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Proteolysis-targeting Chimeras Technology for Selective Protein Degradation

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Abstract: Proteolysis-targeting chimeras technology has emerged as a new therapeutic strategy that take advantage of endogenous ubiquitin-proteasome system (UPS) to selectively degrade oncoproteins. It has been developed from peptide-based PROTACs to small molecule-based ones, with capability to degrade a variety of target proteins with unprecedented advantages compared with traditional inhibitors. PROTACs have attracted the interest of both academia and industry and developed rapidly in the past decades. To date, there has been approximately 13 drugs already entered the clinic. However, as a new technology, it is facing many problems and challenges as well.

This review will begin with introduction of common structure and rational design of PROTACs, with concentration on their significant features compared with traditional inhibitors. Then, different types of PROTACs will be discussed, followed by brief description of PRATACs that have reached the clinical stage.

Keywords: Proteolysis-targeting; PROTACs; UPS;

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1. Introduction

Proteolysis-targeting chimeras (PROTACs) are heterobifunctional molecules consisting of three parts: a ligand that binds to target protein, a ligand that recruits E3 ubiquitin ligase, and a flexible linker that links the aforementioned two parts[1]. The small molecule PROTAC can bring the target protein and E3 ubiquitin ligase in a close distance, or proximity, so that the former can be ubiquitinated and degraded by proteasome into small peptides and amino acids subsequently, just as schematically indicated by Figure 1[2-5].

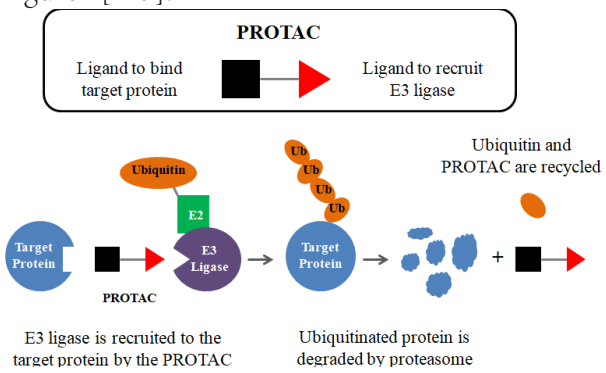

Figure1. Mechanism of PROTACs technology.

PROTACs can recruit the target protein and E3 ligase simultaneously, driving the ubiquitination of target proteins, followed by recognition and degradation by proteasome. It is worth noting that the PROTAC molecule act through a catalytic mode and can be recycled.

Ever since the emerging, PROTACs have shown great potentiality as a novel technology for therapy of disease caused by pathogenic proteins. In contrast to traditional competitive inhibitors that function by inhibiting the activity of specific proteins, PROTCACs perform through a chemical knockdown strategy, namely, degrading the entire target. Steadily increased interest has been attracted to this new technology due to its appealing characters, including:

(1) The ability to degrade undruggable target proteins. Currently, only 20% of known disease-causing proteins can be targeted, including enzymes, G protein-coupled receptors, nuclear receptors, and voltage gated ion channels[6]. The remaining 80% are difficult to target, such as c-Myc, Tau tangles, KSR and Gab family. Blocking the catalytic activity of target protein or PPI (protein-protein interactions) has been perplexing scientists for decades since normally "ideal" pocket structures of proteins are indispensable for the blocking effect, while unfortunately they are not ubiquitous. The arising of PROTACs technology brought light

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to this challenging issue, due to less dependence on the target. Unlike traditional inhibitors, weak binding to target proteins is sufficient for PROTACs to "label" them, so that they can be dissociated and subsequently decomposed. In this sense, those undruggable target proteins in traditional therapy will not be insensate stone anymore.

