Acta Pharmacologica Sinica

REVIEW ARTICLE

Proteomic characterization of post-translational modifications in drug discovery

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Protein post-translational modifications (PTMs), which are usually enzymatically catalyzed, are major regulators of protein activity and involved in almost all cellular processes. Dysregulation of PTMs is associated with various types of diseases. Therefore, PTM regulatory enzymes represent as an attractive and important class of targets in drug research and development. Inhibitors against kinases, methyltransferases, deacetyltransferases, ubiquitin ligases have achieved remarkable success in clinical application. Mass spectrometry-based proteomics technologies serve as a powerful approach for system-wide characterization of PTMs, which facilitates the identification of drug targets, elucidation of the mechanisms of action of drugs, and discovery of biomakers in personalized therapy. In this review, we summarize recent advances of proteomics-based studies on PTM targeting drugs and discuss how proteomics strategies facilicate drug target identification, mechanism elucidation, and new therapy development in precision medicine.

Keywords: proteomics; protein post-translational modification; drug target; off-target; drug mechanism; precision medicine

Acta Pharmacologica Sinica (2022) 43:3112–3129; https://doi.org/10.1038/s41401-022-01017-y

INTRODUCTION

Covalent modification on the amino acid side chain of a protein, also known as post-translational modification (PTM), is a major molecular mechanism to regulate protein activity and function after its biosynthesis. PTMs, such as phosphorylation, acetylation, ubiquitination, play fundamental roles in a great variety of biological processes, such as cellular signaling transduction, protein homeostasis, epigenetic regulation [1–4]. Dysregulation of PTMs is associated with many different types of diseases, such as cancer [5, 6], neurological disorders [7]. So far, there are more than 400 types of literature documented PTMs, which represents the most efficient way to expand the diversity of cellular proteome. Adding or removal of a PTM group on a protein is generally regulated by enzymes, such as kinases, transferases, and ligases. Therefore, targeting PTM enzymes has become increasingly important for drug development. Remarkable success has been achieved for the development of drugs targeting protein kinases [8], histone deacetylases [9], and ubiquitin ligases [10]. As a role model, kinase inhibitors have now played a prominent role in precise cancer therapy.

Despite the progresses, due to the extreme complex and dynamic of PTMs, current understanding of PTM repertoire is by far limited. Recent advancement in mass spectrometry-based proteomics technology, together with PTM peptide enrichment strategy, makes system-wide characterization of PTMs on an unprecedented scale and depth [11, 12]. Thousands or even tens of thousands of PTM sites can be identified and quantified in one experiment. Accordingly, system-wide analysis of PTM substrates and their interaction networks based on proteomics technologies greatly facilitates the characterization of targets, mechanisms of action, and biomarkers of the drugs targeting PTM enzymes. In this review, we provide an overview of the recent progresses on the proteomics-based studies of PTM targeting drugs. We introduce main proteomic technologies applied in drug researches and provide a detailed summary of proteomics-based PTM studies on drugs targeting the enzymes of phosphorylation, acetylation, methylation, and ubiquitination. We discuss how proteomics-based strategies benefit elucidating mechanisms of drug action and drug-resistance, uncovering potential off-target effects, and developing effective drug combination. We also highlight recent advances in targeting PTM crosstalks [13], which supply a drug combination for cancer precise medicine [14].

PROTEOMICS-BASED PTM CHARACTERIZATION TECHNOLOGIES IN DRUG RESEARCH

Proteomics-based PTM analysis supplies high-throughput and systematic technologies for globally identifying and quantifying proteins and PTMs (modification proteins, modification sites) [15, 16], providing a landscape for constructing the temporal and spatial changes of intracellular signal transduction networks in cells, tissues, or the human body under different conditions. Thus, proteomics-based PTM characterization technologies not only facilitate the identification of novel disease mechanisms and novel PTM-based drug targets, but also reveal the global drug targets, mechanisms of action, and potential toxicity. Furthermore, proteome-wide on-target and off-target studies provide potential opportunities for expanding the clinical indications of drugs. In

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Received: 10 June 2022 Accepted: 7 August 2022
Published online: 13 November 2022

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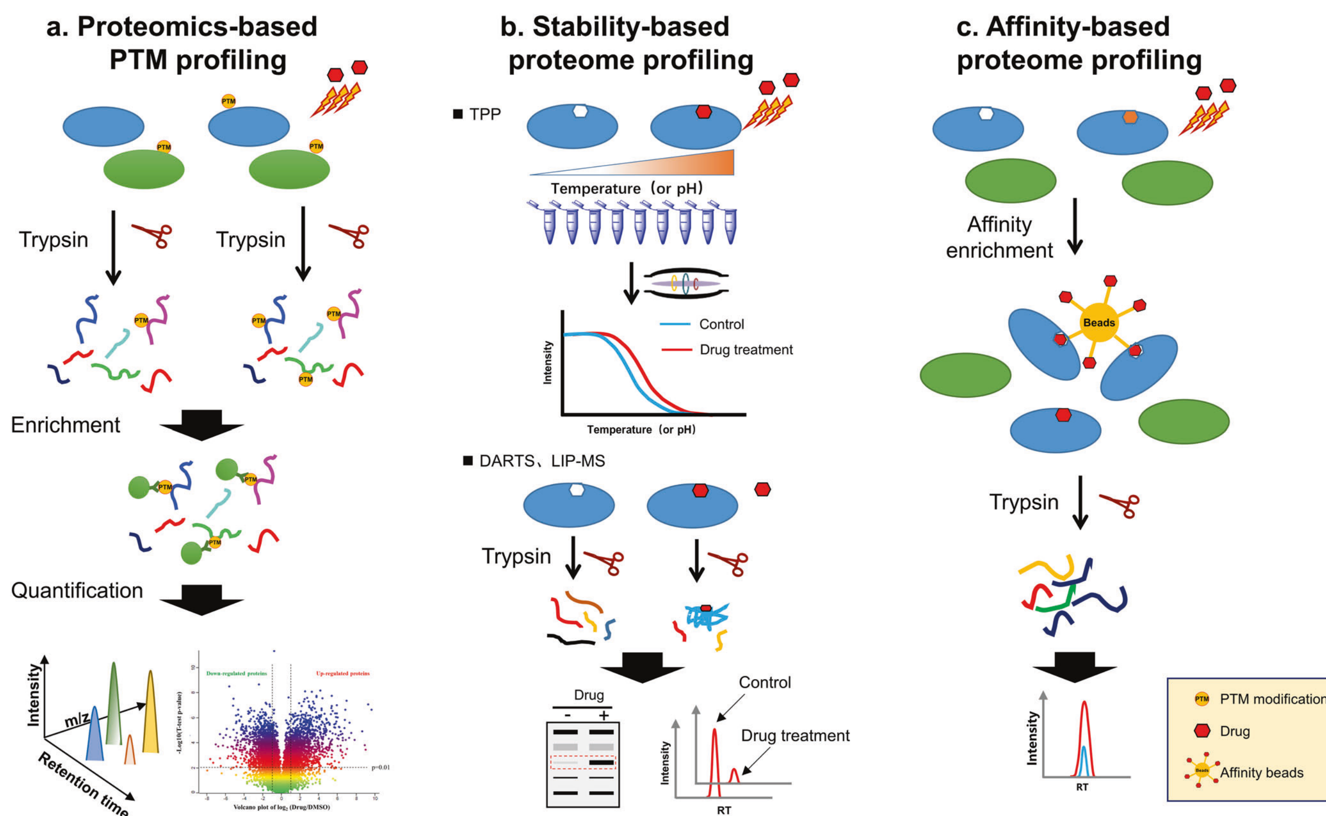

