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Review

Proteomic approaches advancing targeted protein degradation

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Targeted protein degradation (TPD) is an emerging modality for research and therapeutics. Most TPD approaches harness cellular ubiquitin-dependent proteolytic pathways. Proteolysis-targeting chimeras (PROTACs) and molecular glue (MG) degraders (MGDs) represent the most advanced TPD approaches, with some already used in clinical settings. Despite these advances, TPD still faces many challenges, pertaining to both the development of effective, selective, and tissue-penetrant degraders and understanding their mode of action. In this review, we focus on progress made in addressing these challenges. In particular, we discuss the utility and application of recent proteomic approaches as indispensable tools to enable insights into degrader development, including target engagement, degradation selectivity, efficacy, safety, and mode of action.

Harnessing the cellular proteolytic systems for TPD

TPD (see Glossary) eliminates proteins of interest (POIs), such as disease-causing proteins, from cells and tissues, by utilising the natural proteolytic pathways of the cell [1]. Degraders are designed to predominantly recruit an E3 ubiquitin ligase to a specific POI, resulting in the polyubiquitylation and subsequent degradation of the POI via either proteasomal or autophagy pathways (Figure 1). The current portfolio of small-molecule TPD modalities mainly uses two types of small molecule: **MGs** [2] and **PROTACs** [3]. Unlike small-molecule inhibitors, MGs and PROTACs eliminate both enzymatic and non-enzymatic functions of POIs and, hence, are attractive approaches against otherwise intractable targets [4]. Many different types of these modalities are used for TPD (Table 1). Significant progress has been made in the development and application of degraders and understanding the consequences of POI degradation. Ongoing research is aimed at improving degrader design, selectivity, potency, safety, and therapeutic potential, and understanding mode of action. Recent advancements in TPD techniques have accelerated these efforts. PROTACs have been successfully utilised for targeted degradation of the several POIs, co-opted mainly with the E3 ligases VHL (e.g., ACBI2 [5]) and CRBN (e.g., ARV-825 [6]). MGs attach primarily to an E3 ligase component, triggering a conformation change that allows for the recruitment of a neosubstrate [2]. The resulting proximity facilitates the ubiquitylation and degradation of the neosubstrate [7]. The smaller size of MGs makes them favourable for oral bioavailability compared with PROTACs due to their molecular properties, including compliance with Lipinski's Rule of Five for drugs [8,9]. The discovery of **thalidomide** and its derivatives as MGs that bind to CRBN and recruit neosubstrates for degradation has raised the profile of glues in this field [10]. Imide compounds have also been utilised as **E3 warheads** for PROTACs [11,12].

Beyond CRBN, only a few other E3 ligases have recently been identified as candidates for glue development, because systematic approaches for the discovery of novel MGs remain challenging [8]. Several groups have proposed novel approaches for glue discovery, such as the use of scalable chemical screening in hyponeddylated cells coupled to a multi-omics target deconvolution campaign [13], phenotypic screening of a covalent ligand library using chemoproteomics [14],

Highlights

Targeted protein degradation (TPD) is an exciting new modality for inducing target protein destruction in cells by harnessing cellular degradation pathways. However, to develop effective and selective degraders with clinical potential, there are many challenges, ranging from developing an effective, tissue-penetrant, and selective degrader to understanding its mode of action.

Integrating cutting-edge proteomic workflows during degrader development and application can overcome many challenges facing TPD.

Chemoproteomics aids degrader development and refinement through dissection of ligand–protein interactions, ligandability of targets, degrader–target engagement, and specificity of binding.

Quantitative proteomics aids TPD by delineating the specificity of protein-of-interest (POI) degradation, off-target effects of degraders, biological consequences of POI degradation, and mechanism of degradation.

Technological advances in instrumentation, sample preparation workflows, and data-processing power are poised to enable proteomics approaches to be more effective and accessible for TPD applications.

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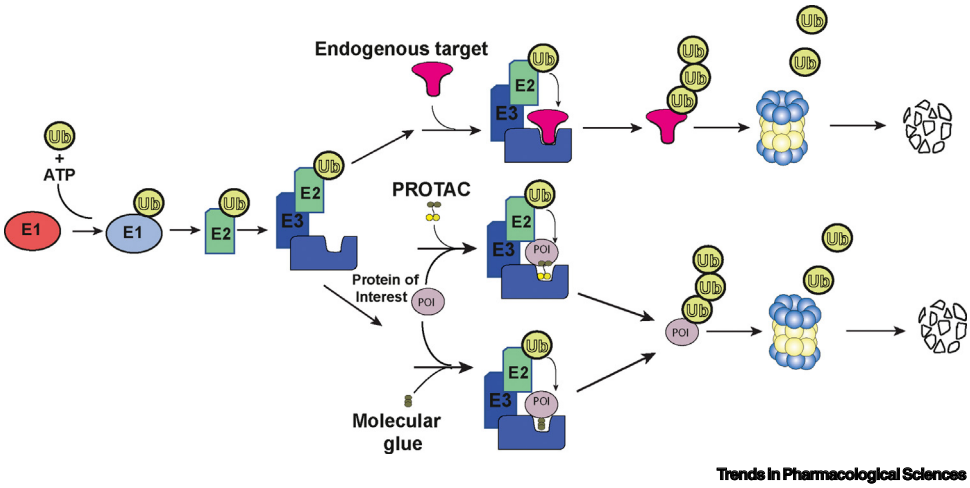

Figure 1. Overview of three mechanisms for protein degradation: natural ubiquitin-proteasome system (UPS), proteolysis-targeting chimeras (PROTACs), and molecular glue (MG)-mediated protein degradation. The natural UPS system involves ubiquitin tagging by E3 enzymes and subsequent proteasomal degradation. PROTACs utilise ligands to recruit E3 ligases for targeted protein degradation. MGs induce protein–protein interactions, leading to ubiquitination and degradation of target proteins. These strategies offer versatile approaches for regulating protein levels with potential therapeutic applications. Abbreviation: POI, protein of interest.

and by measuring CRL substrate receptor abundance following alterations of pharmacologically induced autodegradation [15]. Previously, most studies utilised conventional methods for degrader development and analysis, and often relied on serendipitous discoveries, as in the case of thalidomide. Conventional methods are often biased and low throughput in nature, providing limited insights into degrader efficacy, selectivity, and mode of action. Integrating proteomic workflows for degrader development and application can overcome these limitations. Advances in proteomic approaches have aided the process of designing and developing efficient degraders by identifying disease-associated protein targets, optimising degrader design, and improving efficacy and selectivity. Additionally, the integration of **artificial intelligence/machine learning (AI/ML)** techniques have helped TPD by efficient analysis of proteomic data, predicting ligand–protein and protein–protein interactions (PPIs), and facilitating the design of more effective degraders. In this review, we summarise the state-of-the-art in TPD, outline challenges, and highlight recent breakthroughs that are advancing the field. In particular, we highlight the role of cutting-edge proteomics technologies in addressing some of the challenges facing TPD.

