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Targeted Protein Degradation Technology: A Novel Strategy for Cancer Therapy

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ABSTRACT

PROteolysis TArgeting Chimera (PROTAC) technology is an effective tool to induce targeted degradation of pathogenic protein and is a novel cancer therapy. It uses the endogenous ubiquitin-protease system (UPS), attracting ubiquitin and covering the target protein in it, thus resulting in its complete degradation of the protein interest (POI). The potential advantages of PROTAC technology compensate for the shortcomings of traditional cancer therapy and allow them to target "undruggable" proteins, which promote its rapid development in recent years. The review focuses on the mechanisms of PROTACs, discusses application of PROTACs targeting different oncogenic proteins and analyzes the strategies for designing efficient PROTACs. Collectively, the review provides references for future application of PROTACs in cancer treatment.

Introduction

Cancer is a disease involving the rapid reproduction of mutated cells, often causing assorted physiological problems by disrupting organ functions. It is considered as the second largest contributor to worldwide mortality (Saluja, et al., 2021). Drugs treating cancer include monoclonal antibodies (mAb), siRNAs, and small molecules, which can inhibit activity of oncogenic proteins. However, each of these strategies has its limitations. For example, antibodies can only target cell surface protein and are difficult to achieve cell permeability. siRNAs have off-target issues, and small molecules can lead to drug resistance and compensatory overexpression of target protein (Zou, et al., 2019; Martin-Acosta & Xiao, 2021; Lu, et al., 2015).

PROteolysis TArgeting Chimera (PROTAC) is a novel strategy to treat cancer, overcoming many limitations of cancer therapy with mAb, siRNA or small molecules (Zou, et al., 2019). By utilizing the ubiquitin-protease system (UPS), PROTAC induces target protein degradation in the cell (Zou, et al., 2019; Li, et al., 2021).The normal physiological function of the UPS is responsible for clearing denatured, mutated, or harmful proteins in cells (Li, et al., 2021; Mansour, 2018; Zhou, et al., 2000). In other words, PROTAC takes advantage of the cell's own protein destruction mechanism to degrade specifically disease-causing proteins from cells (Zou, et al., 2019; Li, et al., 2021).To date, the PROTAC technology can be used to target varieties of proteins, including transcription factors, kinases, and nuclear proteins (Martin-Acosta & Xiao, 2021). These targets are often "undruggable" using traditional strategies. PROTAC has other advantages, for example, it usually exhibits improved therapeutic effects, and minimizes drug resistance. Because of these reasons, this technology has drawn great attention in cancer therapy recently and is a hot topic in current research. Many studies have shown that degrading a protein is better than inhibiting a protein for anticancer activities (Neklesa, et al., 2017; Long, et al., 2012).

This review introduces PROTAC, summarizes effective application of the technology in cancer target proteins and elucidates essential considerations for designing an effective PROTAC molecule. The potential challenges of this field are also discussed. Together, we provide a valuable reference for future design and usage of potent PROTACs.

What is PROTAC and How Does It Work?

Ubiquitin-Proteasome System (UPS)

The UPS is one of the major pathways responsible for degrading proteins to maintain cell homeostasis and participates in the degradation of more than 80% of the protein in cells (Li, et al., 2021; Wu, et al., 2005). The UPS system is composed of multiple parts and degrades protein in a multistep process (Qi, et al., 2021; Neklesa, et al., 2011). When the ubiquitin-activating enzyme E1 is present, an ATP is used to activate ubiquitin to create an E1-ubiquitin complex with a Gly-Cys bond. The activated ubiquitin travels to the ubiquitin-conjugating enzyme E2 to make an E2-ubiquitin complex, once again with a Gly-Cys bond. Finally, the ubiquitin goes to the E3 ubiquitin ligase, where it binds to the target protein with its Lys. The protein of interest (POI) is recognized by regulatory particles in the 26S proteasome and transported into the core of 20S. Oligopeptides are released and the target protein is degraded (Qi, et al., 2021; Sakamoto, et al., 2001; Kubota, 2009).

PROteolysis TArgeting Chimera (PROTAC) Components

PROTACs operate by hijacking the UPS (Martin-Acosta & Xiao, 2021; Qi, et al., 2021; Neklesa, et al., 2017; Lai & Crews, 2017). The PROTAC is made of three parts: a protein of interest (POI) ligand or warhead (Figure 1 red triangle), an E3 ubiquitin ligase ligand (Figure 1 blue square), and a linker that connects the two to form a ternary complex (Martin-Acosta & Xiao, 2021). The warhead attaches to the POI, and the ligase-recruiting ligand attracts E3 ubiquitin ligase.