Fig. 1 Different mass-spectrometry-based proteomic technologies in drug research. **a** Proteomics-based PTM profiling supplies the systematic comparison study of the PTM substrates and PTM sites in cells with and without drug treatment; The globally protein and PTM profiling of diseases or drug treatment samples provide accurate molecular subtyping for precision medicine, screening potential biomarkers, revealing drug targets and uncovering systematic mechanisms of drug. **b** Different drug screening strategies such as TPP, DARTS, LiP are developed based on the protein stability under drug binding condition. The stability-based proteomics approach supplies the high efficiency and high throughput for drug target screening. **c** Affinity proteomics technology provides the high-efficiency and high throughput approach to directly screen the drug targets. This affinity-based proteome profiling supplies a high-efficiency technology for directly drug target screening.

fact, proteomics-based technologies are widely used in drug research, including proteome/PTM profiling, stability-based proteome profiling, and affinity-based proteome profiling (Fig. 1).

Proteomics-based PTM profiling

Proteomics-based PTM profiling can systematically identify and quantify the global PTM substrates and PTM sites to uncover the global molecular mechanisms and side effects of drugs. The construction of protein and PTM landscapes of diseases, such as non-small cell lung cancer [17], colon cancer [18], breast cancer [19], hepatocellular carcinoma [20], clear cell renal cell carcinoma [21] and ovarian cancer [22], not only provides accurate molecular subtyping for precision medicine but also uncovers potential druggable targets for disease treatment. In our previous study, we performed multiomics analysis of 103 Chinese patients with lung adenocarcinoma (LUAD) and supplied the global proteome and phosphoproteome characteristics in LUAD cancer tissues compared to nearby normal tissues. As a result, we identified the activation of a series of subtype-specific kinases, as well as potential prognostic LUAD biomarkers and drug targets [17]. Moreover, the Clinical Proteomic Tumor Analysis Consortium (CPTAC) performed a multiomics study of numerous cancers and revealed novel subtypes and biomarkers for precise medicine. For example, CPTAC used a multiomics approach to study 110 clinical colon cancer tissues and uncovered the phosphorylation characteristics of colon cancer. They first found that the phosphorylation on retinoblastoma protein is an oncogenic driver in colon cancer and a potential drug target for colon cancer treatment [18]. The CPTAC also analyzed 108 HPV-negative head and neck squamous cell carcinoma tissues using proteomic and phosphoproteomic technology. As a result, they uncovered significant kinase activation characteristics among the three cancer subtypes. Through phosphoproteomics, they found two modes of EGFR activation pathways that responded to anti-EGFR monoclonal antibodies, and showed that the phosphorylation state of the Rb protein had the potential to indicate the patient's response to CDK4/6 inhibitor treatment [23]. It should be noted that the identification of unexpected drug off-targets not only helps to systematically explain adverse effects, but also provides opportunities for drug repurposing and new strategies of drug combination. For example, the BCR/ABL inhibitor imatinib was reported to target platelet-derived growth factors (PDGFs) and c-KIT kinases, which could modulate gastrointestinal stromal tumor cell apoptosis; thus, imatinib may have been used as a gastrointestinal stromal tumor (GIST) therapy and is now being examined in a clinical trial [24]. Lenalidomide is an immunomodulatory drug and widely used in multiple myeloma (MM) therapy. Ng et al. used tandem mass tag (TMT)-labeled quantitative proteomics and phosphoproteomics to study the dynamic changes in the proteome and phosphoproteome in response to immunomodulatory drug treatment in clinical tissue samples from patients with relapsed MM [25]. The results showed that CDK6 was activated in the relapsed samples, suggesting that CDK6 activation is key to lenalidomide resistance. The study revealed that a combination of the CDK6 inhibitor palbociclib or CDK6-targeting degraders with immunomodulatory drugs can enhance synergy sensitivity to immunomodulatory drug-resistant MM.

