proteins associated with disease. There are several reasons for the fact that most disease-related proteins have no corresponding drugs: first, some proteins may have multiple functions and catalytic domain structures, thus blocking just one function may not be sufficient. Second, some drug targets have no specific active catalytic sites. Therefore, the traditional protein inhibitor approach cannot be applied to these disease-related proteins[83]. Not all molecular targets are enzymes or receptors with druggable "hot spots" accessible to site-directed inhibitors[239]. For these challenging drug targets, PROTAC-induced protein degradation can provide a new solution. PROTAC ligands can be designed to bind target proteins specifically, even if no traditional "hot spot" is available.

The highly selective and potent FAK degrader compound **43**[182] (IC$_{50}$=6.5 nmol/L, DC$_{50}$=3.0 nmol/L, D_{max}=99%) (Fig. 15A)

has been developed based on the most potent clinical FAK inhibitor defactinib. Inducing FAK degradation not only alters kinase-dependent signal transduction, but also affects its kinase-independent signal transduction due to the lack of FAK itself. These results indicate that **43** has great potential in expanding the space for regulating protein function.

5.2. Eliminating the accumulation of drug targets

When inhibitor drugs bind targets, the accumulation of drug targets can be observed even within a short period of time. On the one hand, the accumulation of targets can stabilize proteins and prolong their half-life; this phenomenon can be seen in many inhibitors, such as the BRD4 inhibitor JQ1[200]. In general, the accumulation of drug targets may limit the efficacy of the drug or induce drug resistance. Therefore, target protein degradation induced by PROTAC may be a valuable method for these proteins that may escape by protein stabilization or compensatory upregulation. For example, BRD4 inhibitors rapidly lose efficacy due to BRD4 regulation; compound **54**[200], a heterologous PROTAC, can rapidly and efficiently prolong the degradation of BRD4 in BL cells, suggesting that **52** is particularly suitable for overcoming BRD4's escape from sensitivity to inhibitors by protein stabilization or overexpression.

5.3. Specificity

Developing highly selective molecules is the main goal for many pharmaco-chemical researchers. Good selectivity can be achieved by rational design based on the structure of the compound, and experimental data generation in parallel. It is reported that an amide type SNIPER selectively degraded CRABP-II but had no effect on IAPs[139]. Ideally, some small molecule inhibitors only inhibit pathogenic proteins but have no effect on other proteins. For example, a small molecule inhibitor of BRAFV600E kinase selectively targets the melanoma with the mutant BRAFV600E gene or other mutant BRAF protein changed at codon 600[240], but has no effect on cell lines expressing wild-type BRAF. However, this ideal is not easy to achieve due to limited differences between disease-related proteins and other proteins in the same family.

PROTAC targeting degradable proteins provide a good strategy for high selectivity (Fig. 15B). Compound **36** induces degradation of c-ABL but not BCR-ABL degradation, whereas compound **56** not only retains the ability to induce c-ABL degradation >85% degradation in 1 μmol/L, but also induces BCR-ABL degradation (>85% degradation in 1 μmol/L), indicating that **36** may have higher selectivity[170]. In addition, some PROTACs were developed to induce selective degradation of the target protein among closely related proteins[184]. For instance, compound **45** can selectively degrade p38α in MDA-MB-231 human breast cancer cells (DC$_{50}$=7.16 nmol/L, D_{max}=97.4%), and it has little influence on p38β, γ and δ. Similarly, compound **46** can selectively induce degradation of p38δ instead of p38α, β or γ.

5.4. Substoichiometric catalytic activity

Another feature of PROTACs is their substoichiometric catalytic activity, which reduces the need for target engagement and occupation of traditional inhibitors[163]. Traditional small molecule protein inhibitors regulate protein function in manners highly dependent on concentrations. However, even low concentrations of PROTACs are sufficient to degrade proteins to basal levels and
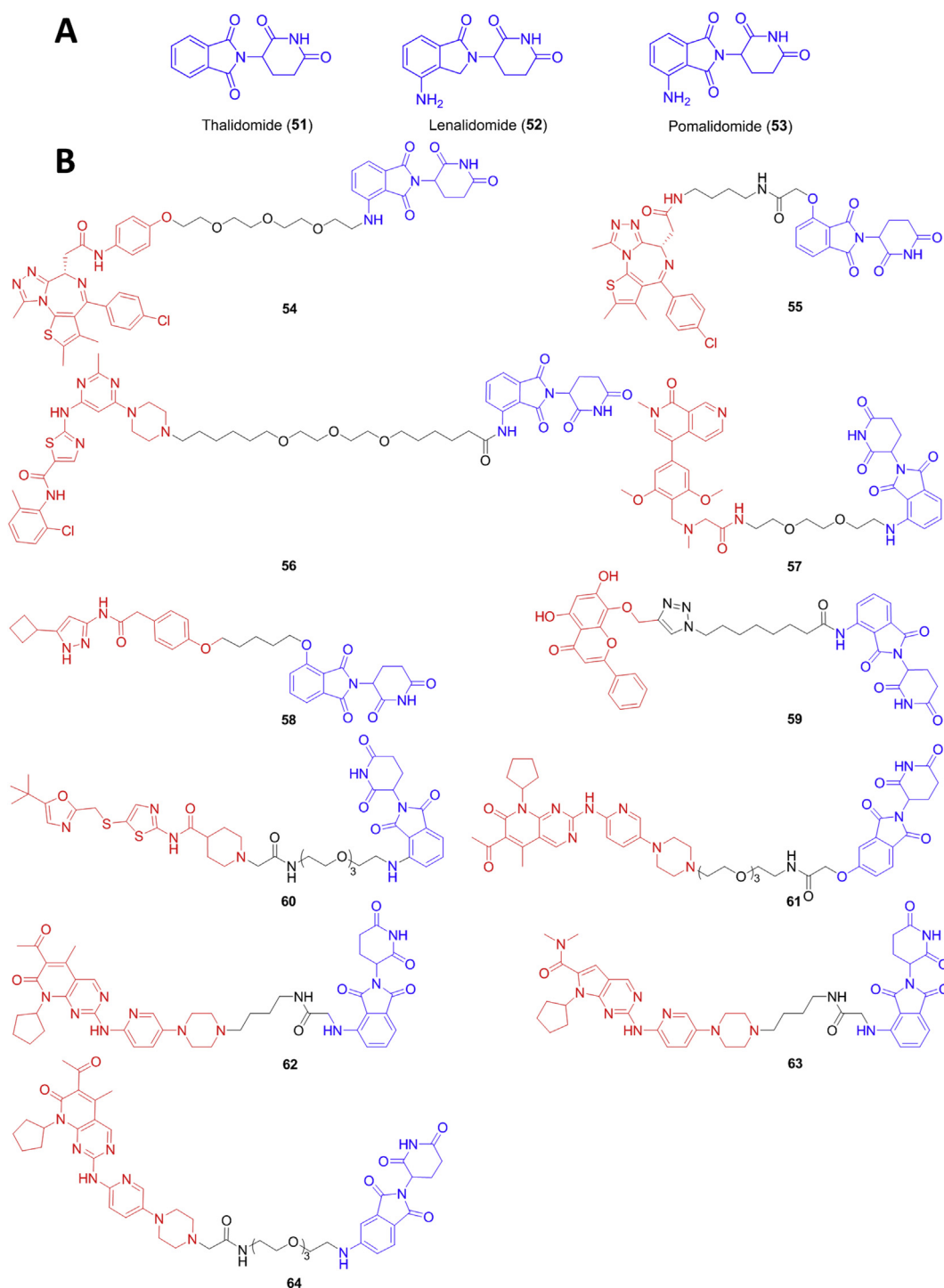
Figure 13 CRBN (cereblon) ligands **51−53** and CRBN PROTACs **54−64**. (A) Development of CRBN ligands. (B) Structures of PROTACs **54−64** based on CRBN as E3.