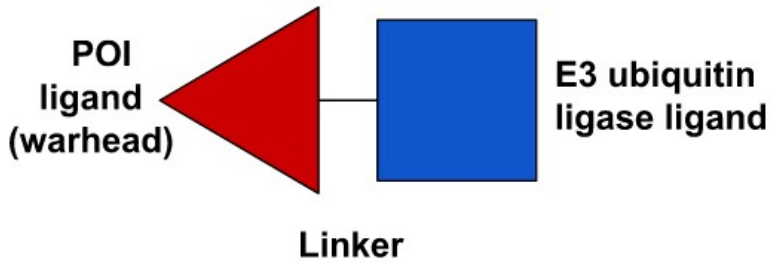

Figure 1. The schematic of PROTAC. The molecule composed of three parts: two ligands – warhead is the protein of interest (POI) ligand and E3 ubiquitin ligase ligand, linked together by a linker

How Does PROTAC Work?

The formation of the ternary complex (Figure 1) promotes ubiquitination because the E3 ligase and POI are brought closer together, increasing the likelihood that it occurs. E3 transfers the ubiquitin over to the POI, tagging the protein for degradation, the proteasome subsequently degrades the POI, then the PROTAC dissociates to catalyze another reaction (Figure 2) (Zeng, et al., 2021). The PROTAC and the ubiquitin are both reusable (Qi, et al., 2021). By completely destroying a protein that contributes to cancer, PROTACs lead to tumor growth inhibition (Li, et al., 2021). PROTACs don't need as much binding affinity as other drugs, and can therefore target a wider variety of proteins (Qi, et al., 2021).

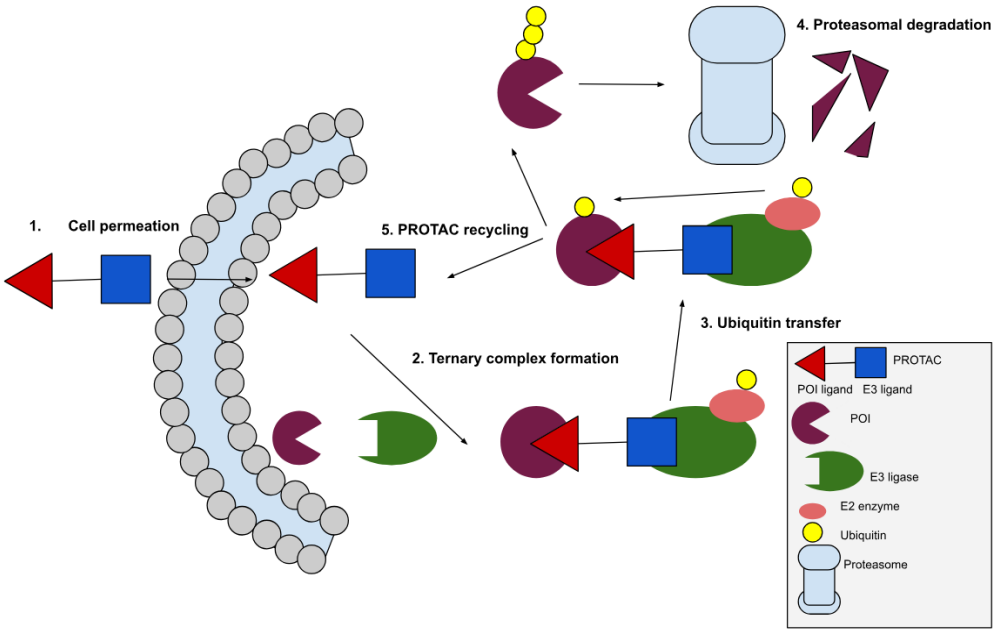

Figure 2. A diagram showing how PROTAC works. (1). PROTAC penetrates cell (2). PROTAC's POI ligand (warhead) interacts with POI on one side, and the E3 ligand engages with E3 ubiquitin ligase on the other side, forming a ternary complex. (3). The E3 ubiquitin ligase will transfer the ubiquitin to the POI via E2 enzyme. (4). The POI will be sent to proteasome, which degrades the protein. (5). PROTAC will be recycled for subsequence rounds of degradation.