(2) Overcoming drug resistance. Kinase inhibitors therapy has achieved excellent therapeutic effects for patients with kinase-mutant malignancies[7-10]. However, the acquired drug resistance through point mutation or overexpression will block drug binding and resultantly impair efficacy, as reported for EGFR[11-12], BCR-ABL[13] and BTK[14] inhibitors. It has been disclosed that PROTACs showed great potential for settling this clinical dilemma[15]. For example, C. M. Crews and co-workers have developed a PROTAC that induced degradation of both wild-type and C481S mutant forms of Bruton's tyrosine kinase (BTK), which caused resistance in more than 80% of chronic lymphocytic leukemia (CLL) patients[16]. The authors also demonstrated that their small molecule PROTAC bound fewer off-target kinases than ibrutinib, and impaired BTK signaling in primary B-cells isolated from C481S patients. Later, S.Wang et al. reported the development of a highly potent and effective PROTAC estrogen receptor (ER) degrader for fulvestrant-resistant breast cancer therapy[17]. Their ER degrader was found to induce more complete ER degradation than fulvestrant, which was the only approved selective ER degrader (SERD), and be more effective in inhibition of cell proliferation than fulvestrant in MCF-7 cells.

(3) Blocking both enzymatic and scaffold functions of specific proteins. Typically, traditional inhibitors are well-known to work by abrogating the enzymatic function of target proteins. In contrast, PROTACs can impair both enzymatic and non-enzymatic roles of proteins of interest. This surprisingly desirable property has conferred it valuable tool in both fundamental scientific research and clinical therapy. For instance, focal adhesion kinase (Fak) is a cytoplasmic tyrosine kinase acting through both kinase-dependent and kinase-independent mechanisms, thus astricting the therapeutic efficacy of traditional inhibitors[19]. By rational designing, Crews's group successfully developed a promising small molecule PROTAC that addressed the kinase-independent function of Fak, thus simultaneously blocked Fak's kinase signaling and scaffolding capabilities, resulting in improved performance than traditional inhibitors (such as defactinib) [20].

(4) Behave via catalytic mode. To date, by large-scale screening and optimization, 52 small molecule inhibitors for kinases have been approved by FDA, which possess high binding affinity against well-defined active sites of specific target proteins and thus can suppress their biological activity. These traditional inhibitors work through an "occupancy-driven" mode, therefore, prolonged binding to the active site of the target protein is necessary for sustained inhibition. However, this strategy is impractical for those "non-druggable" targets proteins other than kinase, such as scaffold protein or transcriptional factor, which have no such targeted active sites. Comfortingly, PROTACs function via "event-driven" degradation of target protein, and each PROTAC molecule is able to degrade multiple target protein molecules[16]. The catalytic nature of PROTACs afford them the ability to degrade target proteins at low exposures. Only sub-stochiometric amounts are needed for PROTACs compared with traditional inhibitors, resulting in decreased side effect (such as off-target toxicity) and increased pharmacological effect[18].

In this review, the development of PROTACs from peptides to small molecules will be discussed. And current molecules that have entered the clinic and their clinical research progress will be summarized. In addition, the main concerns for this new strategy will be briefly discussed.

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2. Development of PROTACs Technology

2.1 Peptide based PROTACs

In 2001, Sakamoto and coworkers[21] reported the first chimeric PROTAC molecule by covalently linking angiogenesis inhibitor ovalicin to 10-aa IκB α -phosphopeptide (Figure 2A). The ovalicin end of the molecule can target MetAP-2, and the IκB α -phosphopeptide end can bind with E3 ubiquitin ligase $SCF^{\beta\text{-TRCP}}$. As expected, MetAP-2 was ubiquitinated and degraded by this hybrid molecule. Using the same strategy, later in 2003, PROTAC molecules that could down-regulate estrogen (ER, Figure 2B) and androgen (AR, Figure 2C) receptors were reported and showed desired functions in vitro and in vivo, respectively[22]. Estradiol (E2) and dihydroxytestosterone (DHT) were linked with IκB α -phosphopeptide, respectively, to form the chimeric molecules to achieve degradation of target proteins.