Overview of the factors affecting the efficacy of TPD

Several factors affect TPD efficacy. Degrader chemical properties, pharmacokinetics (PK) and intracellular delivery, target and E3 ligase abundance, and their subcellular distribution in various biological settings are critical considerations.

The impact of degrader chemical properties on TPD

Target affinity, cellular permeability, compound stability, and linker design determine PROTAC or degrader success. For PROTACs, the linker length and flexibility determine the formation of the productive ternary complex and its spatial proximity for efficient ubiquitination and degradation of the POI [16]. Optimising linker composition, length, and attachment positions is vital for productive ternary complex formation. Advances in computational and structural analyses are key to understanding PROTAC ternary complexes and refining design approaches [17,18]. Systematic alterations of linker and monitoring cellular degradation activity, kinetics, ternary complex

Glossary

Absorption, distribution, metabolism, excretion, and toxicity (ADMET): properties required for an orally active drug that are important beyond its inherent biological activity.
Activity-based protein profiling (ABPP): identifies and confirms binding of small-molecule probes to target proteins. It uses activity-based probes and proteomics technologies to explore mechanisms of binding of compounds to proteins.
Artificial intelligence and machine learning (AI/ML): enables machines to extract knowledge from complex data, learn independently from it, and provide analysis.
Cellular thermal shift assay (CETSA): label-free chemoproteomics technology that measures the interaction of the chemical with its target protein in intact cells and tissues without modifications to the target protein.
Data-independent acquisition (DIA): global MS-based proteomics approach in which precursor ions are isolated into predefined isolation windows and fragmented. All fragmented ions in each window are then analysed on a high-resolution mass spectrometer.
Drug affinity-responsive targets stability (DARTS): proteomics approach used to identify potential protein targets for small molecules based on the principle that, upon drug binding, the target proteins become resistant to protease cleavage.
E3 warhead: targeting ligand for the E3 ligase.
Hydrogen deuterium exchange (HDX) MS: MS-based tool used to study protein conformation and dynamics, PPIs, and protein–ligand interactions.
Isothermal titration calorimetry (ITC): used to determine the thermodynamic parameters of interactions in solution.
Limited proteolysis coupled to mass spectrometry (LIP-MS): structural proteomics method for the analysis of complex proteome structural changes in response to small-molecule binding, based on the restricted proteolysis with sequence-unspecific proteinases.
Molecular glues (MGs): small-molecular degraders that induce or stabilize PPIs between an E3 ligase and a target protein to form a ternary complex,

Table 1. Different TPD modalities

Technology	Classification	Brief description	Refs
PROTAC/peptide PROTAC	Proteasome based	Small bivalent molecules that recruit E3 ligases to POIs for degradation via the UPS. For peptide-based PROTACs, peptides are used as binding moieties for POIs and E3 ligases	[3]
MGs/MGDs	Proteasome based	Small molecules that promote protein–protein interactions between an E3 ligase and POI, leading to its ubiquitination and subsequent degradation through the UPS	[2]
Hydrophobic tagging	Proteasome based	Involves introduction of hydrophobic moiety to target protein, rendering it prone to recognition and degradation by cellular protein quality control systems	[113]
BromoTag/dTAG	Proteasome based	POIs with FKBP12^{F36V} (dTAG) or Brd4BD2^{L387A} (BromoTag) inserted using CRISPR/Cas9 knock-ins can be degraded with PROTACs directed against these tags	[114,115]
Double mechanism degrader	Proteasome based	Many complicated diseases have more than one targets. In such cases, the degrader acts as both PROTAC and MG by altering the linker length	[116]
Affinity-directed protein missile (AdPROM)	Proteasome based	Comprises an E3 ligase or an E3 substrate receptor conjugated to a polypeptide binder (e.g., nanobody) that selectively recognises POIs in cells	[78]
PROTAB	Proteasome/lysosome based	Recruits E3 ligase, such as RNF43 and ZNRF3, to induce cell surface protein degradation via both lysosome and proteasome pathways	[117]
Lysosome-targeting chimeras (LYTACs)	Lysosome based	Small molecules that redirect POIs to the lysosome for degradation, by utilising cell surface receptor-mediated internalisation and lysosomal targeting signals	[118]
Autophagy-targeting chimeras (AUTAC)	Lysosome based	Utilise small molecules that recruit POIs for autophagic degradation, promoting their clearance via the autophagy-lysosomal pathway	[119]
AbTAC	Lysosome based	Target degradation of membrane-bound and cell surface proteins by using multi-specific binders that bind and recruit at least one transmembrane E3 ubiquitin ligase to the cell surface POI intended for degradation	[120]
GlueTAC	Lysosome based	Covalently engineered nanobody chimeras comprising a nanobody conjugated with cell-penetrating peptide and lysosome-sorting sequence (CPP-LSS); recognize membrane proteins and degrade through lysosomes	[1]
AUTOTAC	Lysosome based	Small molecules that target protein degradation through induced-proximity to autosomal cargo protein p62	[1]

leading to protein ubiquitination and subsequent proteasomal degradation.
Multiple reaction monitoring (MRM): targeted proteomics workflow (triple quadrupole MS) used for biomarker discovery.
Nuclear magnetic resonance (NMR): capable of detecting changes in the local electronic environment prompted by binding events, revealing the regions of a protein involved in a binding interface.
Parallel reaction monitoring (PRM): targeted proteomics workflow (high-resolution MS) used for biomarker discovery.
Proteolysis-targeting chimeras (PROTACs): heterobifunctional molecules comprising two ligands connected by a small chemical linker. Each ligand recruits an E3 ligase and a target protein simultaneously; the resulting proximity allows the E3 to ubiquitylate the target protein to trigger its degradation by the UPS.
Stable isotope labelling with amino acids in cell culture (SILAC): quantitative proteomics technique based on MS that detects differences in protein abundance among samples using non-radioactive isotopic labelling.
Surface plasmon resonance (SPR): used to measure interactions in real time with high sensitivity.
Targeted protein degradation (TPD): uses heterobifunctional small molecules to induce degradation of the target proteins in cells via proteasomal or lysosomal pathways.
Thalidomide: compound that acts as a MG by connecting the cereblon (CRBN) component of the ubiquitin E3 ligase complex with specific transcription factors in immune cells.
Ubiquitin-proteosome system (UPS): intracellular proteolytic mechanism that degrades ubiquitylated proteins via the 26S proteasome.

formation, and POI ubiquitination are necessary for the development of efficient degraders [19]. MGs are monomeric, linker-less small molecules and have more favourable physicochemical properties compared with PROTACs [13].