PROTACs Target Diverse Proteins and Represent New Cancer Therapy

PROTACs can target a variety of proteins that contribute to cancer, including those that are not effectively treated by traditional drugs such as mAb, siRNA or small molecules (Zou, et al., 2019). In fact, the majority of proteins such as nuclear receptors, transcriptional regulators, and non-enzyme proteins are considered as "undruggable targets". They lack active binding pockets for small molecules or are not accessible to mAb. Additionally, traditional inhibitor drugs may require high doses and their mutations may cause drug resistance. These features limit the design and development of drugs that inhibit these disease-related proteins (Qi, et al., 2021; Zeng, et al., 2021).

PROTACs broaden the horizon for cancer drug discovery with unique advantages. To date, PROTACs have degreated more than 50 target proteins successfully (Li, et al., 2021; Li & Song, 2020), and 16 molecules have entered clinical trial stages for cancer indications as summarized in Table 1 (https://clinicaltrials.gov/). In this section, we summarized typical PROTAC molecules that have proved effective in degrading activities.

Protein Kinases

Protein kinases belong to the largest protein family. Small molecules are the primary treatment options for cancer driven by kinases. However, drug resistance to small molecules impaired clinical benefit. Bruton's tyrosine kinase (BTK) is an non-receptor cytoplasmic tyrosine kinase that plays a role in the B cell receptor signalling pathway, and is important for B cell development and differentiation (Martin-Acosta & Xiao, 2021; Mohamed, et al., 2009). Mutations or abnormal regulation are capable of leading to B cell-related cancers, more specifically chronic lymphocytic leukemia (Wang, et al., 2019). Irreversible inhibitors (e.g. ibrutinib) generally

work as cancer therapy initially, via irreversible covalent binding to Cys481 in the active site of BTK, before drug resistance occurs (Martin-Acosta & Xiao, 2021; Pfaff, et al., 2019; Buhimschi, et al., 2018). However, mutation in BTK later prevents inhibition via irreversible covalent binding (Martin-Acosta & Xiao, 2021). PROTACs, in this case MT-802, solve the drug resistance problem by using ibrutinib as the warhead with a polyethyleneglycol linker attaching it to pomalidomide (CRBN ligand). It is able to induce degradation in BTK via reversible binding, and is more selective than ibrutinib with fewer side effects (Li, et al., 2021; Buhimschi, et al., 2018). To date, there are four BTK degraders in Phase I clinical trials (Table 1).

Table 1. PROTACs in clinical trials

PROTACs	Target	Type of cancer	Clinical Trial #
NX-2127	BTK	B-cell malignancies	NCT04830137
NX-5948	BTK	B-cell malignancies	NCT05131022
BGB-16673	BTK	B-cell malignancies	NCT05294731
HSK-29116	BTK	B-cell malignancies	NCT04861779
ARV-110	AR	Prostate cancer	NCT03888612
ARV-766	AR	Prostate cancer	NCT05067140
CC-94676	AR	Prostate cancer	NCT04428788
HP518	AR	Prostate cancer	NCT05252364
ARV-471	ER	Breast cancer	NCT04072952
AC682	ER	Breast cancer	NCT05080842
DT-2216	BCR-xL	Blood cancer/Solid tumor	NCT04886622
KT-413	IRAK4	B-cell malignancies	NCT05233033
KT-333	STAT3	Lymphoma/Leukemia/Solid tumor	NCT05225584
CFT8634	BRD9	Advanced or Metastatic cancer	NCT05355753
FHD-609	BRD9	Advanced Synovial Sarcoma	NCT04965753
LNK-01002	Ras GTPase	Myeloid Malignancies	NCT04896112

