

Application of PROTACs in target identification and validation

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ABSTRACT

Proteolysis targeting chimeras (PROTACs), as a novel therapeutic drug model, has received widespread attention from academia and the pharmaceutical industry. PROTAC technology has led researchers to focus on developing chemical biology tool properties due to the unique operating mechanism and protein dynamic regulatory properties. In recent years the rapid development of PROTAC technology has gradually made PROTACs an essential tool for target identification and validation. To further promote the application of PROTAC tools in drug discovery and basic medical science research, this review distinguished target identification and validation concepts. Furthermore, research progress in PROTAC technology was summarized.

Keywords: PROTAC, Probe, Target identification, Target validation

1. INTRODUCTION

In 2001 Crews first proposed the concept of using the inherent protein degradation mechanism (ubiquitin-proteasome system) within the cell to eliminate pathogenic proteins, i.e., proteolysis targeting chimeras (PROTACs) [1]. Since 2017 PROTAC technology has entered an accelerated development period [2]. According to the incomplete statistics from PROTAC-DB [3], there are currently 5388 PROTAC molecules, of which 26 PROTAC molecules have entered clinical trials involving indications, such as solid tumors, hematologic cancers, and autoimmune diseases (Figure 1) [4-6]. Over the past 20 years, researchers have recognized the enormous potential of PROTAC technology and clarified the limitations, such as poor solubility and bioavailability, and potential toxicity to healthy tissues (on-target & off-tumor toxicity) [7, 8]. Therefore, current frontier research focuses on addressing the shortcomings of PROTAC and improving drug availability through other technical methods, such as nanomaterial technology [9-11] and prodrug strategies [12-14].

PROTAC technology has a revolutionary impact on drug therapy and provides new tools for research

in other disciplines [15]. PROTACs achieve rapid and reversible knockdown of target proteins in the form of small-molecule compounds. PROTACs can be used for disease biomarker identification [16] and protein function research [17, 18]. The controllable dynamic regulation of proteins by PROTACs has been applied to study the relationship between liquid-liquid phase separation and biological functions of targets [19]. In addition, our group was the first to propose the concept of targeted degradomics (TGDO), in which the PROTAC probes are combined with quantitative proteomics to identify the targets of natural products [20]. TGDO has received widespread attention and application in the academic community. The continuous deepening of interdisciplinary research will further promote the development of PROTAC technology as a chemical biology tool to solve practical research problems.

The purpose of chemical probes is to explore the role of biological targets in complex living systems, including target identification and validation. The main research objective of target identification is to identify medicinal biological targets. The research methods generally include the discovery of crucial disease biomarkers and drug-based target deconvolution. The main research

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Publication status of PROTAC related literature from 2001 to 2023

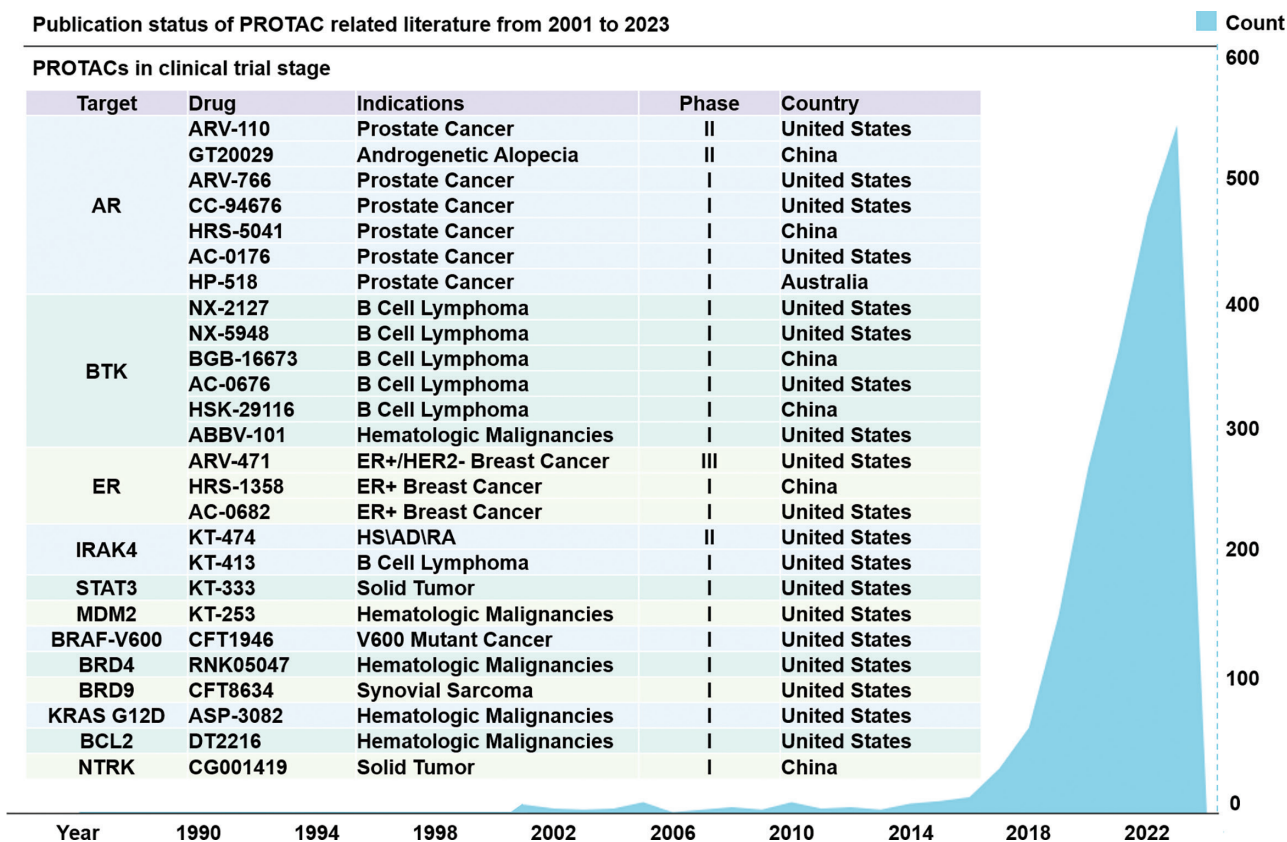


Figure 1 | PROTACs research progress.

The area chart displays the publication status of PROTACs-related literature from 2001–2023. The table shows the basic information about PROTACs in clinical trials.

task of target validation is to demonstrate the function and efficacy of identified targets in diseases, which are generally demonstrated through the reproducibility of drug efficacy in each research stage and different activity evaluation models [21]. Target identification is usually carried out before target validation. Some research content related to target validation will be involved in target identification research. Target identification and validation are performed at different stages of innovative drug development and have crucial roles in drug innovation, safety, and efficacy (Figure 2).

In this review we summarized application examples in target identification using the TGDO technology platform, including target deconvolution of natural products and multi-target discovery of targeted therapeutic drugs. In addition, we proposed a TGDO-standardized workflow to promote the application and development of this technology platform in target identification. We have also collected the methods to improve the activity and selectivity of drugs toward specific targets using PROTAC technology, which will provide more available strategies for developing disease-target correlation research tools and promote the development of a target validation study. Finally, we focused on applying

chemical proteomic-assisted PROTAC technology in target validation.