The impact of ligand–protein binding affinity on degrader efficacy

PROTACs need to bind both the POI and E3 ubiquitin ligase to promote POI degradation. Thus, the binding affinity of a PROTAC to the POI and E3 ligase may affect its efficacy. PROTAC activity

correlates with its binding affinity to the POI and E3 ligase [20,21]. However, steric hindrance or competition with other cellular proteins can impede PROTAC function if the binding affinities are too high [22–24]. ARD-266 is a strong small-molecule androgen receptor (AR) degrader that successfully degrades AR from prostate cancer cell lines despite its limited VHL binding affinity [25]. Achieving selective POI degradation often necessitates meticulous fine-tuning of the chemical properties of the PROTAC [26,27]. The use of promiscuous kinase ligands in PROTACs has revealed some unexpected findings of the role of ligand–protein affinity in determining the extent of PROTAC-mediated degradation [24]. Foretinib, which binds to over 130 kinases, was used in CRBN- and VHL-recruiting PROTACs; the degradation profiles revealed that even low-affinity interactors of foretinib, such as the p38 MAPK, were degraded efficiently [24]. Interestingly, for some targets that foretinib is known to bind to with high affinity, no degradation was apparent [24]. Ultimately, productive ternary complexes in a cellular environment between the E3 and the target determine whether degradation can occur, and *in vitro* target affinity profiles alone do not predict PROTAC efficacy. MGs stabilise or induce E3–protein interactions by forming ternary complexes. MGs frequently bind to their target proteins with modest or even undetectable affinities, while enhanced affinities are usually witnessed with regulatory proteins [28]. Chemoproteomics workflows [29] and structural mass spectrometry (MS) [30,31] have been used to monitor ligand–protein binding.

Impact of degrader PK properties and intracellular penetrance on TPD

Degrader PK and intracellular abundance can dramatically impact TPD. TPD requires understanding of PROTAC/MGD absorption, distribution, metabolism, and excretion. A degrader must degrade its target protein in the intended subcellular compartment at optimal concentrations and for a long duration [32]. Molecular size, lipophilicity, charge, and stability affect PROTAC/MGD PK [33]. PROTAC/MGD effectiveness requires intracellular distribution by passive diffusion, endocytosis, or receptor-mediated internalisation and transport to particular subcellular compartments. The absorption, distribution, metabolism, and excretion of the degrader affect its availability and activity in the body. As such, several approaches using nanoparticles or aptamers for improved delivery of the PROTACs into cells have been explored [32,34–37]. MGs have pharmacological characteristics superior to those of PROTACs [8]. Integrating proteomics workflows in **absorption, distribution, metabolism, excretion, and toxicity (ADMET)** investigations could provide a holistic view and help identify biomarkers of degrader efficacy and toxicity to improve degrader design [38]. As proteomics technologies evolve, they might help uncover degrader behaviour in living systems, further improving the development of novel therapeutic degraders.

Abundance of E3 ligase and its impact on TPD

The abundance and activity of the E3 ligase to be co-opted in the tissue where the POI is expressed must be considered while developing degraders. Targeted degradation efficacy and specificity depend on the abundance of the target E3 ligase [24]. For example, if a specific E3 ligase is abundant in one tissue but not in another, the efficacy of the protein degradation technique may differ depending on the tissue targeted [15,39]. This knowledge directs the selection of appropriate E3 ligases and aids the mitigation of off-target effects, ultimately improving the efficiency and safety of the degrading strategy. Similarly, the tissue-specific abundance of the POI is important to consider because it can minimise unexpected consequences of POI degradation from unwanted tissues [39]. The abundant and active tissue-specific E3 ligases can be utilised in developing PROTACs by developing and optimising ligands for these E3s [40]. Proteomics technologies coupled with subcellular fractionation [41] or immunoprecipitation techniques [42] will allow robust mapping of POIs and E3s in tissues and subcellular organelles.

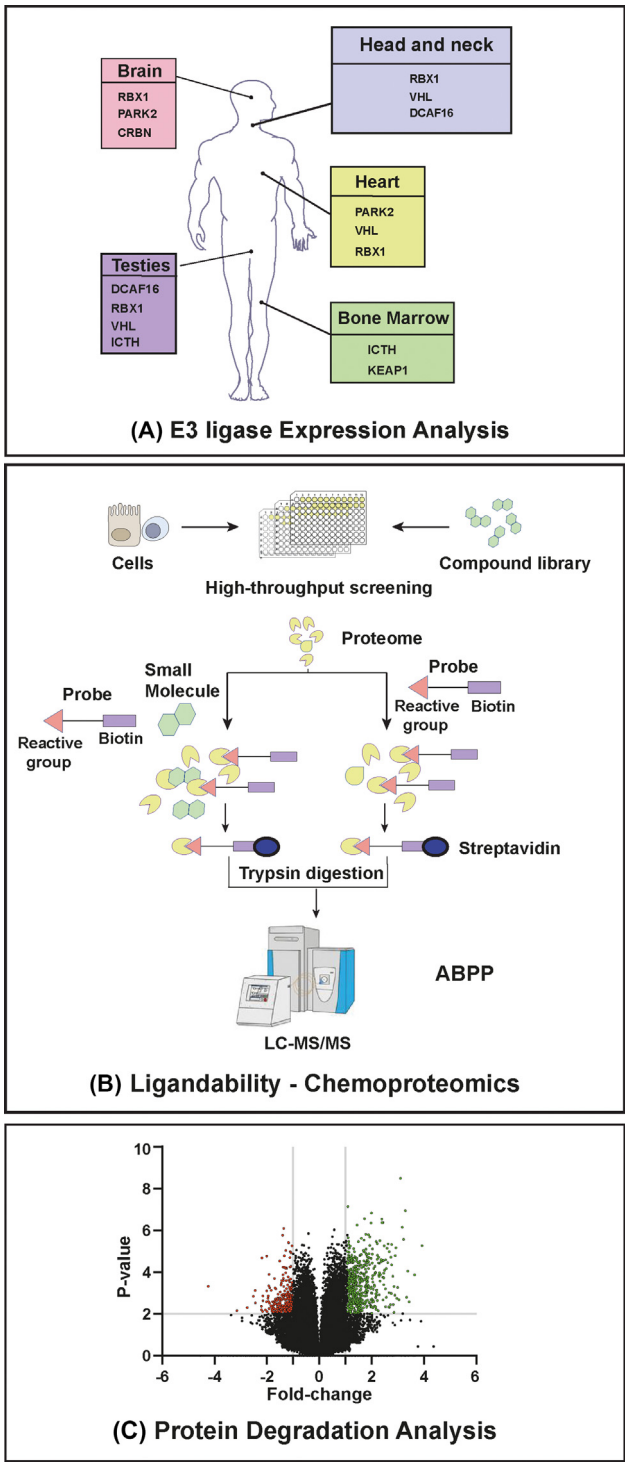

Figure 2. Examples of how proteomics approaches can aid TPD. (A) Illustration of the human body, emphasizing E3 ligases that exhibit enhanced tissue specificities. These E3 ligases can be utilized by targeted protein degradation (TPD) approaches to facilitate the selective targeting of disease-causing proteins in specific tissues and cell types. (B) Ligandability screening using chemo-proteomics platforms to harness these E3 ligases for designing proteolysis-targeting chimeras (PROTACs) and molecular glue degraders (MGDs). Activity-based protein profiling (ABPP) probes are composed of a covalent warhead and a biotin enrichment tag, making them versatile tools for ligand discovery. They can be employed in competitive assays against libraries of covalent fragments to pinpoint potential ligands for target proteins. The binding interactions with these fragments can be directly assessed and quantified using mass spectrometry readouts. (C) Unbiased protein degradation analysis by using proteomics to study the consequences of the degradation of the protein of interest (POI). The figure highlights the importance of understanding E3 ligases in protein degradation and the utility of proteomics techniques in TPD.