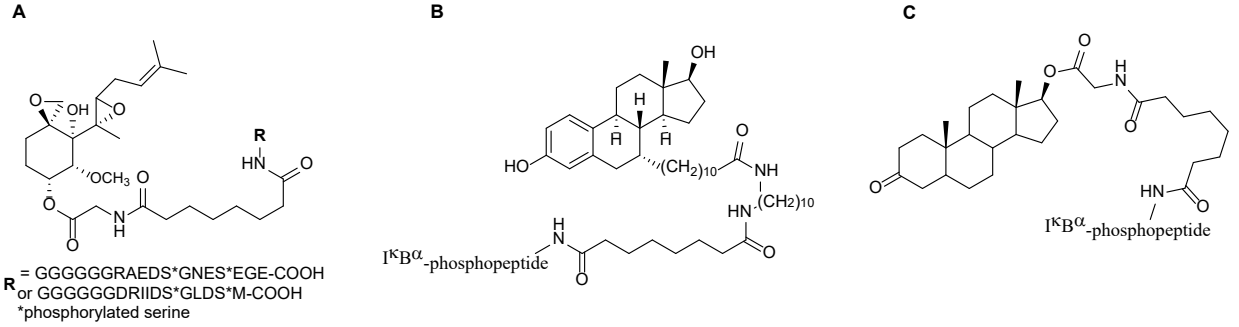

Figure 2. Structures of peptide-based PROTAC molecules to recruit specific proteins and the E3 ubiquitin ligase $SCF^{\beta\text{-TRCP}}$. (A). PROTAC consisting of ovalicin and the IκBα-phosphopeptide to target MetAP-2; (B). PROTAC consisting of the IκBα-phosphopeptide and estradiol (E2) to target ER; (C). PROTAC comprising of the IκBα-phosphopeptide and dihydroxytestosterone (DHT) to target AR.

To further improve cell permeability and uptake of the peptide-based PROTACs, Crews' s group reported a poly-D-arginine tagged 7-aa peptide decorated FKBP12 ligand (Figure 3A) and DHT (Figure 3B) to target FKBP12 and AR in vivo, respectively[23]. The 7-aa peptide used here derived from HIF1 - α and can recognize VHL (von Hippel-Lindau) E3 ligase. They demonstrated that the fusion of poly-D-arginine on the carboxy terminus of the peptide improved the membrane permeability and avoided the need of cell micro-injection[22].

2.2 Small molecule based PROTACs

Early researches focused on the usage of peptide for the recognition of specific E3 ubiquitin ligase. The peptide-based PROTACs proved to be feasible strategy for the selective degradation of target protein. However, poor cell permeability, vulnerable peptide bonds, potential immunogenicity owing to the large size of the molecule and low activity with necessary dose normally in micromolar range[24] hindered its application. Therefore, the development of small molecule based PROTACs is extremely urgent.

The initial small molecule based PROTAC was reported in 2008 by Crews' s group[25]. The PROTAC molecule consisted of an MDM2 ligand known as nutlin, a non-steroidal androgen receptor ligand (selective AR modulator, SARM) and a short PEG linker (Figure 4A). This molecule could recruit AR to E3 ligase MDM2, thus leading to ubiquitination and degradation of AR. Their result showed that the SARM – nutlin PROTAC was cell permeable and the degradation of intracellular AR protein was proteasome dependent in HeLa cells. The report disclosing the binding ability of MeBS (Figure 4B) to the BIR3 domain of cIAP1 (cellular inhibitor of apoptosis protein 1) promoted the development of E3 ubiquitin ligase cIAP1-mediated ubiquitination and degradation

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of the target protein[26]. Bestatin based hybrid molecules have shown their potential for the degradation of a series of proteins, including CRABP-I/-II[27], Er α [28], TACC3[29], and BCR-ABL[30].

Since 2012, small-molecule ligands for VHL ligase cereblon (CRBN)[40,41]. Small-molecule PROTACs based on CRBN ligands have been extensively studied in targeting BRD4[42], CDK6/9[43,44], Sirt2[45], BTK[16], ALK[46], FAK[47], IRAK3[48], etc.