2. APPLICATION OF PROTACs IN TARGET IDENTIFICATION

Target identification is a common issue with natural product active molecules and can hinder the study of mechanisms, drug interactions, and adverse reactions, and even limit the development of new drugs [22]. In recent years the combined application of chemical biology and mass spectrometry has promoted research on target identification of natural products [23]. Chemical probes are small molecule compounds with definite selectivity and biological activity towards the target, which can study the complex biological behavior of the target in a live cell state [24, 25]. Identifying the target of small molecule drugs is crucial for elucidating the mechanisms underlying drug action. Whether or not the probes are used, target identification methods can be divided into non-labeling (using only active drug molecules) and labeling methods (using the probes designed based on active drug molecules). The non-labeling method is used to identify targets by detecting

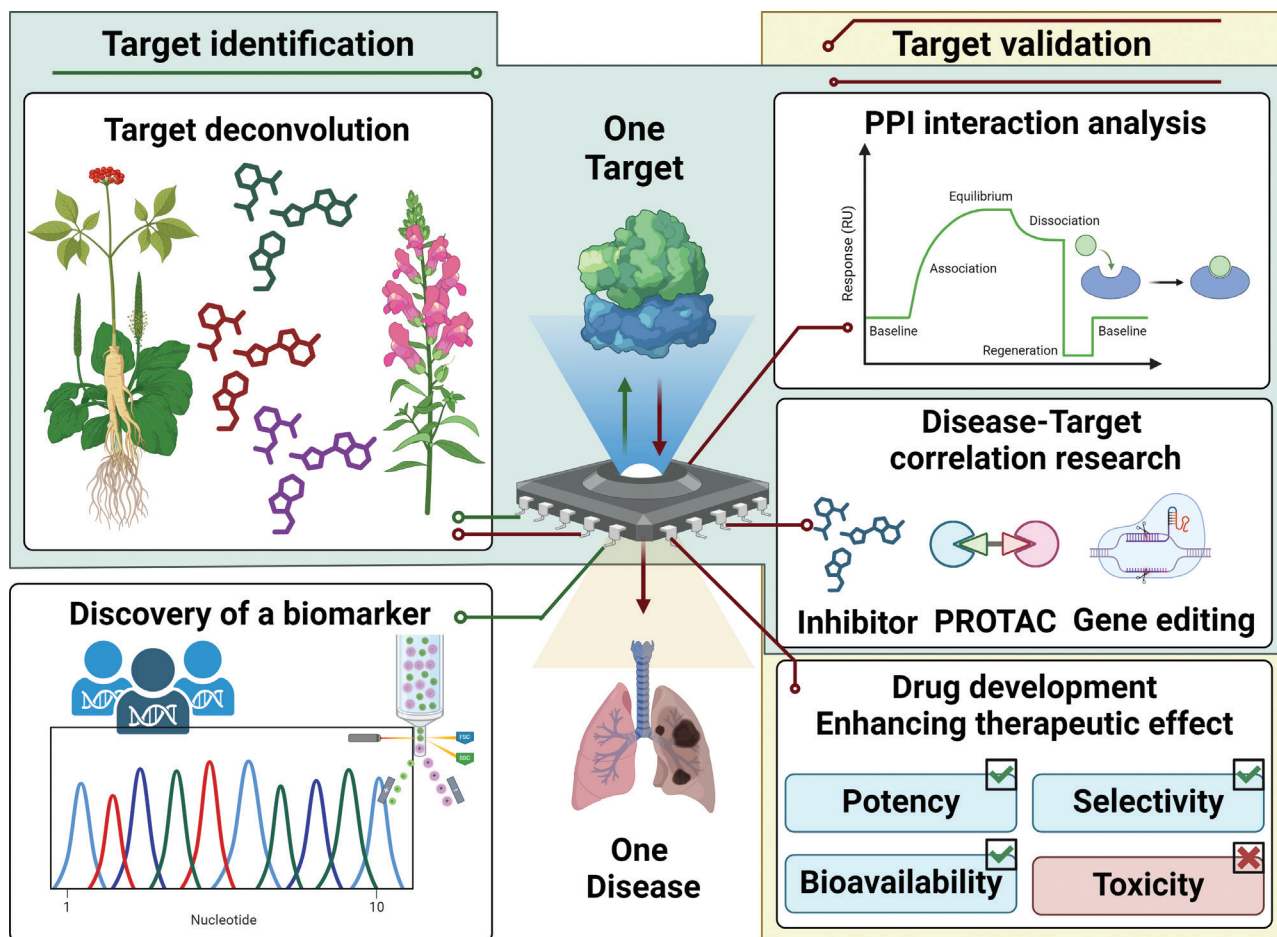


Figure 2 | The difference between target identification and target validation.

the biophysical properties involving changes in a protein of interest (POI) after binding to active drug molecules. The most widely used methods are the cellular thermal shift assay (CETSA) or drug affinity responsive target stability (DARTS) combined with mass spectrometry [26, 27]. The non-labeling method is suitable for natural products that do not have a suitable modification site or the activity may be affected after modification. However, the widespread application is limited by cumbersome experimental conditions and insufficient target sensitivity (Figure 3).

The labeling method utilizes chemical probes to recognize, bind, and enrich target proteins. Probes typically consist of three parts: an active drug molecule; a linker; and a reporting label [28]. According to the different reporting labels, the probes are divided into immobilized (solid-phase carriers as report label) [29] and active probes (biotin [30] and fluorescent groups [31] (Figure 3), bio-orthogonal reaction groups [32], and photoaffinity reaction groups [33] as reporting labels). Immobilized probes are a classic tool for target identification. However, the immobilization process often

causes activity impairment of active molecules and solid-phase carriers also bring significant steric hindrance to the active molecules, which will be unfavorable for binding targets and active molecules. Active probes have developed rapidly, have flexible functional designs, and have been successfully applied in target identification [34], cell imaging [35], and biomarker detection [36]. In the section on target identification, active probes usually require additional enrichment methods. In addition, chemical reactions during chemical modification or labeling processes may cause activity changes in active molecules and induce non-specific target binding.