Impact of the target protein context on TPD

POIs resident in various organelles or subcellular compartments within a cell are degraded differently by the same degrader [43,44]. Most proteins exist in distinct multiprotein complexes that may determine their activity or subcellular distribution. Recruitment of a specific protein by the degrader may inadvertently also degrade other components of the multiprotein complex or, in other cases, the nature of the complex may prevent the degradation of the target itself. Lenalidomide and pomalidomide-induced recruitment of CK1α was shown to degrade its interactor FAM83F [45]. As such, the ability to degrade specific protein complexes with degraders could be beneficial in therapeutics by allowing targeting of specific functions of a multifunctional protein without affecting its other functions. Multi-pass plasma membrane proteins and those resident in some organelles may need endocytosis and trafficking to lysosomes for degradation and may be ineffective candidates for degradation via the **ubiquitin-proteosome system (UPS)**. In these cases, factors affecting endocytosis, vesicle formation, and cytoskeletal transport may affect TPD efficacy.

Integrating proteomics in the development and application of TPD

Integrating cutting-edge proteomics tools within the development pipeline can aid TPD development and application (Figure 2). Here, we discuss some of the proteomic approaches that have been harnessed in TPD workflows.

Protein abundance profiling

Current approaches for detecting POIs and E3 ligases heavily rely on biochemical techniques, such as immunoblotting and ELISA, that necessitate prior generation and characterisation of antibodies against preselected proteins [46]. Proteomic analysis allows unbiased discovery of all TDP players and pathways. MS-based proteomics identifies and quantifies thousands of proteins from complex biological samples in a single experiment [47]. The sensitivity, speed, and precision of MS enables rapid abundance profiling of thousands of proteins in a short time [48]. **Data-independent acquisition (DIA)** approaches [49] and deep neural network tools [50] have improved proteome analysis precision and throughput. Quantitative proteomics of diseased and healthy biological samples can reveal protein abundance, localisation, modifications, and interactions. These outputs can identify potential dysregulated or disease-associated proteins [51], allowing for target and/or E3 selection for TPD. This knowledge may allow AI/ML algorithms to match target proteins with appropriate E3 ligases based on their expression profiles and biological features. Targeting tissue-specific E3s allows more selective and effective degradation of targets. The expression of commonly used E3 ligases is depicted in Figure 3. Generating the expression patterns of E3 ligases and POIs could help select tissue-specific E3 ligases or POIs for degrader development to achieve selective target degradation. TPD relies on subcellular availability of the E3 ligases with the POI for efficient degradation [44].

Subcellular fractionation before MS can reveal resident E3 ligases and POIs in a specific subcellular compartment [41]. To enhance TPD, the objective would be to develop ligands or degraders that can recruit E3 ligases to specific degradation compartments matching with the subcellular distribution of the POIs. Efficient TPD is achieved when the POI is degraded with a rate that is superior to its natural turnover rate. Utilising proteomics-based measurements of protein turnover rates, such as **stable isotope labelling with amino acids in cell culture (SILAC)**, can assist the optimisation of TPD strategies, for example in neuron-enriched and glia-enriched primary hippocampal cultures [52]. The half-lives of short-lived proteins are studied by combining MS-based proteomics with cycloheximide chase assays [53]. Recently, by using surface protein markers, antibody–PROTAC conjugates were used for tissue-specific delivery of PROTACs to degrade BRD4 from HER2-positive tumours [54]. Integrating proteomics to decipher the surface proteome of different target

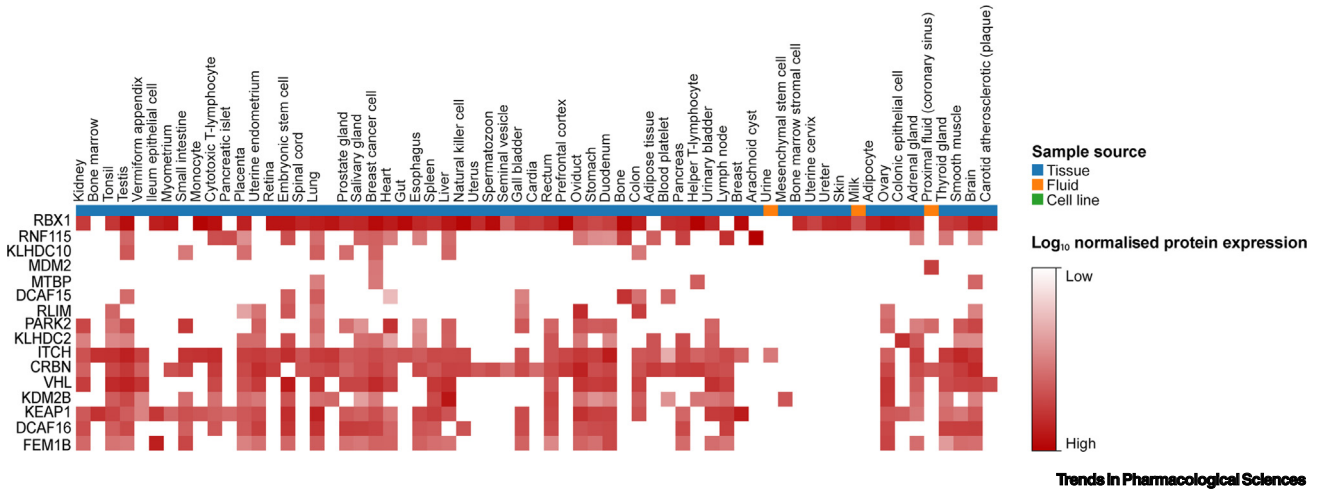

Figure 3. Heatmap of the most commonly used E3 ligases in targeted protein degradation across tissues and body fluids. This heatmap was generated from proteomicsdb (www.proteomicsdb.org/) with gene symbols indicating the abundance of the E3 ligases.

tissues and combining with antibody–PROTAC conjugates has the potential to improve tissue-specific delivery of PROTACs and degraders. This method, which is underutilised in TPD, might allow for personalised medicine and disease-associated cell targeting.