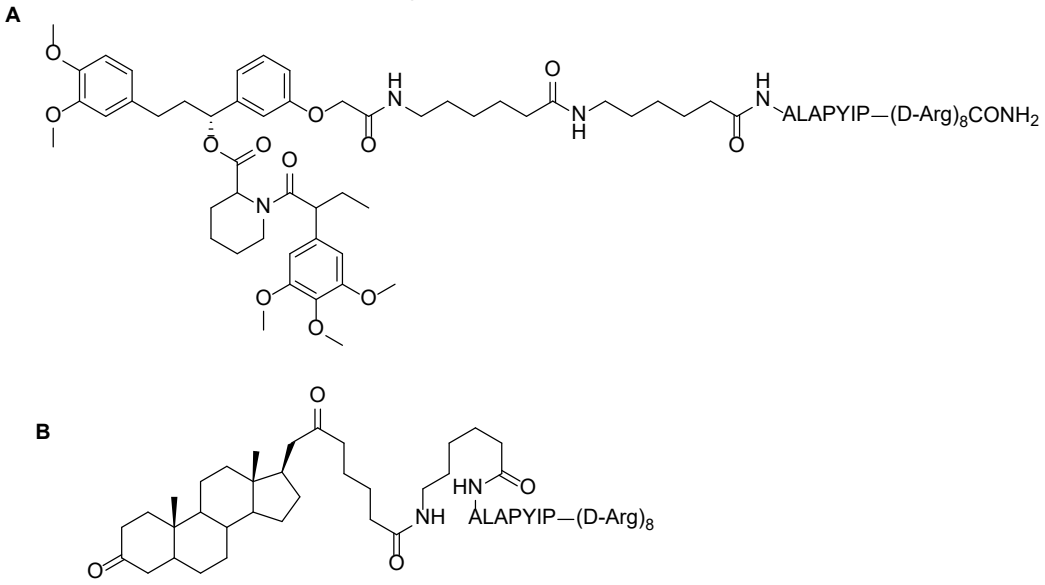

Figure 3. Structures of FKBP12 ligand (A) and DHT (B) based PROTACs to degrade FKBP12 and AR.

which replaced the HIF1 - α peptide, were designed and synthesized[31,32]. This new generation of ligands for VHL reserves the hydroxyproline moiety on transcription factor HIF1 - α , which is crucial for the binding with VHL. And later in 2014, a potential ligand that exhibited improvements in binding affinity and lipophilicity was optimized and described (Figure 4C) [33], which led to the development of VHL-related PROTACs. The small molecule VHL ligands have found wide application in desired degradation of various target proteins, such as ERR α [34], RIPK2[34], BCR-ABL[35], RTK (EGFR, HER2, c-Met)[36], MEK1/2[37], EZH2[38], HDAC6[39], etc. At the same time, the immunomodulatory drugs (IMiDs) thalidomide, lenalidomide, and pomalidomide, have also been investigated as the ligands for E3

2.3 Dual PROTACs

Inspired by the development of small molecule based PROTACs and the idea of dual-targeting drugs, novel dual PROTACs for simultaneous degradation of two different target proteins have been designed and synthesized. In 2021, Li and coworkers reported the first innovative dual PROTAC molecule which combined the E3 ligase and two inhibitors (PARP and EGFR inhibitors) together using a natural amino acid as a star-type linker[49]. This study verifies the concept of dual PROTACs and offers a possibility to replace the current combination therapy.