Nuclear Receptors

Nuclear receptors' main function is to convert external hormonal signals to transcriptional output (Flanagan & Neklesa, 2019). Androgen receptors (AR) is a main nuclear receptor that mediates the biological effects of the androgen hormones. AR controls a variety of physiological and pathological processes, which has made it an attractive therapeutic target for prostate or breast cancer (Zeng, et al., 2021). In men, prostate cancer is the second most important cause of cancer deaths, and metastatic castration-resistant prostate cancer (mCRPC) specifically is difficult to target with inhibiting drugs due to the diversity of AR mutations and the development of drug resistance (Li, et al., 2021; Silke & Meier, 2013). Androgen receptor-targeting PROTAC, ARV-110, is an oral protein degradation agent and is the first PROTAC entering clinical trials. It utilizes an AR antagonist as the warhead and a CRBN as the ligand to degrade the androgen receptor (Li, et al., 2021). ARV-110 can decrease prostate-specific antigen at a lower dose compared with inhibiting drugs. The phase I study showed that it is safe and well tolerated by patients with mCRPC with PSA reduction over 50% at doses greater than 280 mg. It has now entered a clinical phase II trial for prostate cancer (Qi, et al., 2021) (Table 1). As androgen receptors may also play a role in breast cancer, PROTACs may help for this as well (Li, et al., 2021).

Estrogen receptor α (ERα) is another nuclear receptor that has been successfully targeted by PROTAC. ERα regulates gene expression and mediates the effects of estrogens in mammary tissue and female reproductive system (Nilsson, et al., 2011; Jia, et al., 2015). ARV-471 is a PROTAC targeting ERα and can degrade

both wild-type and clinically relevant ERα mutants in multiple ER+ breast cancer cell lines (Qi, et al., 2021). It addresses challenges presented by selective estrogen receptor degraders (SERD), small molecules that could not degrade ER completely and cause drug resistance (Liu, et al., 2022). ARV-471 is in phase II clinical trial to treat ER +/HER2- locally advanced or metastatic breast cancer (Qin, et al., 2022) (Table 1). Results from interim analysis of the trial showed the molecule reduced the expression level of ER in tumor tissues by an average of 62% and up to 90% (Qi, et al., 2021).

Transcriptional Factors

Transcriptional factors are a class of proteins binding to DNA sequence to regulate gene expression. They are historically challenging targets because of the lack of druggable active sites for small molecules. PROTAC technology allows expansion of druggability. Bromodomain and extraterminal (BET) are transcription factors that are related to the expression of various oncogenes (Qi, et al., 2021). They, especially one called BRD4, contribute to various cancers including prostate cancer (Mohamed, et al., 2009), acute myeloid leukemia (AML) and multiple myeloma. However, BRD4 lack active sites for small molecules to bind to, and even if these inhibitors bind to BRD4, they often lead to BRD4 accumulation and fail to inhibit cancer growth entirely (Li, et al., 2021). Therefore, PROTACs are suitable drug candidates. dBET1, using the inhibitor JQ-1 as a warhead and a CRBN ligand (thalidomide), was able to completely degrade BRD4 in two hours in a human AML cell line when standard inhibitors could not (Hines, et al., 2019). Other PROTACs have also been created to target BETs, and they are more effective than small molecule inhibitors at decreasing cell growth and causing apoptosis by reducing BET expression (Li, et al., 2021). This highlights the advantages of PROTAC over small molecule inhibitors.

Design Strategy of PROTAC Structure

When designing a PROTAC, its chemical structure should be carefully considered in order to make it as effective as possible (Jin, et al., 2020). Currently, PROTAC design relies largely on empirical analyses of structure-activity relationship. By analyzing the published data, we provide the following strategy for future design reference.

With two binding sites (one for E3 ligase and the other one for POI), PROTACs are heterobifunctional (Bekes, et al., 2021). X-ray crystallographic structure studies indicated that the formation of a stable ternary complex is critical for ubiquitin transfer, and ultimately determines the occurrence and efficiency of POI degradation (Bekes, et al., 2021). Multiple factors can affect the structure and stability of ternary complexes, including the type of E3 ligase, ligand for E3, the choice of POI ligand and the design of the linker. POI, warhead or POI ligand (red triangle), ligand for E3 ubiquitin ligases (blue square), and recruited E3 ubiquitin ligase (green oval) with success are summarized in Figure 3 (Zou, et al., 2019, Qi, et al., 2021; Bekes, et al., 2021; Liu, et al., 2022; Maneiro, et al., 2021; Zhao, et al., 2019; Paiva & Crews, 2019; Bondeson, et al., 2018; Hu, et al., 2017)

Ligand for E3 Ubiquitin Ligases

Since ubiquitination tags guide the degradation of POI promoted by PROTAC (Jin, et al., 2020), it is very important to rationally choose and design the ligand recruiting E3 ubiquitin (blue square). There are more than 600 E3 ubiquitin ligases encoded in our bodies (Jin, et al., 2020). However, only a handful of E3 ligases, including Von Hippel-Lindau (VHL), Cereblon (CRBN), IAPs, Keap1, and MDM2, can be hijacked by PROTAC *in vivo* (Qi, et al., 2021; Jin, et al., 2020; Zhao, et al., 2019; Paiva & Crews, 2019; Bondeson, et al., 2018; Hu, et al., 2017). The ligand for E3 can be peptides, or small moleculares recognizing a specific E3 ubiquitin ligase.