Stability-based proteome profiling

Protein stability can increase when proteins are combined with ligands, both in vivo and in vitro. For example, proteins will acquire high-temperature resistance and low-protease susceptibility under ligand-combining conditions, which will serve to impart greater resistance to precipitation or proteolysis. Based on this principle, a thermal shift assay (TSA), cellular thermal shift assay (CETSA), and drug affinity responsive target stability (DARTS) and limited proteolysis (LiP) technologies were developed for drug target screening [26–29]. Using the TSA or CETSA method, fluorescence or Western blot analysis is used to detect the protein content change in solution under different temperatures. However, these techniques are commonly used to detect known drug targets and cannot systematically analyze unknown target proteins, and are also highly dependent on the affinity of the antibodies. A novel technology named thermal proteome profiling (TPP) was developed to overcome these shortcomings [30, 31]. TPP is based on the fact that proteins will be denatured and become insoluble following heating. However, the protein's thermal stability will be changed upon interaction with other small molecules, such as drugs and metabolites. TPP uses multiplex-labeling quantitative mass spectrometry-based proteomic technology to conduct quantitative analysis to monitor the melting profile of global proteins. Proteins with melting profiles that are found to be altered after incubation with small molecules are considered potential targets. TPP provides high throughput and unbiased drug target detection technology, which is helpful in the analysis of drug efficacy and toxicity. Based on the TPP strategy, Savitski et al. found that the highly selective PKC/PKA inhibitor staurosporine had more than 50 targets, and also revealed that the clinical kinase inhibitors vemurafenib and alectinib could act on the heme biosynthesis enzyme FECH to induce clinical phototoxic side effects [30]. The DARTS and LiP methods are based on a decrease in the protease susceptibility of the target protein under drug binding conditions. Moreover, both methods directly incubate native small molecules with the native proteome to screen for high-stability proteins (target proteins), with Western blot detection or mass spectrometry-based quantification proteomics analysis. Thus, stability-based proteome profiling provides high efficiency and high-throughput drug target screening.

Affinity-based proteome profiling

Affinity-based proteome profiling involves incubating the whole cell lysate proteome with small molecule inhibitors covalently bound to a solid support. The target proteins are selectively captured before being eluted and detected by mass spectrometry-based proteomics technology. This affinity-based proteome profiling provides a highly efficiency technology for direct drug target screening, which has been extensively used in drug research. Knockaert et al. immobilized glycogen synthase kinase 3β (GSK3β) inhibitor alsterpaullone on agarose beads and incubated the whole cell proteome with the beads, before systematically detecting the captured proteins by mass spectrometry-based proteomics profiling. Hence, they not only validated GSK-3α and GSK-3β as major intracellular targets of paullones, but also revealed that mitochondrial malate dehydrogenase (MDH) as a novel target in cells [32]. Based on the affinity principle, Bantscheff et al. developed a kinobeads technology for kinase inhibitor target screening, in which they immbolized nonselective kinase inhibitors on sepharose beads and incubated the whole cell lysate proteome with the beads. To overcome the interference of highly abundant proteins binding to the beads and improve the accuracy of target protein identification, high-selectivity kinase inhibitors with different concentrations were also added to the incubation system. Finally, the dynamic change in protein concentration after competitive incubation was analyzed using a quantitative proteomic method [33]. In 2017, Klaeger et al. constructed a target landscape of 243 clinically used kinase inhibitors by using kinobeads. The kinase target landscape revealed significant variation in the number of targets of different kinase inhibitors. For example, MEK and EGFR inhibitors have high selectivity, while CDK and PKC inhibitors could target many more proteins. Moreover, 19 CHEKI inhibitors were proven by the landscape, some of which had not been reported previously. The landscape also showed that the SIK2 inhibitor dasatinib could modulate the production of IL-10, and anti-inflammatory cytokine, in mouse bone marrow-derived macrophages, suggesting that dasatinib can be applied to treat inflammatory diseases [34]. Thus, the kinase target landscape not only broadens known targets of existing kinase inhibitors but also expands clinical indications for some kinase inhibitors.

PHOSPHO-PROTEOMICS IN DRUG RESEARCH

Protein phosphorylation is a type of PTM that regulates almost all biological processes in cells, such as proliferation, migration, and differentiation [35]. Phosphorylation is a reversible enzyme-catalyzed reaction which is dynamically regulated by protein kinase and phosphatase. Protein kinase is responsible for catalyzing serine/threonine/tyrosine residues covalently binding with phosphate groups, while phosphatase is responsible for removing phosphate groups from proteins. It has been reported that approximately 80% of phosphorylation occurs on serine, 20% on threonine, and 0.1%–1% on tyrosine residues [36]. The rapid response of phosphorylation modification plays an important role in maintaining the response of cells to various internal and external stimuli. Abnormal phosphorylation often leads to disordered biological processes in cells, which eventually leads to disease development and occurrence [37, 38]. Therefore, kinases and phosphatases are important targets of drugs. It has also been reported that the human genome can translate 556 protein kinases, among which, 98% have catalytic activity and only 55 kinases do not (termed deemed pseudokinases) [39]. Kinases can be classified into three classes according to their amino acid substrate specificity: Ser/Threonine kinases (STKs), protein tyrosine kinases (PTKs), and dual-specificity protein kinases (DSKs) [40]. Most kinases are STKs or PTKs. Phosphoproteomics provides a global identification and quantification approach for the phosphorylation of modified proteins and modified S/T/Y sites in different biological samples (Fig. 2), which will benefit the systematic uncovering of the molecular mechanisms and side effects of kinase inhibitors.

Overview of U.S. Food and Drug Administration (FDA)-approved kinase-targeting inhibitors

Phosphorylation not only changes the hydrophilicity and hydrophobicity of substrate proteins but also affects the structure, interactome, and activity of substrate proteins. The rational design of the kinase inhibitor is occupying ATP binding pocket on the kinase and inhibition of the substrate-binding. Almost one-third of the current FDA-approved clinical drug targets are related to kinases. According to the drugs in the clinical trials database (http://www.centerwatch.com), nearly 400 potential kinase inhibitors are under clinical or preclinical research for cancer treatment [41]. Thus far, more than 80 types of kinase-targetting drugs have been approved by the FDA, with the number continually increasing (Table 1, Supplementary Table S1).

Serine/threonine kinase inhibitor. STK is the most common protein kinase, which plays an important role in the regulation of cell proliferation, differentiation, senescence, and apoptosis. The expression level and activity of STK are altered in many types of cancer [42].