3. Clinical drug

3.1 PROTACs in clinical development stage

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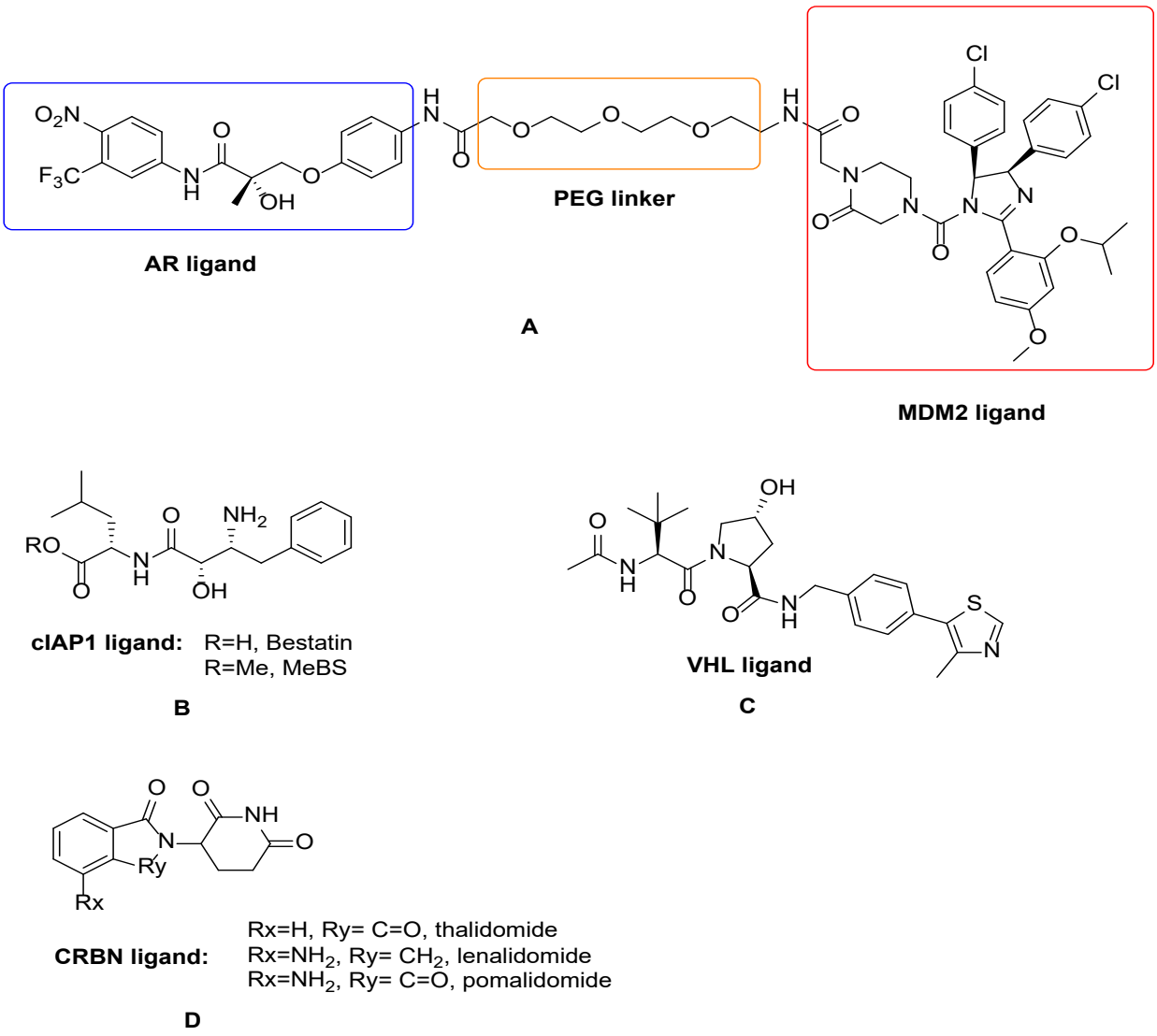

Figure 4. Structures of small molecule-based PROTAC molecules: (A) SARM–nutlin PROTAC; (B) cIAPI ligand; (C) VHL ligand; (D) CRBN ligand.

Within the past decade, PROTACs technology exhibited great development in targeting new proteins and the nonenzymatic functions of established proteins through a different mode of action. Currently, there are approximately 13 drugs already in clinical development stage, and the target proteins including AR, ER, BCL-XL, IRAK4, BTK, STAT3, TRK, BRD9, etc. Among them, six molecules have initiated the Phase I/II trials (Table 1).

In 2019, two PROTAC molecules from Arvinas (ARV-110, ARV-471) became the earliest protein degraders to enter the clinic. This is a milestone in this field, which represents a critical step for PROTAC technology toward a real drug. ARV-110, which targets AR, is the first PROTAC molecule to enter the clinic. AR is one of the most popular targets in the field of protein degradation due to two reasons as follows: (1) AR is a clinically verified target and many drugs have been approved for marketing (including Flutamide, Enzalutamide); (2) AR degraders offer a possibility to overcome the acquired drug resistance to existing AR inhibitors.