maintain this effect to achieve a desirable pharmacological effect, especially effective for some slowly-synthesized proteins. In addition, even if a PROTAC is exhausted or metabolized, the recovery of the target protein may need hours to days. It may be feasible to achieve sustained release of PROTACs *via* certain formulations, *e.g.*, tablets, subcutaneous or intramuscular injection, etc., to treat chronic diseases that need long-term medication[241].

Catalytic degradation induced by PROTAC is usually highly time-dependent and consumes more proteins over time. In addition, this kind of catalysis also has other advantages: PROTAC concentration can be observed in the target protein degradation, and it is far lower than the high level of E3 ligase inhibition.

For example, compounds **9** and **22** can respectively induce AR and BCR-ABL ubiquitination and degradation at micromolar
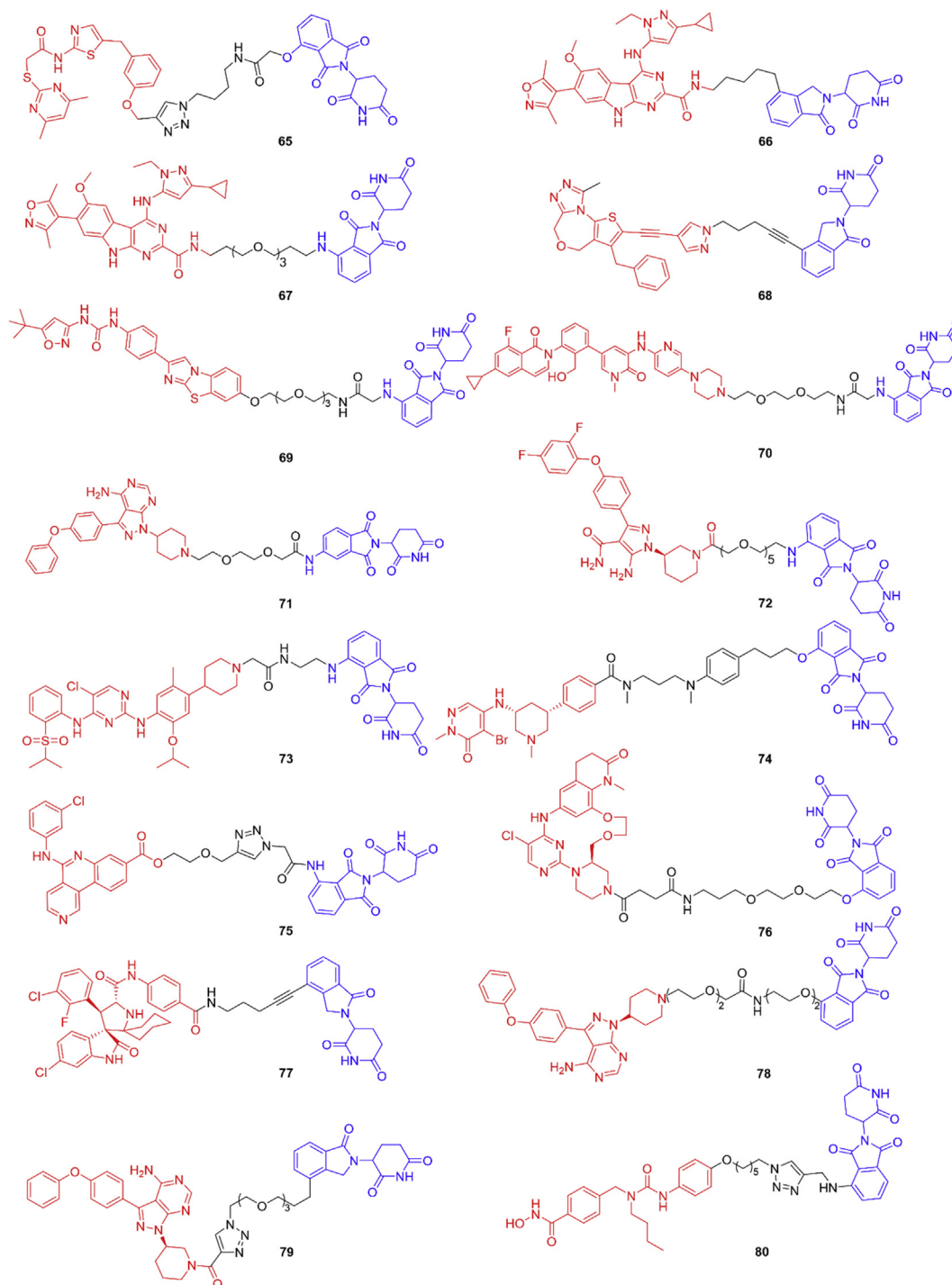
Figure 14   Structures of PROTACs **65−80** based on CRBN as E3.

concentrations[242]. More importantly, PROTACs at lower concentrations also produce less off-target toxicity than traditional small-molecule inhibitors, likely leading to better therapeutic indices.