BRAF is a member of the Raf kinase family, with serine-threonine kinase activity, which regulates the MAP/ERK signaling

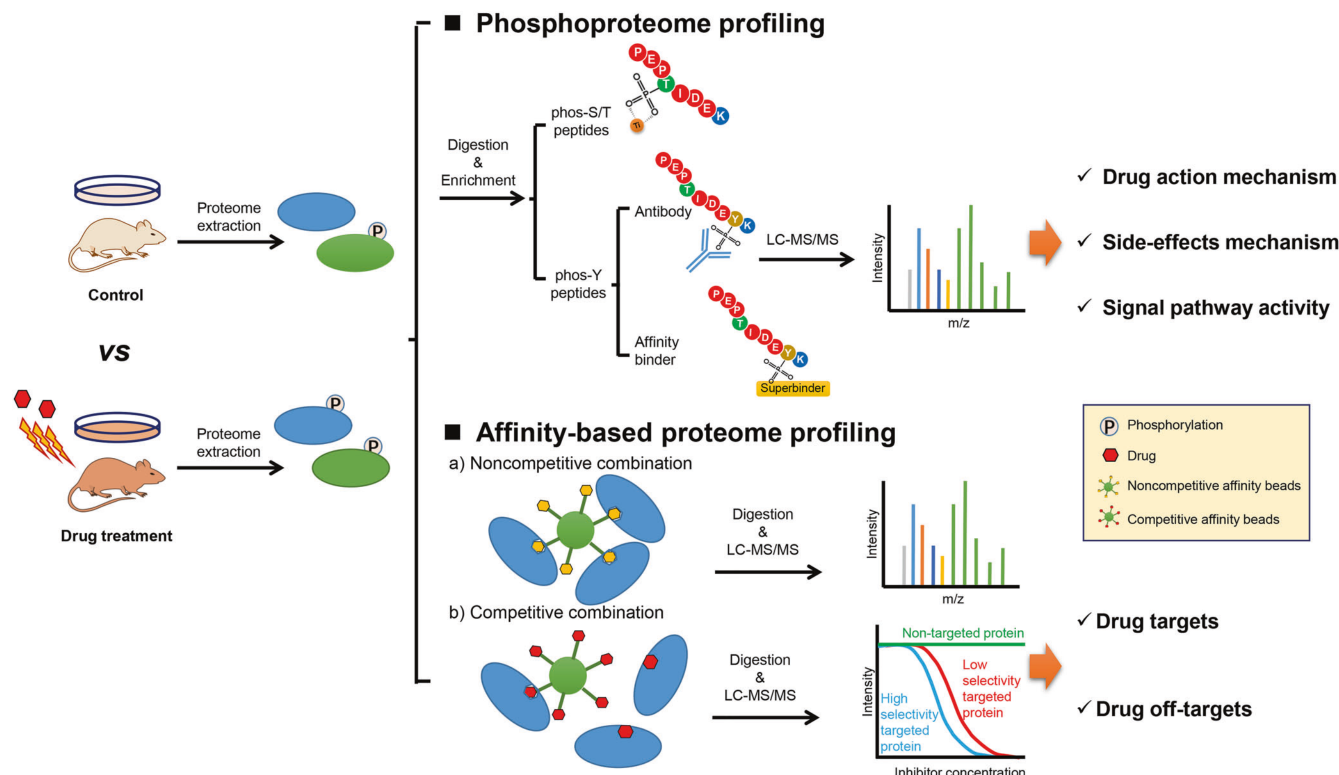

Fig. 2 Schematic diagram of proteomics strategies in study on drugs targeting phosphorylaton. Phosphoproteome profiling is widely used in kinase inhibitor study. Combined phospho-peptide enrichment and LC-MS/MS analysis, the landscapes of altered phosphorylation substrates, phosphorylation sites and kinase network are provided for understanding of signal pathway activity, drug action, and side effect mechanism. Affinity-based proteome profiling provides a holistic view of binding proteins of kinase inhibitors. The targeted proteins would be captured directly by affinity beads or with abundance altered under competitive condition, and then detected by the quantitative proteomics. Both competitive and noncompetitive ABPP can benefit identification of drug targets and off-target effects.

pathway in cell differentiation and cell division, among others. BRAF kinase inhibitors are mainly divided into two types according to their target selectivity: multi-target inhibitors (e.g., sorafenib, regorafenib, pazopanib) and BRAF V600E selective inhibitors (e.g., vemurafenib, dabrafenib). A proteomics approach has been used to perform a more in-depth study of the differences between dabrafenib and vemuratenib-induced dynamic changes in the melanoma proteome, the results of which showed that dabrafenib could selectively target NEK9 and CDK16. Moreover, inhibiting the activity of CDK4 could effectively inhibit the activity of NEK9, which in turn leads to inhibition of cell proliferation. Furthermore, knockdown of CDK16 at the transcriptional level could inhibit the phosphorylation of the downstream Rb protein S780 and highly increase the expression level of the cell cycle inhibition-related protein p27, resulting in the inhibition of cell proliferation. These results reveal a unique mechanism of dabrafenib and vemuratenib, suggesting that a combination of NEK9 and CDK16 inhibitors can effectively overcome their resistance [43].

Sorafenib is the first FDA-approved multi-targeted tyrosine kinase inhibitor that is used to inhibit tumor angiogenesis. However, drug resistance was frequently reported in clinical trials of sorafenib, with mechanisms thought to include metabolic reprogramming, epithelial-mesenchymal transition, and dysregulation of PI3K/AKT and JAK/STAT pathways. He et al. found that PGK1 protein was highly expressed in the sorafenib-resistant OS-RC-2 cell line, and the upregulated expression of PGK1 has been shown to be associated with poor prognosis in patients with kidney renal clear cell carcinoma [44]. Moreover, based on the detection of the phosphorylation level of kinases in the downstream signal pathway, they found that PGK1 could increase the phosphorylation level of AKT and ERK, which was regulated by CXCR4. Thus, they revealed that the cells acquired sorafenib resistance through PGK1 activation of the CXCR4/ERK signaling pathway. Furthermore, Schmitt et al. used stable isotope labeling with amino acids in cell culture (SILAC)-based quantitative phosphoproteomic technology to systematically compare the global protein expression and phosphorylation modification between vemurafenib-resistant and -sensitive melanoma cells [45]. The results showed that the nestin protein was significantly downregulated in drug-resistant cell lines and was closely associated with drug resistance, likely via the resulting increase in metalloproteinase activity. The phosphoproteomic data also revealed that low expression of nestin protein directly affected the activity of the PI3K/AKT/mTOR pathway, resulting in the increased abundance and phosphorylation of focal adhesion kinase.