Apart from AR, drugs targeting other proteins have been developed and entered the clinic as well. For example, DT2216 from Dialectic and NX-2127 from Nurix were designed to target

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BCL-XL and BTK, respectively. KT-474 is the first protein degradation agent to enter the clinic outside the field of tumor. It targets IRAK4, which is difficult to target, and is intended for atopic dermatitis (AD) or hidradenitis suppurativa (HS).

3.2 Clinical research progress of ARV-110 and ARV-471

The clinical progress of two representative drug candidates will be reviewed below.

ARV-110

ARV-110 is a potential orally small molecule degrader of the androgen receptor (AR) developed by Arvinas which is intended for the treatment of metastatic castration-resistant prostate cancer (mCRPC).

Phase 1/2 dose escalation study (NCT03888612) to investigate the safety and tolerability of ARV-110 for mCRPC patients whose disease progressed after being treated with approved standard-of-care therapies (including enzalutamide or abiraterone) showed that four of five (80%) patients with T878/H875 mutations had PSA (prostate-specific antigen) reductions above 30%, and two of them (40%) had PSA reduction above 50%. In addition, one patient had a partial response (PR), and the tumor size decreased by 80%. These results indicated that ARV-110 had a particularly strong response to patients with T878/H875 mutation. What's more, for another group composing of 15 wild-type AR patients, two of them (13%) showed a more than 50% reductions in their PSA protein levels. These results demonstrated the therapeutic potential of ARV-110 for both T878/H875 mutant and wild-type mCRPC patients.

An extension Phase II study (ARDENT) expected to recruit approximately 100 patients is now ongoing. This study will further evaluate the safety and efficacy of ARV-110 at a daily dose of 420 mg. Two groups of patients will be included: one subgroup is for heavily treated patients with T878/H875 mutations to ensure the accelerated approval in this population; and the other subgroup is for patients who received fewer pre-treatments, therefore, more likely to be AR-dependent and more sensitive to ARV-110. The results of the interim data are expected to be available by December of 2021.

ARV-471

ARV-471 is a potential best-in-class oral ER protein degrader for the treatment of advanced or metastatic ER positive / HER2 negative breast cancer.

The Phase 1 interim data (NCT04072952) for ER+/HER2- patients who have received a median of five prior therapies show that ARV-471 can significantly reduce the ER expression level in the patient's tumor tissue, reducing the ER level up to 90%, and 62% on average. Moreover, ARV-471 showed degrading effects on both wild-type and ESR1 mutants.

For 14 metastatic breast cancer patients with high tumor ER-independence, one patient achieved confirmed PR, and the tumor lesion size shrank by 51%. Two patients achieved unconfirmed PRs and one patient had stable disease with over 50% target lesion shrinkage. Additionally, 12 patients were evaluated with CBR and five of them (42%) achieved CBR (CBR is defined as PRs + complete remission + stable disease at 6 months). Three of these five patients have been treated with fulvestrant, and another case has been treated with two investigational selective estrogen receptor degraders.

In summary, the existing clinical data of ARV-471 demonstrates its safety, tolerability, and ER degradation activity for heavily pretreated breast cancer patients. ARV-471 is now undergoing a Phase Ib clinical combination trial with the CDK4/6 inhibitor (palbociclib) and a Phase 2 combination therapy study for the treatment of metastatic breast cancer.