5.5.   Others

PROTACs also have several other advantages apart those mentioned above. Recent studies have shown that PROTACs

have potent *in vitro* and *in vivo* activities. Some examples show that PROTACs, based on the E3 ligand design of VHL and CRBN, have promising medicinal properties, and BET-PROTAC is a typical example of these small molecules. Using BET-PROTAC as a model system for long-term administration, cancer cells showed resistance to PROTAC containing VHL and CRBN ligands. However, unlike other targeted therapeutics (*e.g.*, kinase inhibitors), BET-PROTAC resistance is not due to PROTAC-bound target protein mutations, but due to VHL and

Table 4 PROTACs **54−80** with the ability to degrade target proteins developed based on CRBN as E3[a].

Compd.	Target	Degradation in cell lines		Ref. No. (Year)
		DC_{50}	D_{max} (%)	
54	BRD2/3/4	<1 nmol/L for BRD4	∼100	200 (2015)
55	BRD2/3/4	430 nmol/L for BRD4	>95	41 (2015)
56	BCR-ABL& c-ABL	NA	NA	170 (2016)
57	BRD9	NA	NA	205 (2017)
58	CDK9	NA	NA	208 (2017)
59	CDK9	NA	NA	209 (2018)
60	CDK9	<250 nmol/L	∼100	210 (2018)
61	CDK6	NA	NA	212 (2018)
62	CDK4/6	NA	NA	213 (2019)
63	CDK4	NA	NA	213 (2019)
64	CDK6	NA	NA	213 (2019)
65	SIRT2	0.2−1 μmol/L	90	214 (2018)
66	BRD2/3/4	<0.03 nmol/L for BRD4	∼100	215 (2018)
67	BET	NA	∼100	216 (2017)
68	BET	<0.5 nmol/L	∼100	36 (2018)
69	FLT-3	NA	NA	217 (2018)
70	BTK	NA	NA	217 (2018)
71	BTK	14.6 nmol/L for wild-type BTK; 14.9 nmol/L for C481S BTK	>99	218 (2018)
72	BTK	∼10 nmol/L	NA	219 (2018)
73	ALK	11 nmol/L for NPM-ALK 59 nmol/L for EML4-ALK	>90	221 (2018)
74	PCAF/GCN5	1.5 nmol/L for PCAF 3 nmol/L for GCN5	80	224 (2018)
75	CK2	NA	NA	225 (2018)
76	BCL	NA	NA	226 (2018)
77	MDM2	<1 nmol/L	NA	227 (2018)
78	BTK	NA	85	228 (2019)
79	BTK	29 nmol/LM	NA	229 (2019)
80	HDAC6	1.6 nmol/L	86	230 (2019)

[a]DC_{50}: the concentration at which 50% degradation was observed. D_{max}: the maximal level of degradation. NA: not available.

CRBN-based BET-PROTAC[243]. It is interesting that the resistance to BET-PROTAC is related to the composition of the E3 ligase. In addition, PROTACs provide rapid pharmacological effects on target proteins by C_{max}-driven pharmacodynamic effects and competitive binding sites on target proteins to recruit U ligase[83].

Largely, PROTACs have the advantage of traditional small molecule inhibitors in selectivity, as well as the advantage of siRNA in downregulating the target proteins. Further development of PROTAC technology would include improving cell activity, oral bioavailability and safety.

6. Other options for target protein ligands

The PROTACs we discussed in previous sections of this article were constructed by linking E3 ligase and target ligands with various linkers. However, some molecules are constructed by other methods,
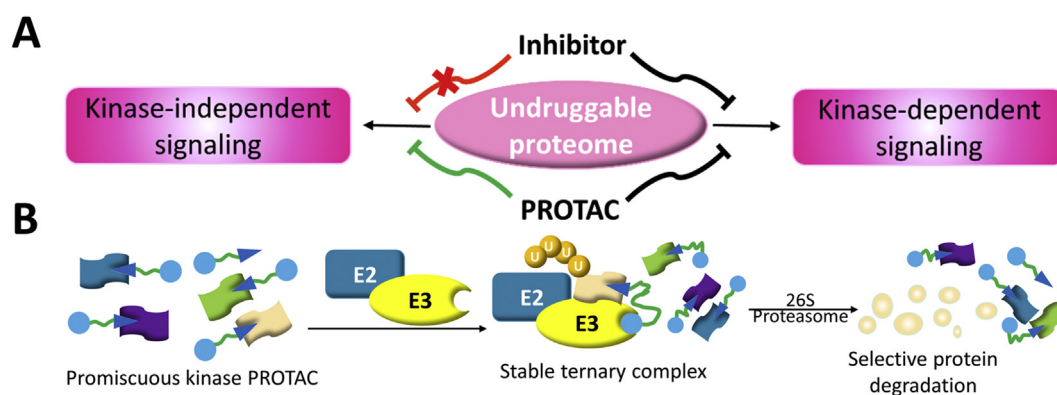

Figure 15 The advantages of PROTACs. (A) PROTAC is advantageous over inhibitors for degrading both kinase-dependent and non-dependent protein. (B) A stable ternary complex between E3 and a potential substrate is required for degradation.

e.g., using "recognition-cleavage" strategy or developing a phosphate-dependent proteolytic-targeting chimera (phosphoPROTAC).

6.1. "Recognition-cleavage" strategy

The "identification-cutting" strategy is another method to regulate protein level. The PROTAC molecule consists of two parts, the recognition group that selectively binds to the target protein, and the cutting group to cut the target protein. Cyclen's Co(III) complex [Co(III)cyclen] is the catalytic center for targeting a selective artificial protease with certain hydrolytic ability. Based on this, a Cu(II)cyclen complex with a recognition group was developed, which was used as the cutting group of a target protein[244].

Compound **81** (Fig. 16) was developed using selective amyloid-β (Aβ) and identifies several groups (KLVFF or curcumin). KLVFF (Aβ residues of 16−20) can be captured with Aβ of copper, using Cu(II) to replace Co(III). It can degrade Aβ and effectively reduce the aggregation of Aβ[245]. In addition, 2 hydrolytic enzyme molecules containing "Tau recognition motif" were reported, *e.g.*, cycln-hybrid artificial "hydrolytic enzyme" i1-Cu(II) (**82**) that can cleave Tau protein *in vitro*, and cell-permeable "hydrolytic enzyme" i2-Cu(II) (**83**) that can also cleave Tau protein[246] (Fig. 16). The soluble oligomer of human islet amyloid polypeptide (h-IAPP) induces the apoptosis of sxin-cells, which leads to the occurrence and development of T2DM[247]. Apo-cyclen is attached to the specific hIAPP recognition motif (NYGAIL) to form the cyclen−NYGAIL−copper complex[248]. The benzothiazole-aniline (BTA) derivative is combined with the new Cu(II) cleavage agent to develop a new molecule[249]. Both can interfere with hIAPP aggregation and cleave hIAPP, providing a good strategy for the treatment of T2DM. Many tissues express the cellular prion protein PrP[C], especially in the central nervous system. Proteolytic cleavage of the cellular prion protein PrP[C] provides a strategy to treat prion diseases[250].