The above methods rely on instantaneous and specific binding between active molecules and potential targets, making it challenging to identify moderate or weak binding targets [37]. In addition, existing methods are influenced by selectivity, efficacy, and physicochemical properties [38]. PROTACs have a similar structure to probes, but the difference is that PROTACs replace the reporting label with the E3-ligase ligand (the triggering unit of protein degradation events). Unlike the occupancy-driven mechanism of traditional small molecule

Chemical probes for target identification

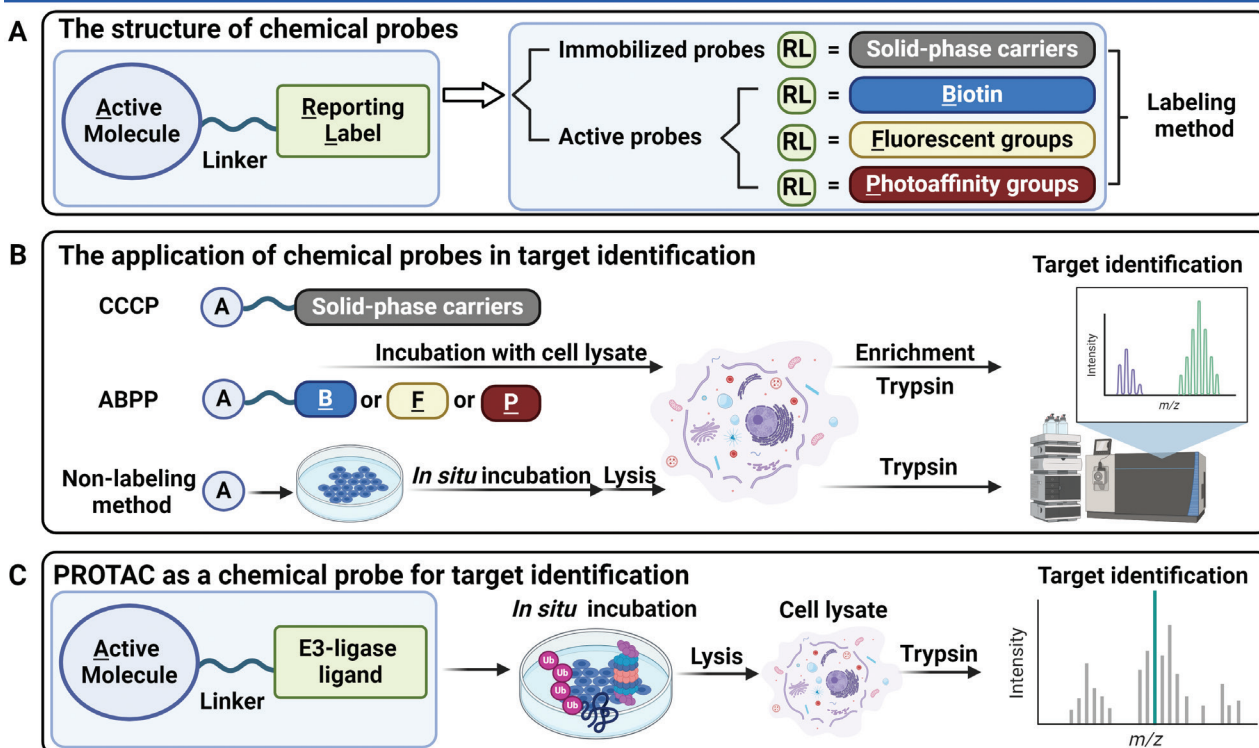


Figure 3 | Chemical probes for target identification.

A. The general structure of chemical probes. B. The application of chemical probes in target identification. Two methods for target identification include the labeling method (compound-centric chemical proteomics [CCCP] and activity-based protein profiling [ABPP]) and the non-labeling method (active compound molecule). C. PROTACs as a chemical probe for target identification.

compounds that rely on sustained high drug concentrations, PROTAC molecules achieve degradation of a POI through an event-driven mechanism (catalyst dosage) [39]. Therefore, PROTACs often exhibit better selectivity than parent compounds and PROTACs even exhibit differential selectivity towards proteins of the same family but different subtypes. This feature gives rise to PROTACs with excellent quality as chemical probes (Figure 3).

2.1 Application of PROTACs in target identification of natural products

Given that PROTAC technology does not require a strong affinity for target proteins and possesses high selectivity towards targets, our research group designed and synthesized the ZCY-PROTAC based on the CRBN-E3 ligase ligand for the triterpenoid compound, lathyrol. Through quantitative proteomics analysis and subsequent pharmacologic studies, we showed that the MAFF protein is a potential target for lathyrene triterpenoid compounds [20]. This study demonstrated the feasibility of using PROTAC technology for target identification of

natural products. It was the first time a systematic and explicit approach had been used to establish an experimental method and workflow for target identification using PROTAC technology. The concept of TGDO was proposed, which is widely recognized by the academic community [40-44] (Figure 4).

In recent years numerous researchers have conducted natural product target identification studies using TGDO workflow, further verifying the practicality and reliability of this method. The research team led by He et al. [40] designed and synthesized the PROTAC molecule, AD4, based on artemisinin. Compared to the parent compound, AD4 has a nearly 12-fold increase in growth inhibitory activity on human acute lymphoblastic leukemia cells (RS4; 11 cells) and revealed that PCLAF may be a potential target for AD4. Mechanistic studies have shown that AD4 causes degradation of PCLAF protein ($DC_{50} = 54.92 \pm 9.55$ nM), leading to upregulation of p21 and downregulation of phosphorylated Rb. As a result, the anti-apoptotic protein, Bcl-2, is downregulated and the pro-apoptotic protein, Bax, is upregulated, which ultimately promotes cell apoptosis. This study achieved