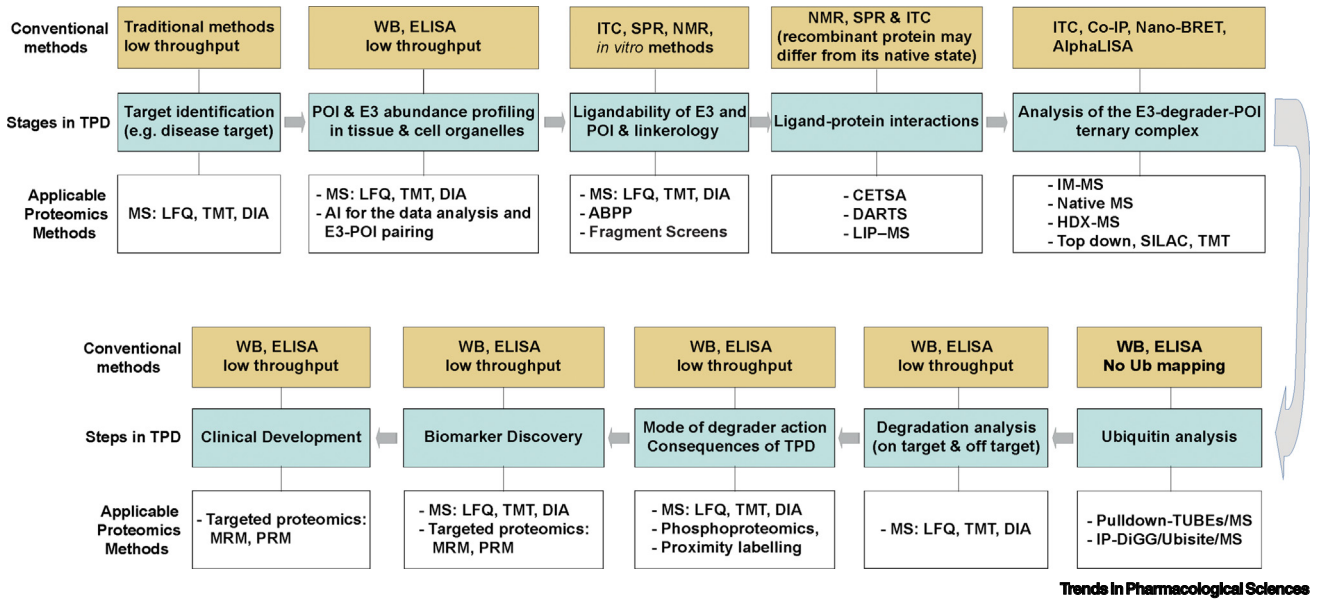

Figure 4. Proteomic and conventional approaches used in targeted protein degradation (TPD). The chart highlights the steps involved in TPD and conventional approaches used in each step. The different proteomic approaches that can be applied, either in conjunction or instead of the conventional approaches, are also highlighted for each step. Abbreviations: ABPP, activity-based protein profiling; AI, artificial intelligence; AlphaLISA, bead-based luminescent amplification assay; CETSA, cellular thermal shift assay; Co-IP, co-immunoprecipitation; DARTS, drug affinity-responsive target stability; DIA, data-independent acquisition; E3, E3 ubiquitin ligase; HDX, hydrogen deuterium exchange; IM-MS, ion mobility mass spectrometry; IP-DiGG/UbSite, immunoprecipitation enrichment of ubiquitinated peptides by antibodies targeted against diGG remnant or C-terminal fragment of ubiquitin; ITC, **isothermal titration calorimetry**; LFQ, label-free quantification; LIP-MS, **limited proteolysis coupled to mass spectrometry**; MRM, **multiple reaction monitoring**; MS, mass spectrometry; Nano-BRET, bioluminescence resonance energy transfer-based assay; NMR, **nuclear magnetic resonance**; POI, protein of interest; PRM, **parallel reaction monitoring**; SPR, surface plasmon resonance; TMT, tandem mass tag; TUBEs, tandem ubiquitin binding entities; WB, western blot.

Chemoproteomics for ligand screening and development

The scarcity of E3 ligase ligands and existing undrugged proteins severely impedes progress in TPD [55]. Traditional approaches to ligand identification focus on recombinant proteins to screen for small molecules that target structural regions or biochemical activity, such as **surface plasmon resonance (SPR)** [21] and fluorescence polarisation [23] (Figure 4). These methods assume that the recombinant protein retains physiological structure and function. Chemoproteomics characterises ligand–protein interactions in intact cells and can inform off-target binding on a proteome scale [56]. Drug-affinity enrichment followed by MS can identify actionable binding sites in a natural protein setting to enable optimisation of efficient small-molecule ligands [57]. Thus, chemoproteomic ligand screening can illuminate small molecule–protein binding affinities, selectivity, and mechanisms [29] (Figure 4). Affinity enrichment is used to identify bioactive compounds by derivatising them with an affinity tag, such as biotin or a biorthogonal reactive group to enable immobilisation on inert beads. Affinity enrichment isolates functional probe-interacting proteins from cellular proteins [58]. Photoaffinity labelling (PAL) using photoreactive molecules converts small-molecule probe–target protein interactions into covalent bonds [59]. These covalent bonds allow probing for target engagement in live cells instead of only cell extracts, because non-covalent interactions tend to re-equilibrate during sample preparation for further analysis. As with any affinity enrichment approaches, considerations should be given for potential binders of chemical linkers, reactive groups, affinity tags, and bead matrices, and contaminating proteins should be excluded from proteomic analyses [60]. Comparative experimental investigation with probes comprising active and inactive epimers of the chemical matter can distinguish true hits from contaminants [61].

Activity-based probe profiling (ABPP) is a viable option when compound-specific probes are unavailable or have too low affinity for the target to allow enrichment. Active site-guided probes measure enzyme activity in complex biological systems [62]. These probes help ABPP identify and characterise active enzymes in their natural environment. ABPP is used widely for determination of enzyme activities in biological processes [63,64] and can reveal covalent ligand reactivity and selectivity [65,66]. ABPP workflows have been utilised for the monitoring of Parkin E3 ligase activity in Parkinson's disease [67] and for identification of the neuron-associated E3 ligase MYCBP2 RING-linked E3 ligase with esterification activity and intrinsic selectivity for ubiquitination of threonine residues [68]. Integrating cysteine or lysine reactive covalent probes followed by MS-based proteomics can also detect proteins bound by the ligands [69,70]. Such information on ligandable sites on target proteins and E3 ligases can prove useful in developing ligands. Several E3 ligases with their ligands have been co-opted for PROTAC development (Table 2).