4. Outlook and challenges

The PROTACs technology witnessed rapid development in the past decades, however, there still are many problems to solve for further

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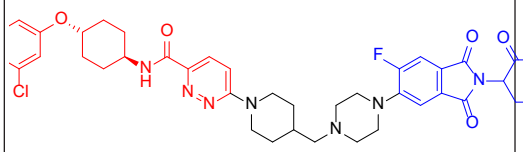
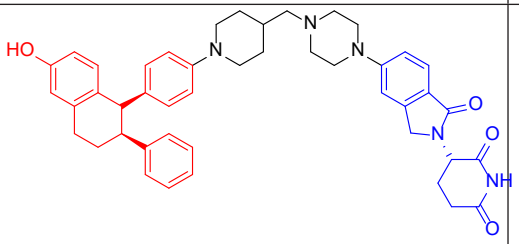
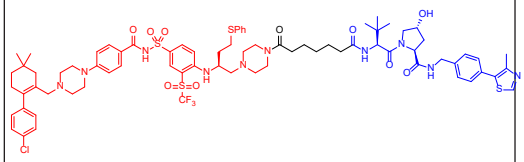
Table 1. PROTAC molecules that have initiated clinical trials.

Drug	company	structure	Target protein	status
ARV-110	Arvinas		AR	Phase II
ARV-471	Arvinas		ER	Phase II
AR-LDD	Bristol Myers Squibb	N.A.	AR	Phase I
DT2216	Dialectic		BCL-XL	Phase I
KT-474	Kymera/Sanofi	N.A.	IRAK4	Phase I
NX-2127	Nurix	N.A.	BTK	Phase I

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development of this technology.

1) Expand the E3 ligases scope. There are more than 600 E3 ligases in the human genome, however, only a few of them have been used in PROTAC design, such as CRBN, VHL, MDM2, cIAPs. The discovery of more suitable E3 ligases for specific target proteins degradation is urgently needed in this field.

2) Explore theoretical support for molecular design. How to rationally design PROTAC molecules, including the selection of the warhead, E3 ligase ligand, linker and the ligand tethering site is still a problem. The factors that influence the physicochemical and pharmacological properties of PROTAC molecules need to be further explored, which can guide rational design and optimization of PROTACs.

3) Solve the safety concerns including on-target and off-target effects. Compared with small molecule inhibitors, PROTACs can degrade the entire protein, destroying both enzymatic and scaffold functions of proteins. This may cause undesired toxicity, since some scaffold functions of proteins are vital for normal cell functions. What's more, prolonged degradation rendered by the catalytic nature of PROTACs may also lead to detrimental effects if the target protein is essential for normal cell function. Off-target effect results from the "Hook-effect" of PROTAC, forming binary complex with either target protein or E3 ligase instead of ternary complex at high concentrations[50]. If a binary PROTAC-E3 ligase complex forms, it can lead to the degradation of non-target proteins and induce off-target toxicity[51]. Therefore, the design of highly selective PROTACs to reduce the on-target and off-target toxicities need to be taken into consideration for future development.

4) Molecule glue: "Molecule glue", which works through the regulation of protein-protein interactions[52], is another promising protein degradation strategy, and has a completely different mode of action compared with PROTACs. The interactions between the two proteins can not occur without the existence of the molecule glue. IMiDs (lenalidomide and pomalidomide) are reported as CRBN E3 ligase modulators and acquire the ability to degrade two B cell transcription factors (IKZF1/3)[53]. Similarly, CC-92480[54] is reported for the treatment of multiple myeloma (MM) through the degradation of IKZF1/3, and CC-90009[55] has shown anti-AML activity through the degradation of GSPT1. In addition, it has been reported that simple structural modifications of an MDM2 PROTAC degrader can convert it into a molecular glue, achieving its antitumor activity through the degradation of GSPT1[56]. Furthermore, Rao's group disclosed a dual degrader GBD-9 that could concurrently downregulate BTK and GSPT1 using PROTAC and molecular glue mechanism, respectively[57]. It is worth noting that molecular glue is small-molecular-weight compound and has better drugability compared with PROTACs. However, the discovery of molecular glue has been serendipitous and more mechanistic insights to distinguish between PROTACs and molecular glue and merge them to work together remains as one of the main challenges in the future.

In conclusion, PROTACs have been demonstrated to possess intrinsic advantages, and have attracted increasing interests from both fundamental scientific research field and clinical drug design. They have shown immense potential as platform for design of drugs, especially for drugs targeting those proteins with poor drugability when using traditional strategy. Meanwhile, as a cutting-edge technology, more comprehensive and thorough study on the mechanism and latent risk is still in urgent need before its wide applications.

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