BRAF V600E mutation occurs in 10% of patients with colorectal cancer. Grbčić et al. compared the differences in proteome expression among BRAF V600E mutant, *KRAS* mutant, and *BRAF/KRAS* wild-type colorectal cancer cells [46]. The study revealed that NPM1 was significantly upregulated in *BRAF* V600E mutant cells. Moreover, for the firt time, they revealed the mechanism of the NPM1/c-myc signaling axis in tumor drug resistance and showed that BRAF inhibitor combined with NPM1 inhibitor had the potential to overcome vemurafenib-resistance. Schmitt et al. used multiomics technology (genomics, proteomics, and phosphoproteomics) to study the mechanisms of vemurafenib sensitivity and resistance in A375 malignant melanoma cells [47]. The results showed abnormal MAPK, PI3K/AKT signaling pathway activation in vemurafenib-resistant cell. Furthermore, in vivo experiments proved that the combined use of an AURKA inhibitor (alisertib) could effectively overcome vemurafenib resistance in malignant melanoma.

Phosphatidylinositol 3-kinase (PI3K) is a class of lipid kinases involved in many cellular functions, such as cell proliferation and

Table 1. The list of FDA approved kinase inhibitors[a].

Target	Inhibitors
ALK	Crizotinib, Ceritinib, Alectinib, Brigatinib, Lorlatinib
BRAF	Vemurafenib, Dabrafenib, Sorafenib,
CDK4/6	Palbociclib, Ribociclib, Trilaciclib, Abemaciclib
MAPK	Binimetinib, Encorafenib
mTORC	Everolimus, Temsirolimus, Sirolimus
EGFR	Gefitinib, Erlotinib, Lapatinib, Vandetanib, Afatinib, Osimertinib, Neratinib, Dacomitinib, Mobocertinib, Panitumumab, Necitumumab, Cetuximab
VEGFR	Sunitinib, Pazopanib, Regorafenib, Axitinib, Nintedanib, Lenvatinib, Cabozantinib, Tivozanib, Ramucirumab
MET	Cabozantinib, Tepotinib, Cabozantinib, Capmatinib
FLT3	Midostaurin, Pexidartinib, Gilteritinib
FGFR	Pemigatinib, Erdafitinib
PDGFRA	Avapritinib, Olaratumab
KIT	Ripretinib
HER2	Tucatinib, Trastuzumab, Pertuzumab, Margetuximab
NTRK	Larotrectinib, Entrectinib
BCR-ABL	Imatinib, Dasatinib, Nilotinib, Bosutinib, Ponatinib, Asciminib
JAK	Ruxolitinib, Baricitinib, Fedratinib, Upadacitinib, Pacritinib, Tofacitinib
BTK	Ibrutinib, Acalabrutinib, Zanubrutinib
RET	Pralsetinib, Selpercatinib
SYK	Fostamatinib Disodium Hexahydrate
PI3K	Idelalisib, Duvelisib, Copanlisib, Alpelisib, Umbralisib
MEK	Trametinib, Cobimetinib
ROCK	Belumosudil, Fasudil

[a]The drug information was obtained from clinical trials database (https://www.centerwatch.com/directories/1067-fda-approved-drugs). Data are to June 2022.

cell differentiation. Alpelisib, copanlisib, and idelalisib are FDA-approved drugs used for breast cancer, relapsed follicular lymphoma, and chronic myeloid leukemia therapy, respectively. Abnormal activation of the PI3K signaling pathway occurs in most triple-negative breast cancers (TNBCs), and PI3K inhibitor drugs only show partial efficacy. To study the mechanisms of primary and acquired resistance to PI3K inhibitors, Mundt et al. studied the TNBC patient-derived tumor xenograft (PDX) models with different susceptibilities to the pan-inhibitor buparlisib through phosphoproteomic and genomic techniques [48]. As a result, they found that upregulated activation of the MAPK/MEK pathway was closely related to drug resistance and reported NEK9 and MAP2K4 as novel drug resistance markers. Guo et al. studied the drug resistance mechanism of the pan-inhibitor BKM120 in the treatment of TNBC [49]. By analyzing the correlation between the protein expression and tumor growth inhibition efficiency of BKM120, they found a series of BKM120 resistance markers, including EGFR, pHER3 Y1197, and PI3Kp85, and also proved the synergistic efficacy of the combination of EGFR inhibitor and BKM120 in vitro.

CDK4/6 is a member of the serine/threonine kinase family and modulates various stages of cell cycle regulation, transcription, and metabolism, among others. FDA-approved CDK4/6 targeting drugs include ribociclib, abemaciclib, and palbociclib, all of which have high selectivity and are used clinically for the treatment of breast cancer. Hafner et al. conducted a multiomics (transcriptomics, proteomics, and phosphoproteomics) study of the CDK4/6 drugs palbociclib, ribociclib, and abemaciclib for the treatment of hormone receptor-positive breast cancer, and found striking differences in the regulation of these drugs at different molecular levels [50]. In particular, abemaciclib was found to inhibit multiple kinases other than CDK4/6, such as CDK2/Cyclin A/E and CDK1/Cyclin B, which have broader kinase inhibitory activity than alvocidid. Sumi et al. used ABPP-based proteomic profiling to systematically compare the targets of palbociclib and ribociclib in H157 lung squamous cell carcinoma cells [51]. As a result, they found that both drugs could target CDK9. Additionally, they discovered new targets of palbociclib, including casein kinase 2 and PIK3R4, both of which can regulate autophagy and uniquely inhibit the AKT signaling pathway. Emily et al. used affinity-based proteomic profiling to systematically study the mechanism of trametinib, BMS-777607, dasatinib, abemaciclib, and palbociclib, and revealed the common and unique kinase targets of abemaciclib and palbociclib [52]. The results showed that abemaciclib could inhibit the activity of specific target GSK3a/b and CAMKIIg/d kinases at lower doses (nanomolar concentrations). Moreover, the activation of GSK3 relieved the inhibition of the WNT pathway and resulted in a negative effect on cells.