Chemo-selective reactions are used in click chemistry to attach chemical matter covalently onto proteins, thereby facilitating discovery of covalent ligands and the characterisation of target proteins [71]. ABPP-based chemoproteomic platforms have been used to identify reactive cysteine residues on several protein targets in pancreatic [72] and breast cancers [73]. ABPPs utilise data-dependant acquisition modes, which requires the use of expensive isotopically encoded quantification reagents. By contrast, DIA-ABPP approaches combine ABPP with DIA MS for high-throughput measurement of the functional cysteinome, and screening of ligandable cysteines with a covalent fragment library. This paves the way for more in-depth and multidimensional profiling of functional proteomes and interactions with bioactive small compounds.

The recently developed streamlined cysteine **activity-based protein profiling** (SLC-ABPP) method improves sample throughput by developing a low-molecular-weight desthiobiotin iodoacetamide probe, multiplexing with tandem mass tag (TMT), and using real-time database search (RTS) [74]. This approach is useful for screening bigger compound libraries more rapidly

Table 2. E3 ligases commonly used in TPD

E3 ligase	Description	Recruiter	Reactive cysteine	Refs
Mouse double minute 2 homolog (MDM2)	E3 ligase ligand that displaces interaction between MDM2 and tumor suppressor p53; used in a PROTAC to recruit MDM2 to degrade the AR	Nutilin	N/A	[121]
VHL	AdPROM section; acts as substrate receptor for Cul2-Rbx1 E3 ligase complex, recruiting HIF1α for its ubiquitination and degradation	VH032	C77	[33]
CRBN	Substrate receptor for the Cullin-RING E3 ubiquitin ligase 4 (CRL4) containing DDB1, CUL4, and RBX1. Recognizes neosubstrates upon binding to thalidomide and other immunomodulatory drugs (IMiDs)	Thalidomide and other IMiD analogs	C188, C287	[121]
Cellular inhibitor of apoptosis protein (cIAP1)	Degrades caspase proteins; methyl bestatin (MeBS) binds to cIAP1 and promotes autoubiquitylation, facilitating its proteasomal degradation	MeBS	N/A	[121]
RNF4	E3 ligase that ubiquitinates SUMOylated proteins for proteasomal degradation; small-molecule cysteine-reactive covalent RNF4 recruiter discovered using activity-based protein profiling (ABPP)-based covalent ligand screens	CCW 16	C132/C135	[121]
RNF114	Covalent RNF114 recruiter derived from the anti-cancer natural product nimbolide; reacts with intrinsically disordered N-terminal cysteine 8 of RNF114	Nimbolide	C8	[121]
DDB1 and CUL4 associated factor 16 (DCAF16)	E3 ligase recruiter; covalent DCAF16 recruiter discovered using cellular covalent PROTAC screening strategy coupled with downstream chemoproteomic target identification	KB02	C177 or C179	[121]
DCAF15	Forms complex with sulfonamide derivatives and coactivator RBM39, leading to induced ubiquitination and proteasomal degradation of RBM39	E7820, sulfonamides	C460	[121]
Kelch-like ECH-associated protein 1 (KEAP1)	E3 ligase recruiter; triterpene derivative bardoxolone methyl (CDDO-Me) activates antioxidant NRF2 pathway by targeting KEAP1	CDDO-Me	C151	[121]
FEM1B	A CUL2 E3 ligase; critical regulator of cellular response to persistent depletion of reactive oxygen species, a condition referred to as reductive stress	EN106	C186	[121]

and with greater depth [75]. Multidimensional proteomic analysis of targeted electrophilic compound libraries can find chemical probes with site-specific functional effects on human cell protein complexes [76]. Affinity-based MS methods are becoming more routine in TPD, with the adoption of chemoproteomics for ligand screening increasing rapidly over time. Improvements in multiplexing methods and DIA can help expedite degrader development and substantially enhance our understanding of cellular processes and protein functions. It is also essential to be able to assess and curate these methods and understand how the data are influenced by many variables, such as the quality of the MS data, chemical ligands, and biological systems, and the robustness of the bioinformatics tools used for data interpretation.

Degradation analysis

Conventional methods focus on the abundance of only the POI for degradation analysis. However, these methods fail to provide an unbiased appraisal of the specificity of target degradation. Proteomics can quantify degraded target proteins, degradation intermediates, target protein degradation dynamics, and biological pathway impacts. TMT-based isobaric proteomics can be used to study TPD specificity. Isobaric labelling with TMT provides multiplexed quantitative proteome analysis for sample comparison [77]. This approach identifies degrader off-target effects and consequences of TPD. TMT-based proteomics has been applied to study the specificity of targeted degradation of K-RAS [78], SGK-3 [79], and LRRK2 [80]. These studies identified not only on-target degradation of the POI, but also other proteome changes, including consequences of POI degradation on downstream signalling. Multiplexing features of these workflows have been utilised in a high-throughput manner where the selectivity of five degraders targeting BRD/BET, FAK, ALK, and BTK kinases was assessed in a single TMT-MS analysis [81]. Although

the accuracy of TMT is limited by reporter ion ratio distortions brought on by co-isolated fragmentation interfering species, this can be overcome by deep fractionation as well as multiple frequency notches for the synchronous precursor selection (SPS)-based MS3 method [82].

Global ubiquitinome profiling is a powerful tool for studying TPD [83]. Analysing globally ubiquitinated proteins in cells or tissues can reveal the mechanism of protein turnover and TPD specificity. Tandem-repeated ubiquitin-binding entities (TUBEs) immunoprecipitate cellular proteins [84]. MS-based proteomics can then identify these proteins or their interactions [85]. Enrichment using TUBEs is a popular approach for ubiquitination analysis, but it is difficult to identify ubiquitylated sites and contaminating proteins co-eluted in immunoprecipitation. Peptide-based antibody immunoprecipitation using the diGly motif [86] and UbiSite [87] antibodies has proven popular for mapping global ubiquitination sites. TMT tags improve analytical throughput [88]. Recently, a sensitive method integrating diGly antibody-based enrichment optimised Orbitrap-based DIA and extensive spectral libraries identified over 90 000 diGly peptides [86]. Neural network-based data processing for DIA-MS analysis speeds up drug mode-of-action profiling [50,89]. The degrader-induced alterations on the POI may be determined by a global ubiquitinome analysis, although it is currently not adopted by most TPD workflows. TPD can utilise these new tools to compare control and degrader-treated ubiquitinome profiles to uncover the degradation mode of action.

We have discussed the various stages of degrader development and the MOA involved in TPD. We have also highlighted the challenges and conventional approaches used to resolve this, along with their limitations. For each stage, we have highlighted the different proteomic approaches that can be applied, either in conjunction with or instead of the conventional approaches, to tackle these limitations (Figure 4).

Proteomics methodologies to study the degrader-target interactions and downstream signalling

The proteomics methods discussed above rely on functionalised small molecules. However, proteomics methods can now detect novel small molecule–protein interactions. These methods use ligand-induced biophysical changes on target proteins, such as thermal stability [90], proteolysis resistance [91], or amino acid side chain exposure under chaotropic conditions [92]. These methods combined with quantitative MS enable proteome-wide small molecule–protein interaction measurements (Figure 5).