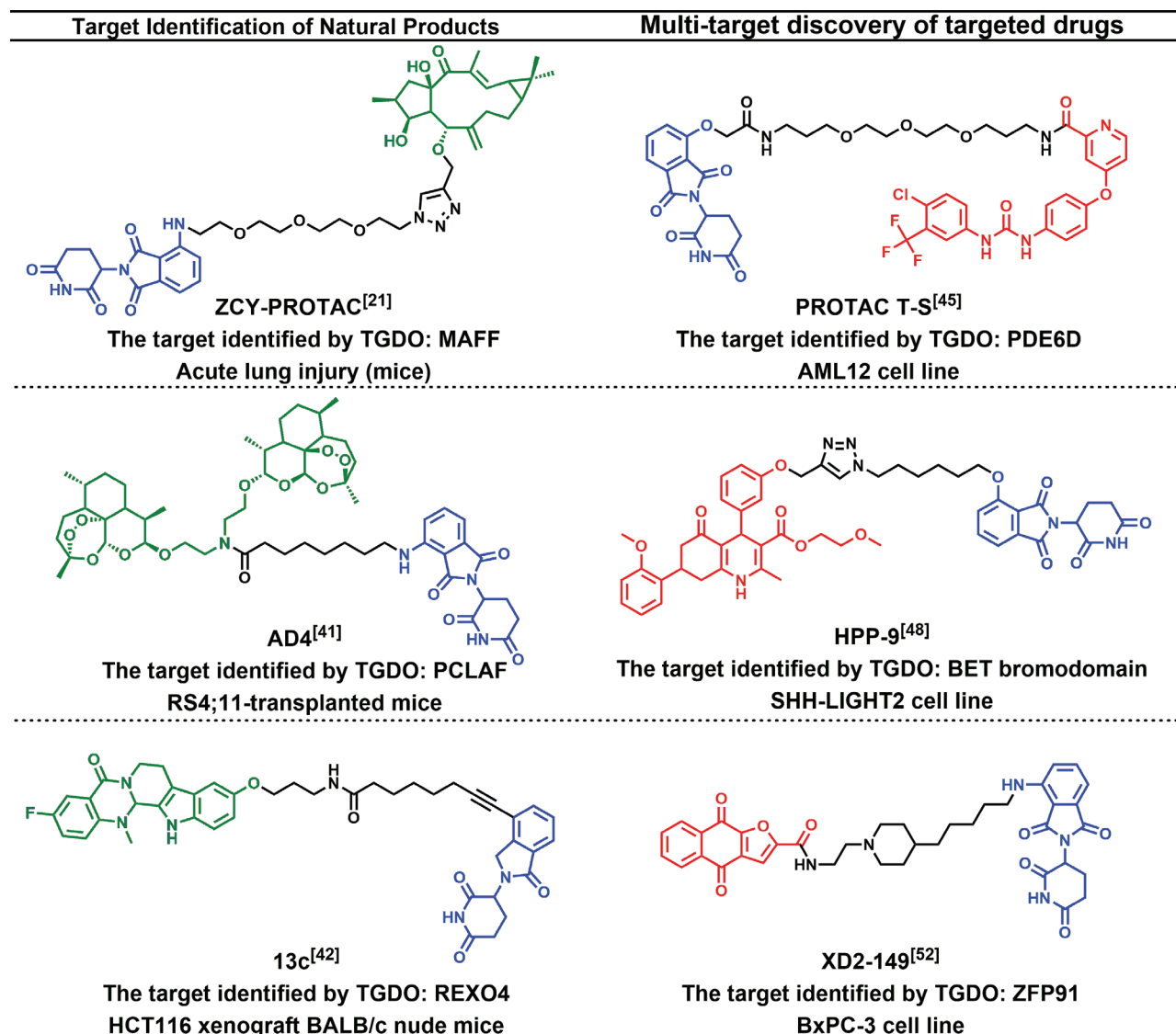


Figure 4 | Target identification research cases based on TGDO.

two goals through PROTAC technology: (1) drug repurposing; and (2) target identification and mechanism determination (Figure 4).

In a recent study researchers designed and synthesized the PROTAC molecule, evodiamine, and identified REXO4 as a potential target through TGDO. This study utilized two active PROTAC molecules with different structures for a TGDO comparison analysis, which significantly reduced the potential target range of the parent compound [41]. The study finding further confirmed the potential of PROTACs as a chemical biology tool for the target identification of natural products. With additional researchers in various fields, TGDO will continue to better address cutting-edge issues, expand the scope of application, and further improve research methods and workflow (Figure 4).

2.2 Application of PROTACs in multi-target discovery of targeted therapeutic drugs

Sorafenib is a multi-kinase inhibitor with significant inhibitory activity against RAF-1, BRAF, c-KIT, FLT3, VEGFR2, and PDGFR [45, 46]. Sorafenib is widely used in cancer therapy. To determine the therapeutic effect and target of sorafenib on liver fibrosis, researchers developed a sorafenib PROTAC molecule based on CRBN-E3 ligase. It was shown that PDE δ is a possible target of sorafenib through quantitative proteomics using tandem mass labeling (TMT) [42] (Figure 4).

The Hedgehog (Hh) pathway is a complex cellular signaling cascade that regulates embryonic development [47]. Dysregulation of signal transduction can lead to developmental disorders and the occurrence and progression of various cancers. The Hh pathway

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inhibitor (HPI-1) is a dihydropyridine compound with excellent anti-cancer activity [48] that is used to treat breast cancer, but the cell target has not been established. Bagka et al. designed a PROTAC molecule (HPP-9) based on the HPI-1 and quantified the protein treated with HPP-9 through label-free quantitative proteomics. The results showed that two members of the bromine domain and the end effector domain (BET) protein family (BRD3 and BRD4) were significantly downregulated [43], further confirming the involvement of the BET bromine domain in regulating the Hh signaling pathway [49]. Based on an evaluation of degradation activity and the underlying mechanism, BET was ultimately determined to be the target of HPI-1 (Figure 4).

Napabucasin is a multi-target drug molecule. Napabucasin is often used as a STAT3 inhibitor in treating various cancers, either as a single drug or in combination with chemotherapy drugs [50, 51]. Hanafi et al. designed and synthesized a series of PROTAC molecules and selected the most active molecules (XD97 and XD2-149) for proteomic analysis. XD2-149 effectively mediates the degradation of ZFP91 through the proteasome pathway. Activity studies showed that XD2-149 exerts

anti-tumor activity by targeting the degradation of ZFP91. This study provided the first evidence that ZFP91 is a potential anti-tumor target [44] (Figure 4).

2.3 TGDO workflow

2.3.1 Design and establishment of the PROTAC library. Using structure-activity relationship analysis, researchers determined reasonable derivatization sites for active drug molecules. The linkers and E3 ligase ligands significantly impact the degradation activity of PROTACs [52]. Therefore, it is necessary to modularize the combination of different types of linking chains, E3 ligase ligands, and active drug molecules to form a PROTAC library (Figure 5).

2.3.2 Target phenotype screening. A target activity evaluation model for phenotype screening of PROTAC molecular libraries identified PROTAC molecules with good therapeutic activity and physicochemical properties as candidate target molecular probes. Selecting 2-3 PROTAC molecules with different E3 ligands as the final probes can improve target identification accuracy (Figure 5).