Peptide-based PROTACs are less permeable and stable, but can be simpler to design and synthesize, less toxic and safer. On the other hand, small molecule-based PROTACs are more effective drugs because they can be better absorbed, more stable and low cost (Zou, et al., 2019). The PROTAC strategy can use light-activated molecules (Zeng, et al., 2021). This is what the most recent generation does; with degradation being triggered by certain wavelengths of light to provide controllability (Li, et al., 2021).

Warhead or POI Ligand

The warhead (or POI ligand, red triangle) is typically small-molecule based (Zou, et al., 2019), but easier-to-design peptide warheads exist as well. Rather than trial-and-error, designing of a peptide warhead is a more accurate process. First, the key interacting residues for POI are analyzed, then the protein epitope mimetic is sequenced, and last, the peptide targeting warhead is synthesized and optimized (Jin, et al., 2020). Compared with small molecules, peptide warheads also possess greater potential in structural modification. However, peptide warheads have not undergone clinical trials.

Table 2. Comparison between peptide-PROTAC and small molecule-PROTAC

Polymer	**Peptide-PROTAC**	**Small molecule-PROTAC**
Ligands for E3 or POI (warhead)	➢ Peptides	➢ Small molecules
Advantages	➢ Target "undruggable" POIs with specificity ➢ Resistance to mutation ➢ Simple design, synthesis and modification ➢ Low toxicity and better safety profile	➢ High cell permeability ➢ High stability ➢ Low cost
Disadvantages	➢ Poor cell permeability ➢ Low stability ➢ Insufficient studies on efficacy	➢ Cannot target "undruggable" POIs with shallow surfaces ➢ Complicated design ➢ More severe side effects

Linker

The linker is hugely important to PROTAC function (Martin-Acosta & Xiao, 2021). The most frequently used linkers are PEG and alkane chains, which often enable good uptake of PROTACs by the cells and promote protein degradation (Liu, et al., 2022). Linkers need to be the right length - too short and it prevents degradation, too long and the complex becomes unstable (Khan, et al., 2020). Bemis et al. found that degraders with longer linkers are more effective. Once the efficient PROTAC is identified, the linker will be shortened step by step to identify the optimal length. Additionally, the composition, flexibility and attachment sites of the linker all affect degradation efficiency.

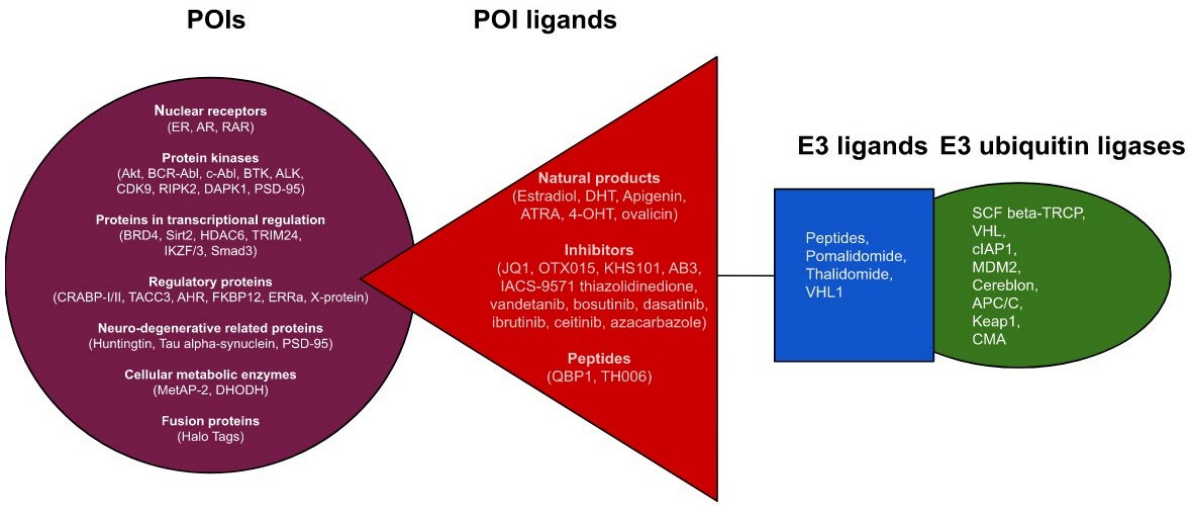

Figure 3. Summary of POIs, POI ligands (warhead), ligand for E3 ubiquitin ligases, and recruited E3 ubiquitin ligases.