Tyrosine kinase inhibitor. Protein tyrosine kinases (PTKs) can modulate cellular signal transduction and cellular activity regulation. PTKs are divided into receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs) [53]. RTKs have roles in transmembrane signal transduction and NRTKs transfer the signals from extracellular receptors to intracellular signal network. More than 90 PTKs have been reported to exist in human cells, including ~60 RTKs and 30 NRTKs.

RTK: EGFR is ubiquitously expressed in human epidermal and stromal cells. EGFR can activate many downstream signal pathways, such as the RAS/RAF/MAPK and PI3K/AKT pathways, which serves to promote tumor cell survival, proliferation, and metastasis. The EGFR kinase activity is based on its tyrosine phosphorylation level; therefore, EGFR inhibitors are designed to selectively compete for the ATP binding site in the intracellular domain, resulting in inhibition of phosphorylation on tyrosine residues. At present, the FDA has approved many EGFR-targeting drugs, such as tucatinib, dacomitinib, brigatinib, neratinib, osimertinib, afatinib, vandetanib, lapatinib, dasatinib, erlotinib, and gefitinib. However, 30%–40% of clinical patients will acquire primary resistance to first- and second-generation EGFR inhibitors (e.g., gefitinib and erlotinib) in the clinic. The most important type of acquired resistance is the EGFR T790M mutation, which occurs in ~60% of cases. Other characteristics of acquired resistance include MET amplification, exon 21 L858R missense mutation, and exon 19 deletion (E746-A750). Osimertinib and rociletinib, as first-line drugs of third-generation irreversible EGFR inhibitors, are effective against EGFR T790M mutant tumors. Zhang et al. used SILAC-based quantitative proteomics and phosphoproteomics to compare the dynamic changes in the protein phosphorylation level between EGFR inhibitor-resistant and -sensitive lung adenocarcinoma cell lines [54]. As a result, they uncovered a series of potential markers of EGFR inhibitor sensitivity. The authors systematically studied the global dynamics of proteins and phosphorylation in osimertinib (third-generation inhibitor) acquired-resistant H1975 cell lines. Consequently, they found that the phosphorylation of SHP2 was significantly downregulated in drug-resistant cells, which led to RAS/MAPK signaling pathway inhibition and PI3K/AKT pathway activation. The study revealed the combined application of the PI3K/mTOR inhibitor dactolisib as a potential new strategy for overcoming EGFR inhibitor drug resistance [55]. Moreover, Terp et al. found that cells could achieve resistance through the activation of the FGFR1-AKT pathway after treatment with EGFR inhibitors (erlotinib, gefitinib, and osimertinib) [56]. The combination of EGFR and FGFR inhibitors could not completely overcome the resistance of cells to the EGFR inhibitor; however, co-inhibition of FGFR, AKT, or PI3K could completely inhibit the FGFR1-AKT signaling pathway and resulted in the

inhibition of cell proliferation. This study provided a novel therapeutic strategy for FGFR1-overexpressing EGFR inhibitor-resistant non-small cell lung cancer (NSCLC) cells.

Activation of bypass signaling pathways is considered a drug resistance mechanism, such as the finding that HGF-stimulated MET pathway activation leads to EGFR inhibitor resistance. Koch et al. used kinobead-based kinase profiling technology to systematically compare the kinase expression differences between gefitinib-sensitive and -resistant cell lines [57]. They not only found that MET amplification was significantly increased in drug-resistant cell lines, but also showed that the significant upregulation of EPHA2 was a novel marker for drug resistance. Silencing EPHA2 in cells using siRNA technology can restore drug sensitivity, which provides a potential drug combination strategy to prevent drug resistance.

FGFRs are similar to EGFRs in that phosphorylation of FGFR activates a series of downstream signaling pathways such as phosphoinositide, MAPK, and PI3K/Akt, which are involved in cell growth, proliferation, differentiation, blood vessel generation, and other physiological activities. The FDA has approved 11 FGFR drugs, such as pemigatinib, erdafitinib, lenvatinib, nintedanib, regorafenib, ponatinib, and pazopanib. Cunningham et al. studied the proteome and phosphoproteome differences between two TNBC cells (MFM223 and SUM52) based on SILAC quantitative proteomic and phosphoproteomic technology [58]. The results showed that the unique proteins in SUM52 cells were mainly associated with cell metabolism, while those in MFM223 cells were mainly associated with cell adhesion and migration. Using phosphoproteomics, the study also revealed the phosphorylation sensitivity differences between MFM223 and SUM52 cells following FGFR inhibitor SU5402 treatment.

To study the factors influencing the clinical efficacy of FGFR inhibitors, Kostas et al. used a proximity-labeling proteomic approach, BioID, to study the proteome dynamics under FGF1 stimulation in osteosarcoma cell lines [59]. The results showed that the sensitivity of cells to FGFR kinase inhibitor was associated with the PTPRG protein expression level, which indicated that PTPRG could act as a pharmacodynamic indicator of FGFR inhibitor treatment. As most FGFR inhibitor drugs are multi-targeting, acquired resistance is the main factors affecting their clinical application. Based on clinical experiments, Krook et al. used the FGFR inhibitor infigratinib (BGJ398) to treat FGFR2-abnormal cholangiocarcinoma and found that numerous single nucleotide variants (SNVs) on the FGFR2 kinase domain could drive acquired resistance to infigratinib [60]. Through proteomic experiments, they also found that the FGFR2 p.E565A mutation leads to significant upregulation of the PI3K/AKT/mTOR signaling pathway in cells, while the combined use of the mTOR inhibitor (INK128) and FGFR inhibitor could overcome the FGFR inhibitor resistance. This study provides a new strategy for clinical drug application for FGFR acquired resistance.