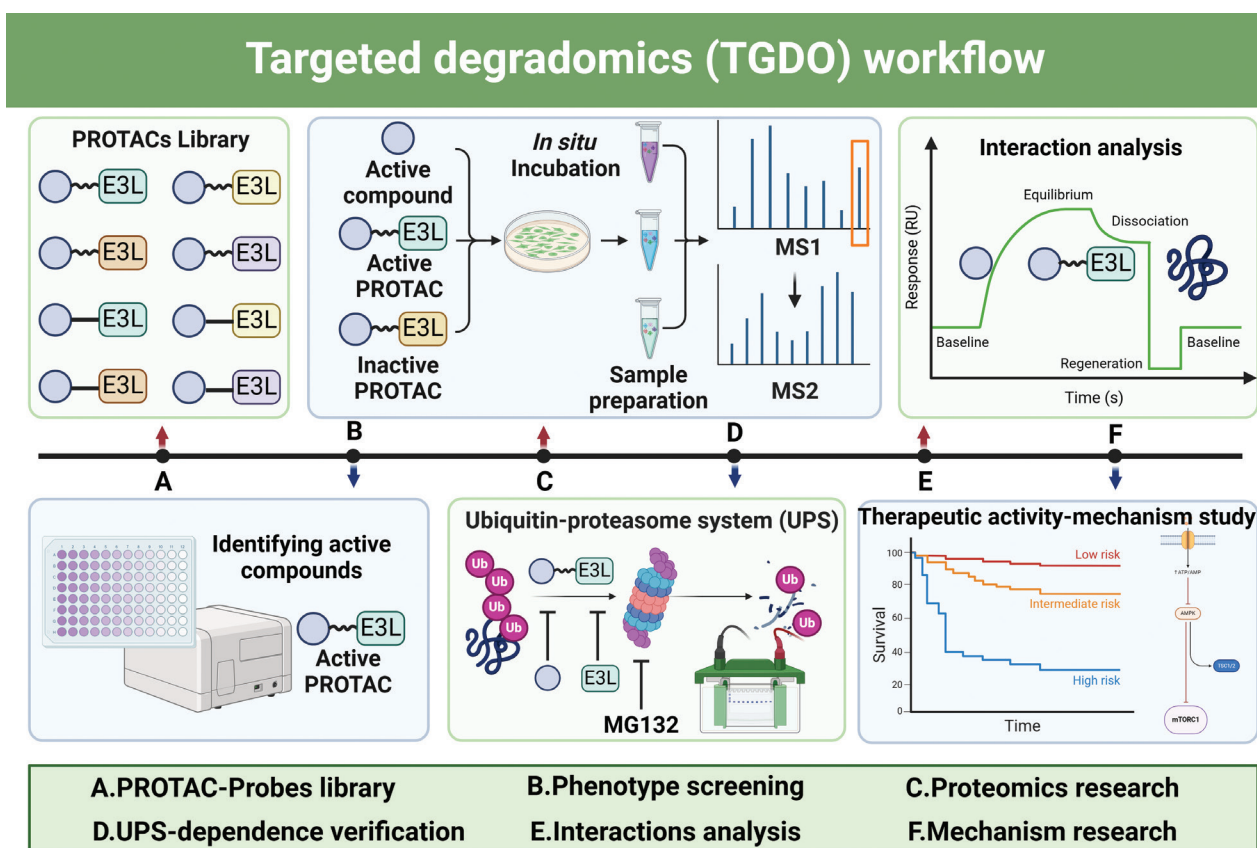


Figure 5 | Targeted degradomics (TGDO) workflow.

Different types of E3 ligands (E3Ls) can be selected for establishing the PROTAC probes library.

2.3.3 Administration and mass spectrometry detection.

Active drug molecules, active PROTACs, and non-active PROTACs were selected to treat the cell model. Cells were collected for mass spectrometry sample processing after incubation. Using the TMT-labeling method for proteomics research, the omics data of active PROTACs were compared with the active drug molecules and non-active PROTACs groups to determine potential targets (Figure 5).

2.3.4 Degradation activity evaluation and mechanism verification.

Immunoblotting technology was used to validate the degradation activity of potential targets and investigate the time- and dose-response relationships of active PROTACs in target degradation. Using methods, such as ligand competition, ubiquitination pathway blockade, and proteasome inhibition [53, 54], it was verified that the degradation of the targets is achieved through the proteasome pathway (Figure 5).

2.3.5 Analysis of interactions between active drug molecules and targets.

The direct interaction between active drug molecules and targets using biotin, fluorescent, or photo-crosslinking probes has been repeatedly confirmed. In addition, it is necessary to conduct affinity evaluation experiments between active compounds and targets, such as surface plasmon resonance (SPR), micro-scale thermophoresis (MST), and isothermal titration calorimetry (ITC). Co-crystallization research will provide more convincing evidence for target identification (Figure 5).

2.3.6 Activity and mechanistic research. First, the therapeutic effects of the original compound and PROTAC molecules should be investigated on target disease models *in vitro* and *in vivo*. Second, the signal pathway research based on identified targets should be conducted to provide further evidence for the correlation between active drug molecules, targets, and diseases (Figure 5).

3. APPLICATION OF PROTACs IN TARGET VALIDATION

The primary purpose of target validation research is to demonstrate the correlation between drug, target, and disease treatment. In addition to the drug-target interaction assay involved in the general process of target identification research, target validation also includes various methods to enhance drug therapeutic activity, selectivity towards targets, and reduce toxicity. These studies will further confirm the therapeutic effects of targeting specific targets on diseases. In this section we summarized the applied PROTAC cases for target validation research and the methods enhancing drug activity and selectivity to provide more chemical tools. We particularly emphasized the significance of chemical proteomics in target validation research based on PROTACs.

3.1 Enhancing therapeutic efficacy

PROTACs exert pharmacologic effects through an event-driven mechanism at a catalyst dosage, which converts traditional small molecule inhibitors into PROTAC molecules and provides a new optimization strategy. At present there have been many successful cases, such as the PROTAC molecules targeting PLK4 [55], SHP2 [56], and BET [57], the anti-tumor activity of which can be increased by 10–1000 times compared to the original drug, while affirming the therapeutic potential of targeting the above three targets for related cancers (Figure 6).

On this basis, the multivalent PROTAC strategy, PROTAC component optimization, and nanotechnology provide alternative solutions for optimizing the activity of PROTAC molecules targeting a single therapeutic target and providing additional tool molecules for target validation, as follows: (1) The multivalent PROTAC strategy involves improving the binding efficiency between PROTAC molecules and target proteins. Researchers have developed trivalent PROTAC molecules targeting BET. Using multiple alkanes as core linkers, an E3 ligase ligand was connected to two molecules of BET ligands to obtain the final compound, SIM1. Compared to the divalent PROTAC molecule, MZ1, the degradation activity of SIM1 was increased 300-fold, which more effectively induced cell apoptosis. It was thought that trivalent PROTAC molecules effectively generate stable ternary complexes and improve protein degradation efficiency by increasing molecular affinity and synergy [58]. (2) PROTAC component optimization was achieved by adjusting the physicochemical properties and molecular conformation, which enhances the activity of PROTACs. Researchers have synthesized PROTAC prodrugs targeting CDK family proteins using prodrug strategies with component 1-CRBN-E3 ligase ligand, which increased oral bioavailability from < 1% to 68% [59]. Component 2-linker connection methods, such as the amide-to-ester substitution method, can improve the solubility, membrane permeability, and degradation activity of PROTAC molecules [60]. Linear linker, circular linker with rigid structure, and linker configuration (component 3-linker type) significantly impact the physicochemical properties and activity of PROTACs. It is necessary to increase the synthesis flux to determine the most active PROTAC molecule [61, 62]. The above technologies enhance the *in vitro* and *in vivo* activity of PROTACs and provide a toolbox for target validation research. Moreover, disease treatment based on relevant targets is promoted (Figure 6).

3.2 Improving selectivity

One of the advantages of PROTACs compared to small molecule inhibitors is the high selectivity towards therapeutic targets. Multiple studies have shown that by optimizing the length of linkers and increasing the stability of ternary complexes, PROTACs can achieve target protein selectivity beyond the original drug. In