6.2. PhosphoPROTAC

Apart from the "recognition-cleavage" strategy, molecules with a protein phosphorylation sequence can also recognize and degrade proteins.

Protein phosphorylation is one of the most common post-translational modifications (PTMs) and plays an important role in cellular signal transductions[251], such as regulating protein activity and subcellular localization[252]. However, an abnormal

phosphorylation process can also cause some serious diseases. For example, several tyrosine residues of RTK are phosphorylated upon activation. The domains of Src homology 2 (SH2) and phosphotyrosine binding (PTB) structures recognize the phosphorylated tyrosine in RTK[253], recruiting and phosphorylating its downstream signaling proteins, further activating the cascade of carcinogenic signaling[254]. HER2 is produced by this activation process and is highly expressed in some breast cancer tissues[255]. Despite significant advances in the development of small molecule inhibitors and monoclonal antibodies for RTK, targeting the RTK signaling pathway remains a challenge in the cancer field[256].

Inducing the degradation of proteins with PTB or SH2 domains may shut down tyrosine kinase signaling, and therefore inhibit tumor cell proliferation. Based on this idea, researchers developed a phosphate-dependent proteolytic-targeting chimera (phosphoPROTAC) which consists of four parts (Fig. 17A): the RTK phosphorylation sequence, the peptide VHL binding fragment, the linker, and the Poly-D-Arg sequence that increases cell permeability. The activated RTK can phosphorylate the Tyr residue of the RTK, then recruit and activate proteins with PTB or SH2 domains. After the hydroxylation of proline residues, the peptide proline-binding fragment recruits E3 ubiquitin ligase CRL2[VHL] to induce polyubiquitination, leading to degradation of proteins with PTB or SH2 domains. These phosphoPROTACs only inhibit the activated RTK signaling occurring in cancer cells, and have little toxicity on normal cells[29].

Researchers have reported 2 PhosphoPROTACs: $^{TrkA}PP_{FRS2\alpha}$ and $^{ERBB2}PP_{PI3K}$[257] (Fig. 17B). $^{TrkA}PP_{FRS2\alpha}$ is formed by conjugating the phosphorylation sequence TrkA (pre-myosin receptor kinase A) to the peptide ligand VHL. The neuregulin receptor ERBB2 (erythropoietin gene B2) was conjugated to the peptide ligand VHL to form $^{ERBB2}PP_{PI3K}$[257]. These phosphoPROTAC recruited neurotrophic signaling effectors, including fibroblast growth factor receptor substrate 2α (FRS2α) and PI3K, which were activated and phosphorylated by these 2 phosphoPROTACs. After phosphorylation, FRS2α and PI3K were ubiquitinated and degraded. $^{TrkA}PP_{FRS2\alpha}$ (**84**) reduced FRS2α protein in a dose- and time-dependent manner. After treating PC12 cells with nerve growth factor (NGF) and **84** for 60 min, the FRS2α protein was reduced by approximately 90%, indicating the high efficiency of this PROTAC. In addition, the mouse xenograft model showed that the tumor size was significantly reduced by 40% after treatment with $^{ERBB2}PP_{PI3K}$ (**85**) daily, indicating that this phosphoPROTAC had good therapeutic efficacy *in vivo*. PhosphoPROTAC can target and inactivate RTK signaling, but the molecular weight of these
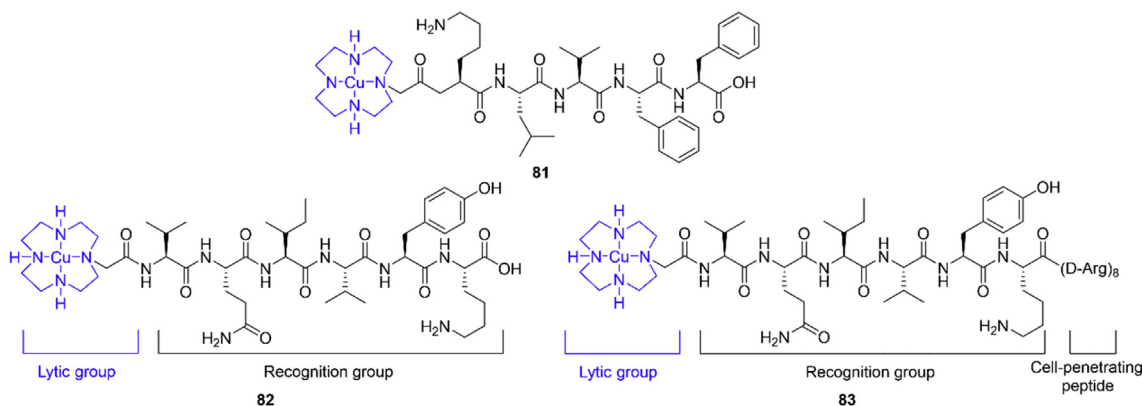

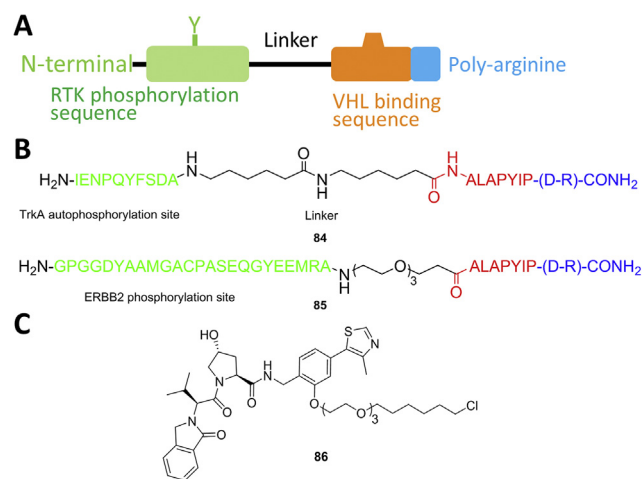**Figure 16** Structures of compounds **81**, **82** and **83**.