Thermal protein stability profiling

Binding of degrader molecules to cellular proteins is needed to understand the specificity of engagement with the target protein. **Cellular thermal shift assays (CETSAs)** are useful for studying the interaction of small molecules, such as PROTACs and MGs, with their target proteins. The basis of CETSA is exposing cell or tissue extracts to different temperatures, leading to denaturation and aggregation of the unfolded proteins. Compound-bound proteins are more stable and need more energy to denature. CETSAs quantify soluble proteins at each temperature using high-resolution MS, creating denaturation curves. This determines thermal stability and ligand-induced shifts [93]. Initial CETSA protocols were limited to detecting solely soluble intracellular targets, but recent methods have expanded analysis to membrane proteins [94]. Thermal changes depend on POI structure, chemical concentration, and affinity. This creates two major issues. First, ligands binding closely to big, multidomain proteins may not change thermal stability, resulting in false negatives [95]. Second, most proteins melt at 40–75°C, but some need higher temperatures [96]. 2D thermal proteome profiling (2D-TPP) has been developed to overcome these limitations. This new approach combines temperature-dependent and isothermal ligand concentration-dependent experiments into a single analytical scheme [97]. CETSA MS with

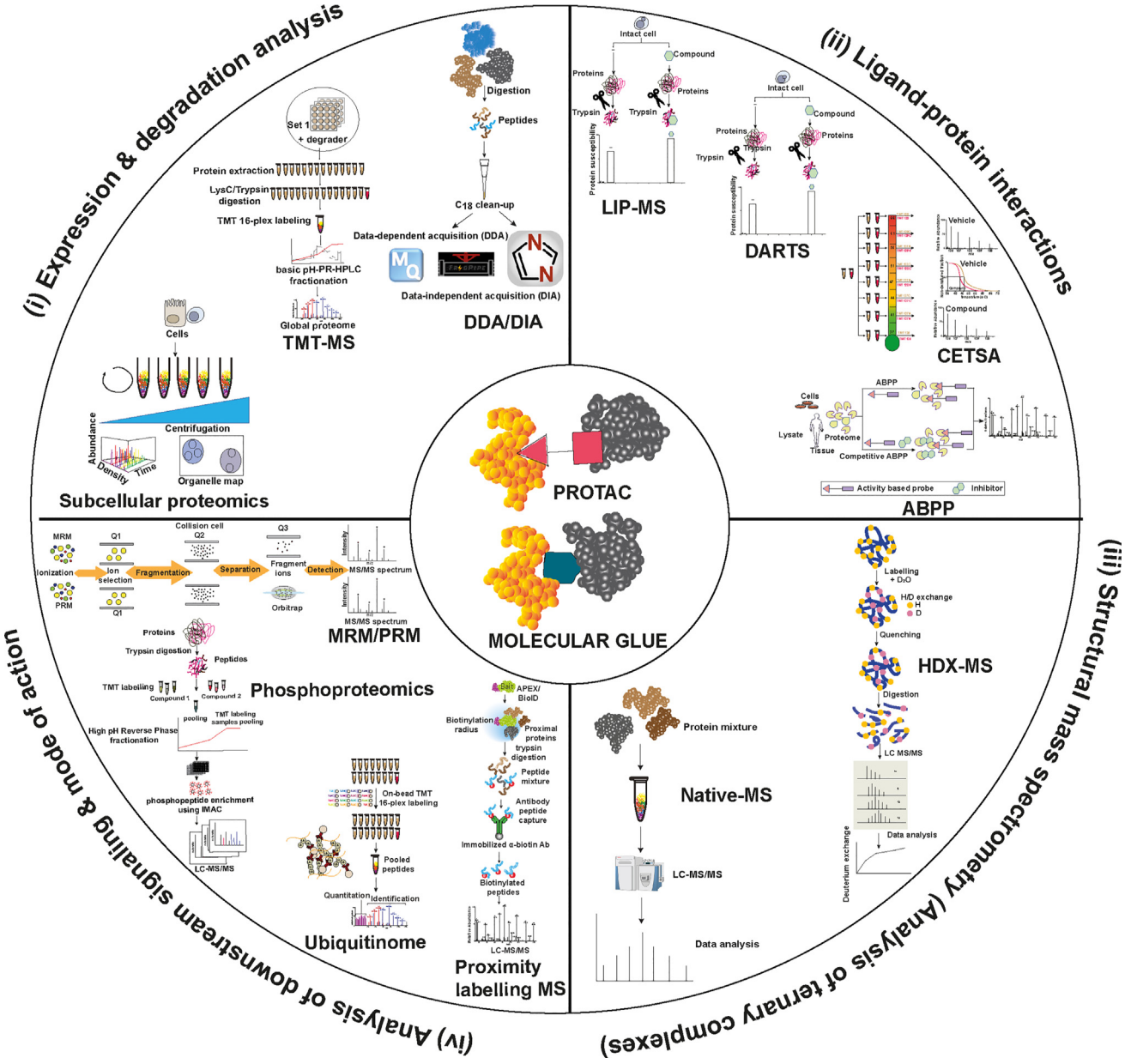

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Figure 5. Comprehensive proteomics technologies for advancing targeted protein degradation (TPD). The segregation is based on the specific steps and areas of TPD. (i) Expression and degradation analysis: approaches include data-dependent acquisition (DDA) and label-free data-independent acquisition (DIA) analyses for expression across the samples. Tandem mass tag (TMT) mass spectrometry (MS) is used for accurate quantification across samples in a multiplexing format. Subcellular proteomics is used to understand spatial expression of E3s and proteins of interest (POIs) across cell organelles. (ii) Ligand–protein interactions: MS methods include drug affinity-responsive target stability (DARTS) and limited proteolysis (LIP) for identification of compound binding to POI, thermal protein profiling (cellular thermal shift assay; CETSA) for assessing the binding of the compound to POI, and activity-based protein profiling (ABPP) for identifying active E3 ligase and the ligandable sites. (iii) Structural mass spectrometry (MS): methods used to study ternary complexes includes native MS and hydrogen deuterium exchange (HDX)-MS. (iv) Analysis of downstream signalling and mode of action: MS methods include targeted proteomic analysis using multiple reaction monitoring (MRM)/parallel reaction monitoring (PRM), phosphoproteomic analysis for investigating downstream signalling events, ubiquitinome analysis for identifying ubiquitination sites modified by degraders, and proximity labelling-based MS methods for understanding degrader-induced protein–protein interactions.

quantitative proteomics was used to study target and degradation specificity of several IMiDs in intact cells and cell lysates [98]. The capacity of CETSA to reveal small molecule–target protein interactions and degradation of proteins is not yet widely used in TPD. CETSA requires skill and multiple days of MS time for a single experiment. Nevertheless, TPD research may optimise CETSA or its variants for protein degradation studies, and its refinement, accessibility, and standardisation may boost its use and adoption.