Application of PROTACs in Target validation

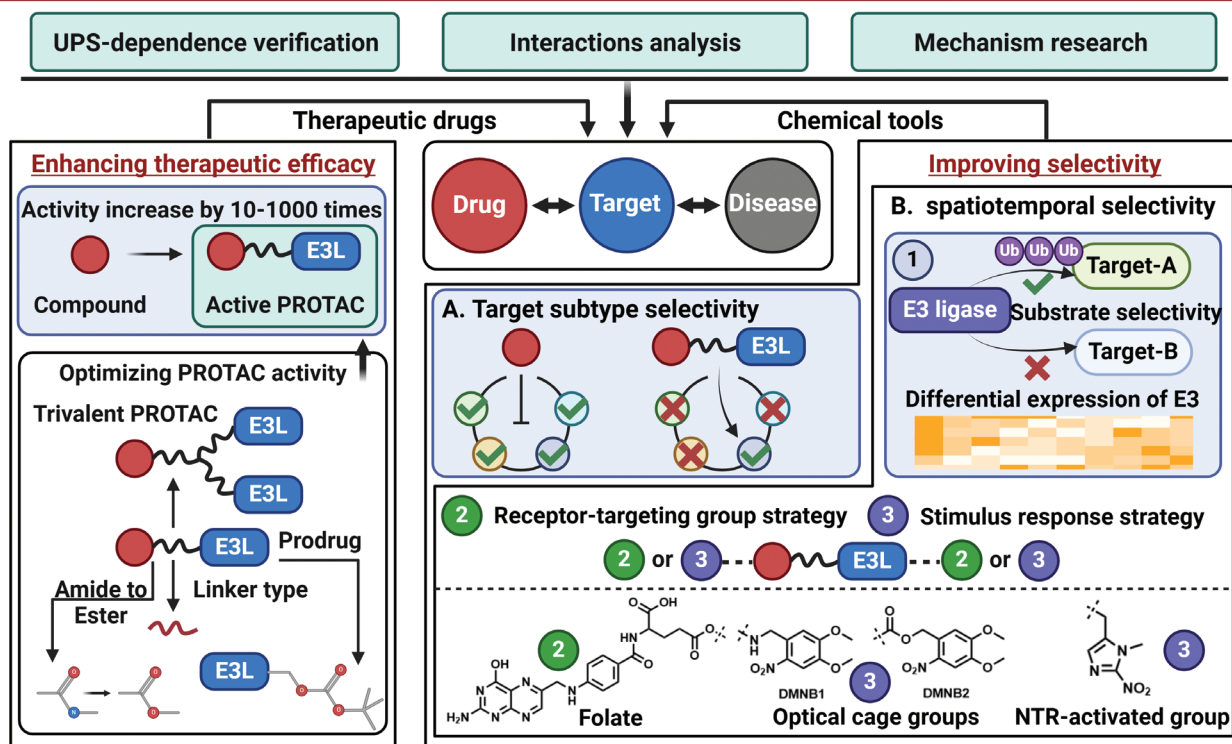


Figure 6 | Application of PROTACs in target validation.

The main task of target validation is to determine the correlation between the target and the disease. In addition to UPS-dependence verification, interaction analysis, and mechanism research, these three parts are also involved in target identification. The target validation research also includes using PROTAC technology to enhance drug activity and selectivity, providing new therapeutic drugs for target-based disease treatment, and more precise chemical tools for studying the relationship between targets and diseases.

addition, the spatiotemporal selective PROTAC molecules targeting specific cells and tissues can be developed by utilizing the substrate selectivity of E3 ligases, the differential expression in cells and tissues, and receptor-targeting groups or stimulus-responsive strategies [63]. Target selectivity enhancement achieved through PROTAC technology not only reduces the toxic side effects caused by the drug itself but also more accurately identifies the positive role of the target in disease treatment (Figure 6).

3.2.1 Improving selectivity for specific target subtypes.

Relevant studies have reported selective PROTACs targeting specific subtypes [63]. Cyclin-dependent kinases (CDKs) are a class of serine/threonine kinases that have critical roles in cell cycle regulation and transcription processes. Due to the highly-conserved ATP binding pockets of different CDK subtypes, it is challenging to find ATP-competitive and highly selective CDK inhibitors [64]. To avoid potential toxicity induced by inhibiting CDK13, Tian et al. developed a CDK12 selective

degrader (PP-C8) using non-covalent CDK12/13 dual inhibitors. Proteomics analysis showed that PP-C8 exhibited high selectivity towards CDK12. In addition, PP-C8 combined with a PARP inhibitor showed a synergistic anti-proliferation effect on triple-negative breast cancer [65]. Other research teams designed and developed the CDK12-PROTAC, BSJ-4-116. BSJ-4-116 selectively degrades CDK12, as assessed through quantitative proteomics. It is worth noting that BSJ-4-116 has a significant anti-proliferative effect on CDK12-resistant tumor cells [66]. CDK9 is an essential regulatory factor for transcriptional elongation and a crucial target for cancer therapy. Olson et al. designed and synthesized THAL-SNS-032, a selective CDK9 degradation agent. Proteomics analysis showed that THAL-SNS-032 induces rapid degradation of CDK9 without affecting the levels of other SNS-032 targets. Compared to NVP-2, a selective ATP-competitive CDK9 inhibitor, the compound elution experiment revealed that THAL-SNS-032-induced degradation of CDK9 and showed a more prolonged cytotoxic effect [67] (Table 1 and Figure 6).

Table 1 | List of selective PROTACs for target subtype.

PROTAC	Target binding selectivity	Degradation selectivity	E3 ligase
PP-C8	CDK12/13	CDK12	CRBN
BSJ-4-116	CDK12/13	CDK12	CRBN
THAL-SNS-032	CDK1/2/7/9	CDK9	CRBN
ACBI2	SMARCA2/4	SMARCA2	VHL
A947	SMARCA2/4	SMARCA2	VHL
PRT3789	SMARCA2/4	SMARCA2	Undisclosed
AK2292	STAT5/6	STAT5	CRBN

SMARCA2 and SMARCA4 are two ATP-dependent proteins in the SWI/SNF complex, the primary function of which is to regulate chromatin structure, thereby affecting gene expression. Therefore, SMARCA2 and SMARCA4 have crucial roles in DNA repair, cell cycle regulation, and cell differentiation. When SMARCA4 mutates, tumor cells rely on the homologous protein (SMARCA2) to maintain survival [68]. Due to the simultaneous knockout of SMARCA2 and SMARCA4, which can lead to synthetic lethality, developing selective SMARCA2 is a highly promising cancer treatment strategy. Farnaby et al. utilized structure-based drug design and co-crystallization research to conduct multiple rounds of optimization on degradation agents from various aspects, such as target protein ligands, linkers, and VHL ligand binding sites, and obtained a selective orally utilized SMARCA2 PROTAC molecule, ACBI2. In human colon adenocarcinoma cells, the degradation efficiency of SMARCA2 was 30-fold higher than SMARCA4 and the oral bioavailability was also increased by 22%. Whole-cell proteomics analysis further confirmed that ACBI2 exhibits high degradation selectivity towards SMARCA2 [69]. The same year, Yauch et al. developed a potent and selective SMARCA2-PROTAC (A947). In addition, compared to the wild-type, A947 displayed a more robust *in vitro* growth inhibitory activity and *in vivo* therapeutic effect in the SMARCA4mut model. The global ubiquitination map and proteomics analysis showed no relevant off-target effects when treated with A947, which provided a new potential therapeutic opportunity for cancer patients expressing SMARCA4^{mut} [70]. In addition, the SMARCA2 selective degrader, PRT3789, which was developed by Prelude Therapeutics, has entered the phase I clinical trial stage [71, 72]. Therefore, the development of SMARCA2 selective targeted drugs may have therapeutic effects on multiple types of cancer, further affirming the enormous potential of PROTAC technology in target selectivity and providing a research tool for clarifying SMARCA2 as a cancer treatment target (Table 1 and Figure 6).