Advantages and Disadvantages of PROTAC

Advantages

PROTACs have many advantages as a novel cancer therapy. They are ideal for dealing with otherwise difficult-to-bind to "undruggable" proteins because the POI ligand does not need high binding affinity nor long lasting occupancy to induce degradation (Zeng, et al., 2021; Gao, et al., 2017). It is also capable of overcoming drug resistances, by degrading the proteins produced by drug resistance mutations (Zeng, et al., 2021). Moreover, as catalyst, PROTAC can be repeated by recycling the molecules, which are not used up and therefore only a small dose is required (Zeng, et al., 2021; Coffey, et al., 2016). They thus have less toxicity and side effects compared to other drugs (Martin-Acosta & Xiao, 2020). Additionally, PROTAC is capable of selectively degrading only certain proteins, thus having the potential for high selectivity and specificity (Zeng, et al., 2021). The specificity may be contributed by the synergistic effects of POI, E3 ligase and linker, especially the stringent conformational requirement of ternary complexes. PROTAC also has tissue specificity because expression of E3 ubiquitin ligases vary in different tissues (Martin-Acosta & Xiao, 2020; Bray, et al., 2013; Kissopoulou, et al., 2013). Last, light-controlled PROTACs are more controllable than other drugs (Li, et al., 2021).

Disadvantages

The development of any new technology comes with not only opportunities but also challenges, and PROTAC is no exception. Although PROTAC technology has many unique advantages over other cancer drugs, they have potential limitations. PROTAC need to form a stable complex in order to properly function (Zeng, et al., 2021). However, designing and creating a table ternary PROTAC complex is difficult (Zeng, et al., 2021). Optimization of PROTACs is a long and complex process that is difficult, expensive, and not necessarily successful (Li & Song, 2020).

PROTAC can sometimes cause irreversible binding that leads degradation to fail, as the degrader is incapable of separating from the POI and prevents degradation from happening (Zeng, et al., 2021). They sometimes fail to degrade targets for unknown reasons (Zhou, et al., 2020). In addition, some targets are only partially degradable or can not be degraded effectively such as tubulin (Martin-Acosta & Xiao, 2020). They are not ideal for degrading cell-surface proteins because the ubiquitin-proteasome system is inside the cell (Bekes, et al., 2021).

PROTACs may also have toxic side effects because they may degrade normal proteins, and have off-target effect (Liu, et al., 2022). PROTACs can be incapable of permeate the cell and have low bioavailability due to their relatively large molecular weights (Zhou, et al., 2020). However, this can be improved by giving the PROTAC an alpha-helical structure and positive charge, or by using cell-penetrating peptides (Jin, et al., 2020).

Conclusion

PROTAC has developed significantly in recent years and shows high potential as a novel cancer therapy. Many PROTACs have successfully degraded their target proteins safely and efficiently. To date, at least 16 of them have entered clinical trials for cancer therapy, with some appearing promising (Qi, et al., 2021; Bekes, et al., 2021; Liu, et al., 2022). However, they have their issues that need to be solved before more extensive clinical applications, including off-target, low permeability and stability, and large molecular weight. Another issue is the difficulty of synthesis and optimization of the hybrid molecules (Li, et al., 2020). The good news is that many groups started to overcome these challenges in different ways (Zou, et al., 2019; Bekes, et al., 2021). For example, PROTAC design and optimization can potentially accelerated by deep learning and molecular simulations (Zheng, et al., 2022). The next few years will be a critically important time for the PROTAC development, with more PROTACs entering preclinical and clinical studies to further investigate their therapeutic effect of PROTAC. It is worth believing that PROTAC will become another important cancer therapeutic after monoclonal antibodies, siRNA and small molecule inhibitors, as well as herald a new era of biopharmaceutical innovation.

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