Drug affinity responsive target stability and limited proteolysis-coupled mass spectrometry

Drug affinity-responsive target stability (DARTS) has been used to directly study ligand–protein interactions [91]. DARTS comprises proteolytic enzyme treatment of cell lysates with and without drug molecules and restricted protein digestion. SDS-PAGE and staining identify proteolysis-resistant protein bands, which are analysed by MS [99]. DARTS has been successfully used to identify the binding of small molecules with their target proteins [100]. It has become useful to identify MGs by taking advantage of the capacity of small molecules to maintain PPIs. DARTS can aid understanding of molecular interactions and make it easier to create targeted therapeutics by identifying the regions of proteins that interact with small molecules and may help maintain the stability of protein complexes.

Limited proteolysis-coupled MS (LIP-MS) has been used for the identification of structural features of the purified proteins [101]. A broad-specificity protease is applied to the lysate in non-denaturing conditions with or without the small molecule. The bigger fragments are then cleaved with trypsin and analysed by MS. Small molecules binding to proteins change their proteolysis susceptibility, which is reflected in MS analysis [102]. The ligand-binding peptide sequence can be mapped onto the protein structure to reveal topological conformational changes. This technique can identify pharmacological targets in complex proteome combinations using machine learning.

Despite their widespread use in drug research, it is surprising that neither of these approaches have been adopted extensively in TPD.

Proximity labelling

Strong PPIs in extracts are detected using immunoprecipitation (IP) or other affinity purification approaches followed by MS. Cell-based tools, such as yeast two-hybrid systems and fluorescent colocalisation microscopy, can also capture PPIs. To capture weak or transient interactions, cross-linking MS approaches can be applied, although such assays inevitably introduce false positives. Furthermore, conventional IP/MS approaches are unable to establish interactions with proteins that are resident in complex cellular environments, such as membranes and some organelles [103]. Recent developments in proximity-labelling followed by MS can capture PPIs in live cells. Typically, the POI is tagged with an enzyme, such as biotin ligase, which modifies the interacting protein when brought into proximity to it. The modified proteins can be enriched and identified by MS. Examples of proximity-labelling techniques include BioID, TurboID, and APEX2 [104]. In TPD, such approaches have been used to identify POI–E3 ligase interactions induced by PROTACs and MGs [105]. Proximity labelling methods will be very useful for TPD in establishing PPIs induced by degraders.

Structural mass spectrometry

The determination of the POI–degrader–ligase ternary complex can reveal the mechanism of action involved. Native MS is a powerful tool that was used to analyse PROTAC GNE-987-induced ternary complexes between Brd4 bromodomains 1 and 2 and VHL [30]. Native MS requires a very low amount of input sample and can screen large number of samples rapidly.

Hydrogen deuterium exchange (HDX) MS can reveal structural insights into PPIs. HDX exchanges hydrogens for deuterium atoms in the protein backbone amide moiety in solution and MS can quantify deuterium uptake over time. PPIs that reduce solvent exposure shield deuterium absorption. HDX-MS was used to identify the degrader-induced PPI interface for the characterisation of the BRD4 degraders CFT-1297 and dBET6, which had distinct deuterium uptake patterns [31].

Proteomics for analysis of PTMs and consequences of TPD

Most intracellular signal transduction pathways are controlled through post-translational modifications (PTMs) on proteins. Any stable PTM-modified peptide can be detected by MS. For degrader-induced changes, beyond analysis of ubiquitination and neddylation of proteins, other global changes in PTMs, such as phosphorylation, methylation, and acetylation, that could result from TPD can be determined by MS. Phospho-proteomic analysis could reveal motifs for recruitment of certain E3 ubiquitin ligases [106] as well as signalling pathways that are affected by POI degradation [107]. Acetylation has been reported to lead to degradation of some transcriptional regulators and there is a competitive interplay between stabilising acetylation and destabilising ubiquitination [108]. The workflows for identification of most PTMs usually include enrichment steps using matrices with appropriate physical properties [77] or enrichment with antibodies recognising specific PTMs [109]. Proteomics workflows have been instrumental in revealing the molecular consequences of TPD; for example, the cytotoxicity caused by BRD4-L degraders was revealed to be a consequence of the accumulation of toxic proteolysis peptide products resulting from a constitutive BRD4-L proteasomal degradation [110].

AI/ML in the development of the TPD

AI in drug research and development has grown exponentially due to the availability of high-quality biological data [111]. Large storage systems and graphics processing units (GPUs) have made parallel computing widespread, accelerating computationally heavy operations. AI, particularly ML, uses mathematical algorithms to find complicated correlations within existing information and reliably predicts outcomes for new samples. Traditional approaches rely on explicit physical models [112]. Big proteome data will also benefit from AI/ML. AI/ML algorithms can process tissue-specific expression profiling of POIs and E3 ligases, their pairing based on subcellular localisation, ligandability, and degradation analysis, and help with every element of TPD.

Concluding remarks and future perspectives

Despite numerous challenges at every stage of the degrader development process (see Outstanding questions), the field of TPD has seen significant recent advances, from development of potent degraders and their application in cellular and tissue contexts to their use in clinical settings. The incorporation of state-of-the-art proteomic methodologies has been at the forefront of recent advances in TPD and holds enormous potential to enhance every facet of the degrader development and application process. This ranges from the initial stages of ligand conception and refinement, all the way to unravelling the intricacies of kinetics and mechanisms governing the degradation of POI within cellular and tissue environments. Although proteomics lags behind in terms of sensitivity and coverage compared with DNA/RNA-sequencing technologies, the current pace of technological revolution in the instrument sensitivity and efficiency of mass spectrometers as well as data-processing power are making proteomics an indispensable tool for TPD. Advances, such as single-cell proteomics, are poised to reveal cellular response heterogeneity and potentially contribute to personalised degrader therapies. Proteomics will remain instrumental in unveiling the dynamic proteome landscape, guiding rational drug design and clinical adoption of more targeted and potent next-generation degraders. Although proteomics is a powerful tool for TPD research, it is currently underutilised in TPD workflows. Lack of technical skills, robust

Outstanding questions

What are the key factors that affect the efficacy of degraders?

What new E3 ligases can be co-opted for TPD modalities?

Does the POI and E3 cellular, subcellular, and tissue context matter in TPD? If so, how do we develop degraders that are effective in specific contexts?

How can we improve the selectivity and efficacy of degraders?

How do we dissect the precise molecular mechanisms by which degraders lead to POI proteolysis?

Are there consequences for the natural substrates of E3s when degraders redirect the E3s to neosubstrates?

Can cutting-edge proteomic tools address many of the TPS challenges highlighted above?

bioinformatics pipelines, and computational tools for massive data processing, and high hardware and software expenses contribute to this low adoption rate. Nevertheless, improved instrumentation, sample preparation procedures, and data-processing tools are making proteomics methods in TPD more accessible.

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Declaration of interests

None declared by authors.

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