Signal transduction and transcription activating factor 5 (STAT5) activation typically occurs in chronic myeloid leukemia (CML), acute myeloid leukemia (AML), T-cell leukemia, and lymphoma [73]. However, STAT5 inhibitors with high activity and selectivity have yet to be developed [74]. Wang et al. used PROTAC technology to synthesize the selective STAT5 degrader, AK2292, for the first time. AK-2292 effectively induced degradation of STAT5A, STAT5B, and phosphorylated STAT5 in AML cell lines in a dose- and time-dependent manner ($DC_{50} = 110-160$ nM). AK-2292 cannot induce degradation of STAT6 and other STAT proteins in cells. AK-2292 exhibited effective inhibitory activity on AML cell lines with high-level phosphorylation of STAT5 and effectively inhibited cell proliferation in six CML cell lines. In addition, the *in vivo* activity evaluation results further confirmed the therapeutic efficacy and STAT5 selectivity of AK-2292 [74] (Table 1 and Figure 6).

3.2.2 Improving spatiotemporal selectivity.

3.2.2.1 Substrate selectivity and differential expression of E3 ligases. Currently, most PROTACs are developed based on four E3 ligases (CRBN, VHL, IAP, and MDM2), often producing significantly different degradation activities in different cell models [75]. There may be two possible reasons for this finding: (1) E3 ligase has substrate specificity [76]. Researchers are increasingly interested in the interaction between E3 ligases and their substrates, such as using the BioE3 technology platform to identify specific substrates for E3 ligases [77] and determining the E3 ligase substrate relationship through multiple CRISPR screening [78]. These studies will greatly promote the construction of E3 ligase-substrate interaction networks. UbiBrowser 2.0 (http://ubibrowser.bio-it.cn/ubibrowser_v3/), which was developed by Beijing Protein Research Center, has been integrated into the network to retrieve known or predicted unknown E3 ligase-substrate interactions. These E3/DUB substrate interactions are derived from five data sources: manual cleavage; protein GO annotation; protein domain; protein motif; and network topology

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[79]. This tool will provide reference information for the rational design of PROTACs, reduce unnecessary development costs, and accelerate the research process. (2) E3 ligases exhibit differential expression in different tissues and cells. Therefore, the cellular and tissue distribution characteristics of E3 ligase will promote selective PROTAC development for different diseases [80]. Due to the low expression of VHL in platelets, DT2216 achieved selective degradation of Bcl-XL in cancer tissues without causing significant platelet reduction [81]. In addition, the selective expression of E3 ligases in specific tissues, organelles, or cancer cells provides a research basis for the selective degrader [82]. FBXO41, FBXL16, RNF167, TRIM9, TRAF3, and TRIM2 are expressed in brain tissues. TRIM54, TRIM63, FBXO40, and KLHL31 are muscle tissue-specific E3 ligases. The muscle tissue-specific E3 ligases may provide new selective targeting strategies for treating related diseases (Figure 6).

3.2.2.2 Receptor-targeting group strategy. Folate receptor α (FOLR1) is highly expressed in many cancer cells. Wei et al. utilized a folate coupling strategy to transport folate-PROTAC to cancer cells with high FOLR1 expression. After entering cancer cells, folate-PROTAC was cleaved by endogenous hydrolytic enzymes and released active PROTAC molecules. Folate-PROTAC can degrade the POI in a folate receptor-dependent manner. This method can be applied to the VHL-recruited PROTACs, providing a universal strategy for achieving targeted degradation of target proteins in cancer cells [12] (Figure 6).

3.2.2.3 Stimulus-response strategy

3.2.2.3.1 Photo stimulus-responsive PROTACs

In 2019 Pan et al. introduced the photo-controlled group (DMNB1) into BRD4-PROTAC (dBET1) and synthesized the first photo stimulant-responsive PROTAC (pc-PROTAC) [83]. In addition to the photo-controlled group (DMNB1), UV-responsive groups, such as DMNB2, NPOM, and DEACM, have also been successfully applied in the design of photo-controlled PROTACs, which expand the application of photo-cage strategies in PROTAC molecular design [84] (Figure 6).

UV-activated PROTACs commonly cause limitations of phototoxicity and poor tissue penetration. Therefore, to overcome the above limitations, researchers have designed and synthesized PROTAC molecules that can be activated by near-infrared light based on self-assembly nanotechnology [85]. BRD4-PROTAC (ARV-771) was selected to connect with near-infrared light photosensitizer groups in the active sites of VHL ligands and prepared as a self-assembled nanoparticle. This nanoparticle can be efficiently enriched in tumor tissues and the liver. After being irradiated by near-infrared light, the photosensitizer in PROTAC molecules will release singlet oxygen, leading to chain breakage of linker and ultimately releasing active PROTACs [85]. This technology has successfully constructed PROTAC nanoparticles

that can be activated by near-infrared light, providing a more accurate research tool for exploring the therapeutic effect of targeted BRD4 in specific cancers. It is worth noting that this technology also provides a feasible technical platform for constructing other stimulus-responsive PROTAC drug delivery systems.

In 2022 X-ray-responsive groups were introduced into the design of PROTAC molecules (RT-PROTAC). After X-ray radiation, RT-PROTACs can be activated in specific tumor tissues and synergistically inhibit tumor growth *in vivo* [13]. Compared to UV-responsive PROTACs, this study provided a new combination therapy strategy for cancer patients who cannot undergo surgery.

3.2.2.3.2 Hypoxia-responsive PROTACs based on nitroreductase (NTR)

NTR is generally highly expressed in the hypoxic environment of solid tumors. Nitroimidazole is an NTR substrate and can be selectively cleared by NTR. In 2021 Zhang et al. introduced the hypoxic-responsive group (nitroimidazole) into the target protein-ligand and synthesized Ha-PROTAC [86]. In 2022 researchers reported a novel hypoxia-responsive PROTAC by introducing nitroimidazole groups into the section of E3 ligands. Compared to Ha-PROTAC, this study provided a universal technical platform for other target proteins [87] (Figure 6).

3.2.2.3.3 Chemical reaction-responsive PROTACs

Overexpression of NAD(P)H:quinone oxidoreductase 1 (NQO1) is closely related to the occurrence and development of cancer. NQO1 catalyzes the double electron reduction reaction of quinones. Wang et al. designed two Pro-PROTAC molecules (NQO1-PROTAC and ROS-PROTAC), which can be activated by the reduction products formed by NQO1 catalysis, providing a new approach to enhance the selectivity of PROTACs [88].

$\alpha\beta$ integrins are a common target for drug delivery [89]. In 2023 Wang et al. [90] successfully designed a biologically orthogonal reaction-activated PROTAC (crPROTAC). With ARV-771 as the focus of research, Wang et al. first coupled the bio-orthogonal trans-cyclooctene (TCO) group to the active site of the VHL ligand. RGD peptide c modified with tetrazine (Tz) specifically entered tumor cells with high expression of $\alpha\beta$ integrins, reacted with TCO groups, then released the active PROTACs. This technology has been validated in another study [91].