Figure 17 Chemical structures of phosphoPROTAC and HaloPROTAC-3 (**86**). (A) The PhosphoPROTAC is composed of an RTK phosphorylation sequence, VHL-binding sequence and a Poly-D-Arg sequence connected *via* a linker. (B) PhosphoPROTACs: compound **84** and **85**. Green: RTK phosphorylation sequence; Red: ligand to recruit E3 ubiquitin ligase is shown in red color; Blue: poly-arginine. (C) Chemical structure of compound HaloPROTAC-3 (**86**).

two phosphoPROTACs is too high for ideal cell permeability. For this reason, phosphoPROTACs need further improvements on pharmacological properties.

Several PROTACs containing a small molecule VHL ligand were developed to induce the degradation of HaloTag7 fusion protein. Among them, HaloPROTAC-3 (**86**) was one of the most effective PROTACs (Fig. 17C) to induce GFP-Halotag degradation (DC$_{50}$ = 19 nmol/L, D_{max} = 90%)[67]. These PROTACs with specific protein recognition motifs demonstrate that this is a good protein degradation strategy.

7. Outlook

7.1. PROTAC application in aggregated protein (degradation of Tau, etc.)

The amyloid beta protein plaques and the phosphorylated Tau neurofibrillary tangles (NFTs) are the major pathological signs of some neurodegenerative disease, *e.g.*, frontotemporal dementia (FTD), Alzheimer's disease (AD) and Parkinson's disease (PD)[258]. Many approaches to regulating Aβ and Tau levels have been reported, including UPS, autophagy lysosomal degradation, proteasome degradation[259,260], and microglia phagocytosis[261]. More importantly, inhibitors of the protein refolding pathway can prevent the accumulation of abnormal proteins by enhancing the activity of the associated proteasome (26S proteasome, HSP90)[262]. PROTACs may provide a possible way to selectively remove these protein aggregates[263].

A series of multifunctional PROTACs have been reported: they contain Tau recognition sequence and VHL E3 ligase binding ligand to induce Tau degradation and therefore prevent cytotoxicity[35] (Fig. 18A). Meanwhile, these PROTACs effectively induced Tau degradation in primary nerve cells and 3xTg-AD mice. In addition, Lu et al.[34] synthesized some peptide PROTACs to recruit Tau into KEAP1−Cul3 U E3 ligase complex for ubiquitination and subsequent proteasome-mediated Tau degradation

(Fig. 18B). Among them, peptide 1 strongly bound to KEAP1 and Tau *in vitro* and has K_d values of 22.8 and 763 nmol/L respectively. Using PROTAC to recruit KEAP1 to induce Tau degradation instead of inhibiting NRF2 may provide a new strategy to treat neurodegenerative diseases. This demonstrates the capability of PROTACs to prevent the aggregates of Aβ, Tau and other proteins.

Hydrophobic tag-conjugated peptide (HyT−Tau−CPP)[264] (Fig. 18C) was reported to selectively accelerate Tau degradation in a concentration- and time-dependent manner. 5(6)-Carboxyfluorescein-hydrophobic tag-conjugated peptide (CF−HyT−Tau−CPP) specifically binds Tau (K_d = 0.77 ± 0.19 μmol/L). HyT−Tau−CPP specifically interacted with Tau and permeates the cell membrane *in vitro*. HyT−Tau−CPP also reduced the level of Tau in the brain of AD mouse models. In conclusion, a hydrophobic marker-conjugated peptide may be a potential therapeutic strategy for AD by degrading Tau. In addition to these methods, the "Recognition-Cleavage" strategy may also effectively degrade aggregate proteins such as Aβ and Tau[244]. Most of these molecules use either selective Aβ recognition groups (KLVFF or curcumin) as recognition groups[245] or contain 2 "Tau recognition" motifs[246]. Recently, Silva et al.[265] synthesized a small molecule Tau protein degrader using the Tau positron emission tomography (PET) tracer as Tau protein ligand. Among them, QC-01-175 (**87**) (Fig. 18D) exhibited superior Tau protein scavenging effects. Compound **87** was able to promote Tau clearance in FTD neurons expressing Tau-A152T or Tau-P301L in a concentration-dependent manner, subsequently rescued Tau-mediated neuronal stress vulnerability. When there was only about 50% of Tau expressed with variants, the effect of **87** in A152T and P301L heterozygous neurons would result in more than 70% and 60% Tau clearance respectively on average. It is worth noting that **87** can remove Tau from FTD patient-derived neuronal cell models specifically, with minimal effect on Tau of healthy controls. The
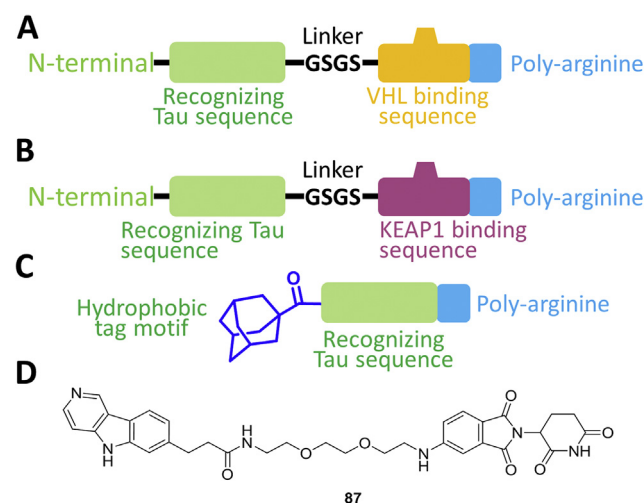

Figure 18 A series of multifunctional PROTAC molecules hijacking Tau. (A) Design of the peptide PROTAC contain Tau recognition part and VHL E3 ligase binding part to enhance Tau degradation. (B) Design of the peptide PROTAC (peptide 1) contain Tau recognition sequence and KEAP1 E3 ligase binding ligand to enhance Tau degradation. (C) Design of the hydrophobic tagging-conjugated peptide induces degradation of Tau. (D) Chemical structures of QC-01-175 (**87**).

affected neurons provide an unprecedented advantage for targeting pathologically related Tau species.

In summary, the application of PROTACs is very extensive. PROTACs not only induce degradation of certain tumor proteins, but other aggregates such as Aβ and Tau as well. However, in clinical settings for treating neurodegenerative diseases such as AD, PROTACs need to be delivered into the central neutral system. Many of the reported PROTACs have large molecular weights, which are unfavorable for permeability through the blood–brain barrier (BBB). The "Lipinski's Rule of Five" indicates that membrane permeability of a compound is related to its molecular weight, lipophilicity, polar surface area, hydrogen bonding and charge[266].