FLT3 is a type of receptor tyrosine kinase that is mainly expressed in the hematopoietic compartment. FLT3 mutation, such as FLT3 internal tandem duplications (FLT3-ITD), is common in leukemia. FLT3 gene mutation leads to the dimerization of the FLT3 receptor and then actives the downstream signaling pathways. Cucchi et al. used phosphoproteomics technology to study 35 clinical patient samples with AML (17 FLT-WT vs. 18 FLT3-ITD) [61]. The results showed that patients with FLT3-ITD mutants had increased sensitivity to gilteritinib and midostaurin. Moreover, combination with the MEK inhibitor trametinib could completely inhibit the MEK-ERK axis and significantly improved the efficacy of the FLT3 kinase inhibitor. Joshi et al. found that Fibroblast Growth Factor 2 (FGF2) and early hematopoietic growth factor Flt3 ligand (FL) could activate the MAPK/PI3K/AKT signaling pathway, enabling leukemia cells to escape the growth-inhibitory effects of gilteritinib [62]. They revealed the important role of the tumor growth microenvironment in gilteritinib resistance in the treatment of acute leukemia. They also found that the combination of protein kinase Aurora B (AURKB) inhibitor and gilteritinib could synergistically exert a significant anti-leukemia effect and effectively overcome the early drug resistance of leukemia cells.

NRTK: BCL-ABL is a fusion oncogene that is formed by the combination of the ABL oncogene on chromosome 9 and the BCR gene on chromosome 22. The fusion gene expresses a BCR/ABL fusion protein with high tyrosine kinase activity and promotes the activation of multiple downstream signal pathways that interfere with the normal regulation of cells and induce excessive proliferation. The fusion gene frequently occurs in chronic myelogenous leukemia (CML). The FDA has approved several BCL-ABL-targeting drugs, such as imatinib, bosutinib, dasatinib, ponatinib, nilotinib, and asciminib, all of which can inhibit cell proliferation and induce apoptosis by selectively blocking the ATP binding pocket in the BCR-ABL fusion protein and inhibiting its activity. Imatinib (also known as Gleevec) was the first small-molecule kinase inhibitor approved by the FDA for BCR-ABL-positive CML therapy in 2001. Bantscheff et al. used kinobeads in combination with iTRAQ-based quantitative proteomics technology to systematically quantify the protein phosphorylation-level changes following treatment of K562 cells with imatinib, dasatinib, and bosutinib [33]. Their results globally uncovered the phosphorylation modification events on more than 200 kinases in K562 CML cells under imatinib treatment and confirmed the known targets, such as ABL and SRC family kinases. The results also revealed epithelial discoidin domain-containing receptor 1 (DDR1) and NQO2 (ribosyldihydronicotinamide dehydrogenase) as novel targets of imatinib. Moreover, Kayoko et al. systematically compared the phosphorylation-level differences between imatinib-sensitive and -resistant gastrointestinal stromal tumor cell lines by using quantitative phosphoproteomics [63]. As a result, they found that the activity of KIT was reversed and both EGFR and MAPK were abnormally activated in drug-resistant cell lines. These results suggested that the activation of EGFR involved in the molecular mechanism of imatinib was associated with the acquisition of drug resistance. In vitro, the combined application of the EGFR inhibitor gefitinib and imatinib was shown to significantly inhibit the proliferation of imatinib-resistant cell lines.

BTK belongs to the non-receptor tyrosine Tec family, which is the second largest family of human non-receptor kinases and is widely expressed in all hematopoietic cells (except T cells). Abnormal activation of BTK is closely related to the pathogenesis of B cell lymphoma; thus, BTK is an important target for treating hematological malignancies. At present, three BTK kinase drugs have been approved by the FDA, including zanubrutinib, acalabrutinib, and ibrutinib, all of which show high BTK selectivity and are commonly used for treating B-lymphocyte tumors. Ye et al. used an affinity-based proteome profiling strategy to systematically compare kinase activity between cells treated with ibrutinib and the ibrutinib analog MM2-48 [64]. The results showed that MM2-48 could inhibit tumors more effectively than ibrutinib, specifically the key protein BCCIP of the DNA repair pathway, thereby inhibiting DNA repair pathway activity. They also found that co-inhibition of BTK and DNA repair signaling pathways could improve the therapeutic efficacy of B-cell lymphoma treatment. Moreover, the activity of ibrutinib significantly decreased due to the mutation of cysteine at position 481 of BTK protein to serine (C481S). Clinical data have proven that this mutation is the fundamental cause of ibrutinib resistance, although other mutations at position 481 of the BTK protein are also known to cause ibrutinib resistance.

Protein degradation targeting chimera (PROTAC) is a unique protein degradation technology that can link E3 ligase and target protein ligands to ubiquitinate ligand-targeted proteins, which are then further degraded by the proteasome [65]. PROTAC technology can target and degrade both wild-type and mutant BTK

proteins, which result in ibrutinib resistance. It has been reported that PROTAC degrader can rapidly degrade BTK in cells in only 1 h in a dose-dependent manner [66]. Rao et al. reported the first PROTAC degrader that targeted BTK. In this study, they used an ibrutinib derivative and PROTAC technology to synthesize a new BTK degrader, L181, which can target and degrade the C481S mutant BTK protein to overcome the mutation-induced ibrutinib resistance [67]. They also successfully developed a novel soluble PROTAC degrader for BTK that efficiently degraded different mutant BTK proteins [68].

JAK is a family of non-receptor tyrosine kinases, which are reported to be related to hematological tumors; specifically, JAK2 is related to the production of erythrocytes and platelets, and JAK1/3 is related to immune regulation. The JAK-STAT signaling pathway is associated with the signal transduction of inflammatory cytokines and tumor cells, and is also one of the immune regulation-related pathways that is widely involved in cellular activities related to human health and disease. Five drugs targeting the JAK-STAT signaling pathway have been approved by the FDA, including ruxolitinib, fedratinib, upadacitinib, baricitinib, and tofacitinib. Parra-Izquierdo et al. compared the platelet biochemical and physiological responses of five JAK inhibitors in vitro [69]. The results showed that baricitinib and ruxolitinib, both of which target JAK1/2, not only significantly reduced the phosphorylation level of AKT and activated glycoprotein VI protein, but also regulated the phosphorylation level of DAPP1, thereby effectively regulating the GPVI-mediated reduction of platelet adsorption and aggregation. Eberl et al. used kinobeads-based kinase profiling technology to study different JAK inhibitors and used a broad-JAK inhibitor (tofacitinib) as a competitor [70]. They revealed the same target characteristics among these inhibitors through cluster analysis, and also constructed the first off-target profiles of JAK kinase inhibitors in peripheral blood mononuclear cells.