The receptor-targeting group and stimulus-response strategies provide many groups for developing the PROTACs with spatiotemporal selectivity. However, introducing these groups can also significantly increase the molecular weight of PROTACs, which may affect pharmacokinetic properties and oral bioavailability.

3.3 Chemical proteomics promotes the application of PROTACs in target validation

3.3.1 Protein degradation analysis. Currently, most studies utilize immunoblotting for POI degradation analysis. The limitation of this method is the specific degradation

Chemical proteomics promotes the application of PROTACs in target validation

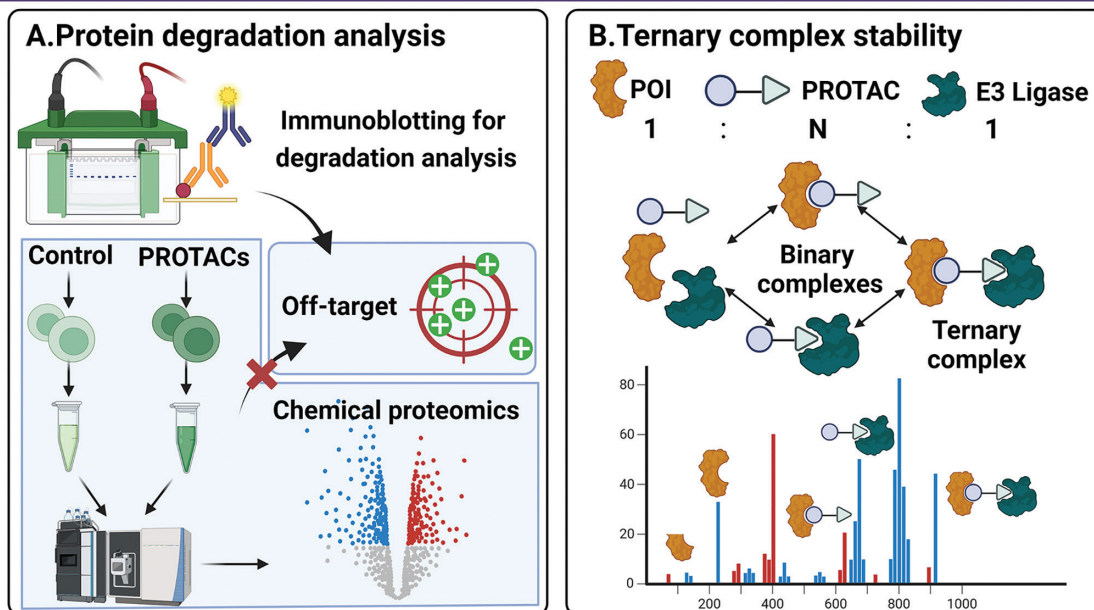


Figure 7 | Applying chemical proteomics in protein degradation analysis and ternary complex stability studies.

of degraders cannot be elucidated. To avoid off-target effects, some researchers employed mass spectrometry-based proteomic techniques to study the degradation specificity of PROTACs. TMT-labeled quantitative proteomic analysis is the most often used method and has been successfully applied to specifically targeted degradation studies involving K-RAS [92], SGK-3 [93], LRRK2 [94], SMARCA2 [70], BRD/BET, FAK, ALK, and BTK [95]. This method has also driven the development of molecular glue discovery platforms [96]. In addition, a few studies have applied label-free quantitative proteomics methods to determine the on-target degradation activity of NAMPT [97] and NTMT1 [98]. These studies identified the targeted degradation of POI and investigated the effects of degradation agents on other proteins, including the impact of POI degradation on downstream signal transduction (Figure 7).

3.3.2 Ternary complex stability studies. Forming a stable ternary complex (E3 ligase-PROTAC-target protein) has always been considered a critical step for PROTACs to exert a degradation effect. Detection methods for the formation of ternary complexes currently include X-ray crystallography [99, 100], homogeneous time-resolved fluorescence (HTRF) [101, 102], the AlphaLISA assay [103], isothermal titration calorimetry (ITC) [104], and surface plasmon resonance (SPR) [105]. In addition, western blot analysis can demonstrate the formation of ternary complexes by detecting the HOOK effect [106]. Recently, native mass spectrometry (Native-MS)

has become a reliable method to characterize PROTAC-mediated ternary complex formation. Beveridge was the first to use Native-MS to detect the formation of E3 ligase-PROTAC-POI ternary complexes [107]. Other researchers further validated the reliability of the technology [108]. Native-MS only requires a shallow input sample size and can quickly screen many samples. Native-MS is a high-throughput screening platform for developing PROTACs. In addition, the combination of hydrogen deuterium exchange mass spectrometry (HDX-MS) and computational modeling can also be used to study the formation of ternary complexes induced by PROTACs [109] (Figure 7).

4. SUMMARY AND OUTLOOK

In this review we summarized the application progress of PROTACs in target identification and validation. We summarized the cases of target identification in natural products and targeted therapeutic drugs using the TGDO proposed by our research group. In addition, we have provided a recommended operational workflow for TGDO. This section will promote the widespread application and development of TGDO. With respect to target validation we have paid particular attention to the instrumental properties of PROTACs. With the help of PROTAC technology, the activity and selectivity of compounds towards identified targets can be improved. This provides better therapeutic drugs for disease treatment and efficient chemical biology tools

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for studying the relationship between related targets and diseases.

PROTACs have shown many advantages in target identification and validation. Because PROTACs do not require strong affinity to effectively and specifically degrade target proteins using PROTACs as a chemical probe for target identification may reveal more potential targets [20]. In addition, PROTACs utilize the intracellular ubiquitin-proteasome system to induce the degradation of target proteins without additional triggering factors, such as UV light or chemical reactions that can avoid the interference caused by external factors in target identification. With respect to target validation, PROTACs can achieve spatiotemporal and highly selective knockdown of the POI. In addition, POI degradation induced by PROTACs is reversible, which may provide an effective tool for conducting dynamic studies in the same research system. PROTACs are small molecules that make it easy for activity optimization and synthesis through pharmaceutical chemistry methods.

PROTACs are similar to conventional chemical probes. Use of PROTACs as chemical probes in target identification and validation require corresponding quality standards. Although some researchers have proposed referential quality standards for PROTAC probes, including physical and chemical properties, activity, selectivity, and control compound settings [110], the above parameters focus on target confirmation research. For the target identification of natural products based on TGDO, it is necessary to consider the actual research situation, increase the application cases of this technology, and objectively summarize the quality standards of this type of PROTAC probe.

In recent years artificial intelligence (AI) has undergone rapid development. To effectively guide the rational design of PROTACs, deepPROTACs have been proposed to predict and design PROTAC molecules with better degradation activity [111]. AI will also promote the rational design and application of natural product PROTACs, including the selection of derivative sites, determination of evaluation models, and extensive data analysis generated by testing. The PROTAC biochemical detection method developed based on proximity binding assays [112-114] may provide a high-throughput target identification and validation screening platform. PROTACs, as a probe, have shown promising tool properties and require long-term investment from more researchers.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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