For effective central nervous system drugs: 1) Lipophilicity is generally higher, with log$P$ between 2 and 5[267]. Also, molecular weight is usually less than 450 Da[268]. In addition, most are neutral or weakly basic molecules with a pKa between 7.5 and 10.5[269]. 2) Lower molecular polar surface area is beneficial, generally less than 90 Å[268]. A low hydrogen bond donor number, generally less than 3[268]; what's more, lower hydrogen bonding capacity, Δlog$P$ is usually less than 2.3) Most are spherical molecules, and increasing the branching will reduce the ability to cross the BBB[268]. In addition, molecular flexibility is lower, and with fewer spin-fast bonds[268].

Therefore, to ensure permeability across BBB, an ideal PROTAC should have good lipophilicity, a small polar surface area, a suitable number of hydrogen bonds, and a molecular weight of not more than 450 Da[270].

In the future development of PROTACs attempts can be made to combine the PROTAC with some drug delivery systems (such as nanocarriers), which not only improve the drugability of PROTACs, but also deliver those PROTACs that degrade aggregated proteins into brain.

7.2. Homo-PROTAC (dimers), degradation tag (dTAG) systems and endosome targeting chimeras (ENDTACs)

E3 ubiquitin ligase is a key player in the U–proteasome pathway and it appears as an attractive drug target, particularly in cancer[271]. Ciulli's team envisions using the PROTAC method, and E3 ligases themselves hijack each other, thereby inducing E3 ligase degradation rather than blocking E3. In 2017, Ciulli's team[272] described Homo-PROTAC, a small molecular approach that effectively dimerizes E3 ubiquitin ligase and induces its own degradation. Based on this assumption, they link the same ligand of the ubiquitously expressed VHL protein through a linker. This bifunctional small molecule induces VHL dimerization, which causes ubiquitination and subsequent degradation of the VHL protein. Among them, the symmetric homo-PROTAC, CM11 (**88**) (Fig. 19), has ∼20-fold higher affinity (cooperative), resulting in effective, complete and prolonged degradation of VHL in different cell lines.

Subsequently, Krönke, Gütschow and their colleagues reported a homologous PROTAC of CRBN ligase[273], which showed that homo-PROTAC **89** (Fig. 19) can reduce intracellular CRBN levels for a long time even at low concentrations. In addition, PROTAC **89** specifically induced CRBN degradation, had little effect on the new pomalidomide substrates IKZF1 and IKZF3, and had no effect on other members of the CRL4 ligase family. In 2019, Ciulli's team[274] extended the homo-PROTAC method by combining 2 different E3 ligases using a heterobifunctional PROTAC to form VHL-CRBN heterodimeric PROTAC. The results showed that

CRBN was preferentially degraded, and the most potent PROTAC compound **90** (Fig. 19) induced CRBN degradation with high potency (DC$_{50}$=200 nmol/L) and at a high level (D_{max}>98%) and rapidly (within 1 h of treatment). In summary, the homo-PROTAC may provide a good strategy for the degradation of E3 ligases.

In addition, the degradation tag (dTAG) protein degradation technology can be used as an effective tool for target discovery and confirmation. This technology uses CRISPR to fuse a variant non-native protein FKBP12 (F36V) to a target protein, and then uses a series of PROTAC compounds (dTAG compounds) that have a good membrane permeability and can selectively bind to FKBP12 (F36V) and CRBN to degrade this fusion protein[275].

The dTAG technology uses non-endogenous proteins that do not theoretically affect proteins other than the target protein. Once this highly active, highly selective ligand is found, the tagged protein can be fused to many target proteins to degrade these target proteins. This single dTAG system can be used as a broad-spectrum, universal protein degradation tool. PROTAC and dTAG are 2 more broad technologies, a combination of bifunctional molecules and chemically induced proximity (CIP) technology. Although dTAG cannot be used as a drug, the significance of highly selective target confirmation techniques is noteworthy.

PROTACs can only degrade the proteins in cells by means of UPS. Dr. Crews' team[276] developed a new method to degrade extracellular proteins through receptor-mediated endosome pathway internalization and degradation, using a heterodimeric molecule of "endosome targeting chimera" (ENDTAC). Both ends of the ENDTAC molecule bind to extracellular target proteins and receptors, which mediate cell surface endocytosis. ENDTAC can trigger the endocytic process by attaching an extracellular POI to receptors on the cell surface, which are subsequently degraded by lysosomes. Compared to PROTAC, ENDTAC technology provides a strategy to degrade disease-associated extracellular proteins, such as cytokines.

7.3. Choices of PROTAC warheads and linkers

Currently, the design and subsequent optimization of PROTACs has been focused on the binary interactions between the "warhead" and the respective target protein. This requires a large amount of crystal structures of binary complexes derived from the Protein Date Bank (PDB). However, in order to develop a PROTAC with protein degradation capability, the following aspects need to be noted:
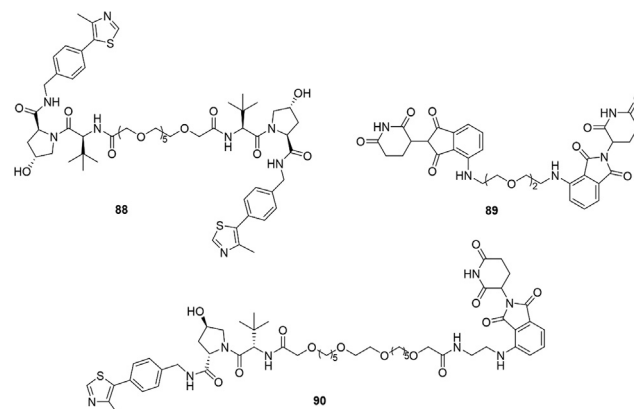

Figure 19   Chemical structures of Homo-PROTACs **88**–**90**.

1): Type of linkers (Table 5): The linker has an important influence on the biological activities and physicochemical properties of the PROTAC molecule. Researchers initially used peptide linkers, and in the development of PROTACs, it was found that the linker preferably has a certain flexibility, thus varying lengths of PEG and (un)saturated alkane chains are commonly used. Of course, there are also some relatively rigid linkers. The groups on the linker, such as oxygen atoms, may also interact with the protein to stabilize the orientation of the protein. In order to increase the solubility of PROTACs, a piperidine group is also a good choice as a linker[183]. Amide bonds, ether bonds, alkyl-amines, carbon−carbon single bonds, and carbon−carbon triple bonds are also generally used in PROTACs. In addition, the chemical bonds described above are linked to an alkyne or azide linkage and then joined by a "click chemistry" reaction.

The cyclic addition reaction between 1,1,3-dipole acetylene and azide was first proposed by Huisgen in the 1960s[277]. Sharpless and others subsequently developed the concept of "click chemistry" in this reaction, which has a high yield and simple reaction conditions[278]. In order to achieve high molecular weight, lipophilicity and permeability, researchers explored the application of this bio-orthogonal click chemistry for rapid synthesis of PROTAC.

Currently, the latest applications of "click chemistry" are focused on copper-catalyzed azide−alkyne cycloaddition (CuAAC) and the Diels−Alder (DA) reaction (Fig. 20A and B). However, PROTACs based on "click chemistry" are mostly synthesized by CuAAC reaction.

A Cu(I) catalyzed azide and an alkynization inhibitor from thalidomide are linked by a "click chemistry" reaction to form a PROTAC and the highly effective inhibition of SIRT2 was detected with an IC_{50} value of 0.25±0.02 μmol/L[214]. In addition, researchers designed a tetrazine-labeled thalidomide derivative that reacts rapidly with the *trans*-cyclooctene-labeled ligand of the target protein in the cell to form a CRBN-based PROTAC[279]. The in-cell click-formed proteolysis targeting chimeras (CLIPTACs) (compounds **91** and **92**) were successfully used to reduce two key targets of oncology, BRD4 and ERK1/2, respectively (Fig. 20C). "Click chemistry" reactions provide a new strategy for the selection of synthetic PROTAC connection chains and lay a solid foundation for the development of PROTACs.
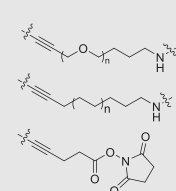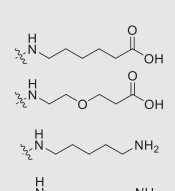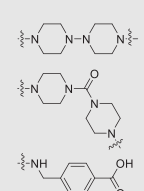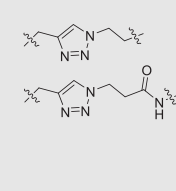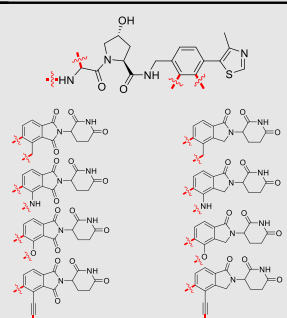
2): Length of linkers: the length of the linker also plays a key role in the degradation of the targeted protein. In fact, the optimization process of PROTAC molecules mainly focuses on the study of the structure−activity relationship of the length of linkers. First, if the linker is too short, the 2 ligands cannot simultaneously bind to their targets due to steric effects, resulting in the failure of ternary complex formation. Conversely, if the linker is too long, the 2 proteins cannot be pulled in to form PPI, nor will the target protein be ubiquitinated.

3): Site of attachment (Table 5): The site of attachment is typically based on analysis of solvent-exposed areas on ligand-protein binding structures. Generally, the end of the ligand is in the solvent-exposed area of the POI target. If the linker of the PROTAC has no flexibility for extension, the E3 ligase and the protein surface of the link site cannot form an effective PPI. Recent studies have also shown that increased affinity does not necessarily increase target protein degradation, most likely because E3 ubiquitin ligase, target protein and ligand did not form a suitable ternary complex. In a recent crystal structure of PRO-TAC **33**[167], Gadd et al. elucidated that **33** is "sandwiched" between VHL and the BRD4[BD2] bromodomains, inducing a wide range of new protein−protein and proteins−ligand interactions, with hydrophobic and electrostatic properties. This indicates that the PROTAC not only binds to the target protein and E3, but also promotes the interaction between E3 and the target protein. Furthermore, the isoform-specific cooperativity of ternary complex formation is determined by the surface complementarity between E3 and the target protein. More importantly, a highly stable ternary E3−PROTAC-target complex is critical for PRO-TAC's ability to degrade proteins, especially for ligands with inherently weak binary binding affinities.

4): Type of E3 ubiquitin ligase: Most of the current studies are targeted to E3 ligases CRBN, IAP and VHL for PROTAC development, but these E3 ligases show restricted substrate specificities[217,280]. Crews used different E3 ligands and 3 target protein ligands, as well as four different linkages to design chemically spatially diverse PROTACs with the goal of degrading the fusion protein BCR-ABL[146]. The results indicate that an imatinib-based PROTAC cannot induce degradation of ABL or BCR-ABL; a bosutinib-based PROTAC can induce degradation of ABL, while a dasatinib-based PROTAC can induce degradation of BCR-ABL. In addition, E3 ligase types also determine protein degradation efficiency. CRBN can simultaneously degrade BCR-ABL, while VHL can only degrade ABL. There is no convincing explanation for these observations, and we believe that E3 ligases can only

Table 5 The common linker for construction of PROTACs and lead-out position of E3 ligase ligand.

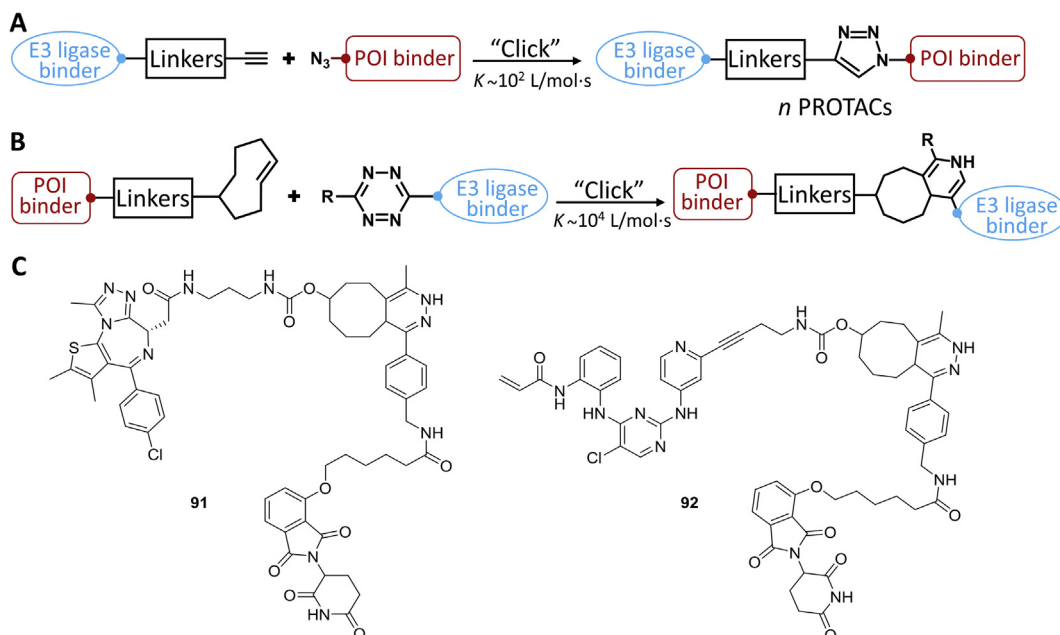| Example of linkers				E3 (VHL-CRBN) ligand
Alkyne PEG linkers	Flexible linkers	Relatively rigid linkers	Triazole linkers	

Figure 20 General strategy using "click chemistry" for PROTAC synthesis. (A) Cu(I)-cataylzed alkyne−azide cycloaddition (CuAAC). (B) Diels−Alder (DA) cycloadditions between tetrazines and stained alkenes. (C) Chemical structures of the in-cell click-formed proteolysis targeting chimeras (CLIPTACs), **91** and **92**.
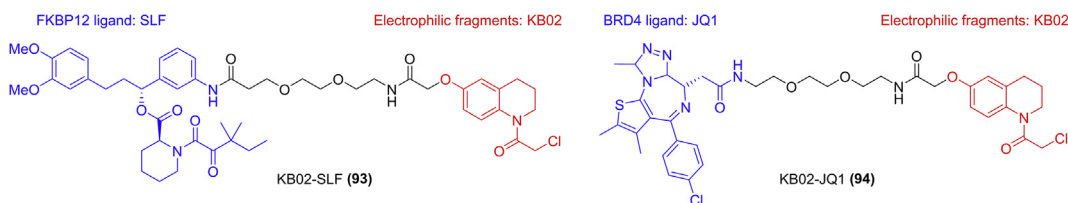

Figure 21 Chemical structures of KB02-SLF (**93**) and KB02-JQ1 (**94**).

recognize specific types of target proteins. To discover additional ligandable E3 ligases, Cravatt's team[281] presented a chemical proteomics approach to discover E3 ligases. This strategy leverages cysteine-directed electrophilic fragments (chloroacetamide and acrylamide) in order to couple the target protein ligands with the screen which was designed for heterobifunctional degrader compounds operated by covalent adduction of E3 ligases. This approach identified the E3 ligase subunit DCAF16 as a target of electrophilic PROTACs that promote the nuclear-restricted degradation of proteins. Compound KB02-SLF (**93**) (Fig. 21) promoted a substantial reduction in nuclear FKBP12, which was sustained across a 4−72 h time frame. In addition, DCAF16 could also support the degradation of the nuclear protein BRD4. KB02-JQ1 (**94**) (Fig. 21) bifunctional compound induced degradation of BRD4 in HEK293T cells in a concentration-dependent manner. More importantly, the study underscores the value of broadly reactive electrophilic fragments as tools to discover ligandable sites on poorly characterized proteins such as DCAF16.

Ciulli's team[168] has analyzed and elaborated the crystal structure of the ternary complex BRD4−MZ1−VHL, and structure-based-designed compound **34** exhibits highly BRD4 degradation activity. Structure-based drug design (SBDD) can provide a reference for the length of the linker chain, which rationalizes the PROTAC molecular design, thus accelerating the discovery of active PROTAC molecules. The use of HADDOCK for

protein−ligand−protein simulation docking will provide a selection basis for the molecular link chain, and preliminary prediction of different solvent exposure areas. However, this may not reflect the mode of action of the protein complex, not to mention the presence of an artificial ligand. For the rapid synthesis of PROTACs, in addition to "click chemistry", a new method for preparing PROTAC conjugates has recently been reported. The desired PROTACs are rapidly synthesized by linking TPR with a kinase inhibitor or its compact derivative having a reactive amino function through an ethylene-oxy linker. This method is highly versatile. In addition to protein kinases, it can be applied to other proteins that can be used to conjugate inhibitors/modulators/ligands.

8. Conclusions and future perspectives

Although PROTACs have many unique advantages, they still have several inherent flaws. For example, the pharmacokinetic parameters of the PROTAC molecule may not be particularly desirable, although some studies have confirmed good metabolic stability and tissue distribution. Recently, GlaxoSmithKline reported a review of the *in vivo* study of PROTAC, linking *in vitro* cell experiments with *in vivo* experiments to better guide the design and development of PROTAC animal and clinical trials[282]. Cellular permeability, tissue distribution, and metabolism can be improved by optimizing the molecular structure of the linker region of the

PROTAC. However, due to the high molecular weight and complex structure of PROTACs, their distribution and metabolism within the tissue must be limited by the transmembrane permeability of the PROTAC.

To date, from peptides to small molecule PROTACs, there have been significant improvements in the stability, cell permeability, solubility and tissue distribution of PROTACs. A variety of synthetic techniques, such as click chemistry, further enriches the structural diversity of PROTACs. In just three years since the discovery of E3 ubiquitin ligase-specific small molecule ligands, PROTACs have entered a period of rapid development, creating a new direction in biopharmaceutical industry. Arvinas, founded by Professor Crews, and C4 founded by Dr. Bradner, have entered co-development with major pharmaceutical companies such as Merck, Pfizer and Roche to study the pharmacological properties of PROTACs. Among them, Arvinas' ARV110, a PROTAC molecule targeting AR, has been approved by the FDA for clinical research. Further, Arvinas has announced that its new ER drug-targeting agent ARV-471 for the treatment of locally advanced or metastatic ER-positive/HER2-negative breast cancer has received FDA approval. Phase I clinical trials of ARV-471 are scheduled to begin in the third quarter of 2019[283,284]. Kymera's KYM-001 is a class of orally effective IRAK4 selective degrading agent that can cause tumor retraction in the MYD88 mutant ABC DLBCL model[285]. The degradation of KYM-001 can simultaneously remove the kinase activity and scaffold function of IRAK4, so its efficacy may be better than the IRAK4 kinase inhibitor. KYM-001 is expected to be tested clinically in 2019. Meanwhile, several pharmaceutical companies, including Novartis and AstraZeneca, launched PROTAC-related research projects.

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Author contributions

Haopeng Sun, Wenyuan Liu and Yang Wang were responsible for writing the whole passage. Haopeng Sun, Wenyuan Liu and Feng Feng were in charge of checking and revision. All the figures in the article were made by Xueyang Jiang.

Conflicts of interest

The authors have no conflicts of interest to declare.

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