Protein phosphatase inhibitor

Protein phosphatases can be distinctly assigned to three families according to their catalytic domain sequence similarity: PTPs (protein tyrosine phosphatases), PPPs (phosphoprotein protein phosphatases), and PPMs (protein phosphatase metal-dependent) [71]. The active site of phosphatases is usually positively charged, so the ligands that bind to the active pocket of phosphatases should also be polar, which will lead to unsuitable administration and low bioavailability. Additionally, as the catalytic domains of phosphatases are highly similar, the selectivity of their inhibitors has become a major problem. Because of the above problems, phosphatases have always been considered undruggable target proteins. Recently, many compounds that bind to the allosteric binding site on the target phosphatase have been developed, such as the SHP2 inhibitors SHP099, SHP389, SHP294, and TNO155, and the PPP1R15A inhibitor IFB-088 [72].

The SHP2 protein is a member of the PTP family and is encoded by the *PTPN11* gene. SHP2 plays an important role in the regulation of the growth factor receptor signaling pathway [73]. Our previous study found that the activity of SHP2 is significantly increased during the occurrence of osteoarthritis. Using 10-plex TMT labeling-based quantitative proteomics, we performed a thorough analysis of the global differential expression of intracellular proteins in osteoarthritis lesions after treatment with SHP099. We found that the downstream protein UPP1 of the SHP2 regulatory pathway changed significantly during the disease process. We also used SILAC labeling-based quantitative proteomics and tyrosine phosphoproteomics to systematically analyze the changes in intracellular tyrosine phosphorylation modification levels before and after treatment with SHP099. We finally confirmed that SHP2 promoted UPP1-mediated uridine degradation through DOK1 Tyr397 dephosphorylation. Our study revealed that uridine supplementation can improve the occurrence and development of osteoarthritis by maintaining the balance of anabolism and catabolism [74].

ACETYL-PROTEOMICS IN DRUG RESEARCH

Lysine deacetylase inhibitors

Pan HDAC inhibitors. The histone deacetylase (HDAC) family catalyzes the removal of acetyl groups from lysine. HDACs are classified into two superfamilies according to their catalytic mechanisms: metal-dependent HDAC classes I, II, IV; and NAD^+ dependent class III. The former class is usually referred to as HDACs, which are among the best-studied drug targets in epigenetic enzymes (Fig. 3). To date, five HDAC inhibitors, predominantly pan-HDAC inhibitors, have been approved for clinical use in cutaneous T-cell lymphoma (CTCL) and MM, including vorinostat, romidepsin, belinostat, panobinostat, and tucidinostat [75].

HDAC inhibitors (HDACi) modulate histone PTMs and regulate protein expression at the epigenetic level. A detailed molecular mechanism of HDACi was uncovered by combined quantitative genomic and proteomic analysis, in which H4 acetylation increased under HDAC inhibition, and served as a binding substrate for the bromodomain and extra-terminal (BET) family, particularly BRD4. Recruited BET proteins enhanced transcription initiation and elongation, and further regulated protein expression [76]. Histone methylation also responds to HDAC inhibition such as that by epigenetic regulation, a classic mechanism of HDAC inhibition; indeed, in the past decade, numerous studies have used proteomics to reveal HDACi-altered proteins [40, 77]. In NSCLC, HDAC inhibition regulates proteins involved in the metabolism pathway [78]; in bladder cancer, the prominent HDAC inhibition pathways are cell cycle, apoptosis, oxidative stress, and autophagy[79]; in SWI/SNF-deficient SCCOHT, HDACi exhibits a synthetic lethal effect and shows synergism with EZH2 inhibitors [80]; in cervical cancer, HDACi reverses epithelial-mesenchymal transition by modulating the ubiquitination pathway [81].

Besides histone acetylation, HDAC inhibition also modulates non-histone substrates. Combined SILAC-based quantitative proteomics and affinity-based enrichment strategies, a resource of altered acetylome by HDAC inhibition was provided by the Chunaram Choudhary group in 2015. The research covered 19 HDAC inhibitors and revealed distinct groups of acetylomes altered by different inhibitors [82]. In CTCL, quantitative acetylome analysis also detected non-histone substrates under HDAC inhibition, such as oncogenic MYC protein, glycolysis-related phosphoglycerate kinase 1 and ATP-dependent RNA helicase DDX24 [83]. Other types of acylation have also been shown to be regulated by HDACi. HDAC inhibition increases histone butyrylation in neuroblastoma [84]. Ultradeep proteomics analysis revealed that HDAC inhibition upregulated hundreds of lysine crotonylation and 2-hydroxyisobutyrylation sites [85, 86]. The catalytic activities of HDAC on non-histone acetylation and rare acylation revealed new insights into the mechanism of HDAC inhibition in cancer therapy.

Despite numerous studies indicating the potential of HDACi in tumor treatment, their clinical application is currently limited in hematological cancers, and clinical trial results in solid tumors have been largely unsuccessful. Therefore, integrative proteomics and phosphoproteomics have been applied to uncover the molecular differences of HDAC inhibition in sensitive and resistant cell lines. Quantitative proteomics data revealed that the glycolysis/gluconeogenesis pathway was activated in resistant cells and two upregulated hexokinases (HK1 and HK2) were promising targets for combined administration. Further proteomics and phosphoproteomics data showed that in the combinational treatment of HDACi and HKi, transcription- and protein homeostasis-related pathways were suppressed but several kinase-related pathways were activated. These results suggest that an additional introduction of kinase inhibitors and HK inhibitors could benefit HDACi therapy [87].

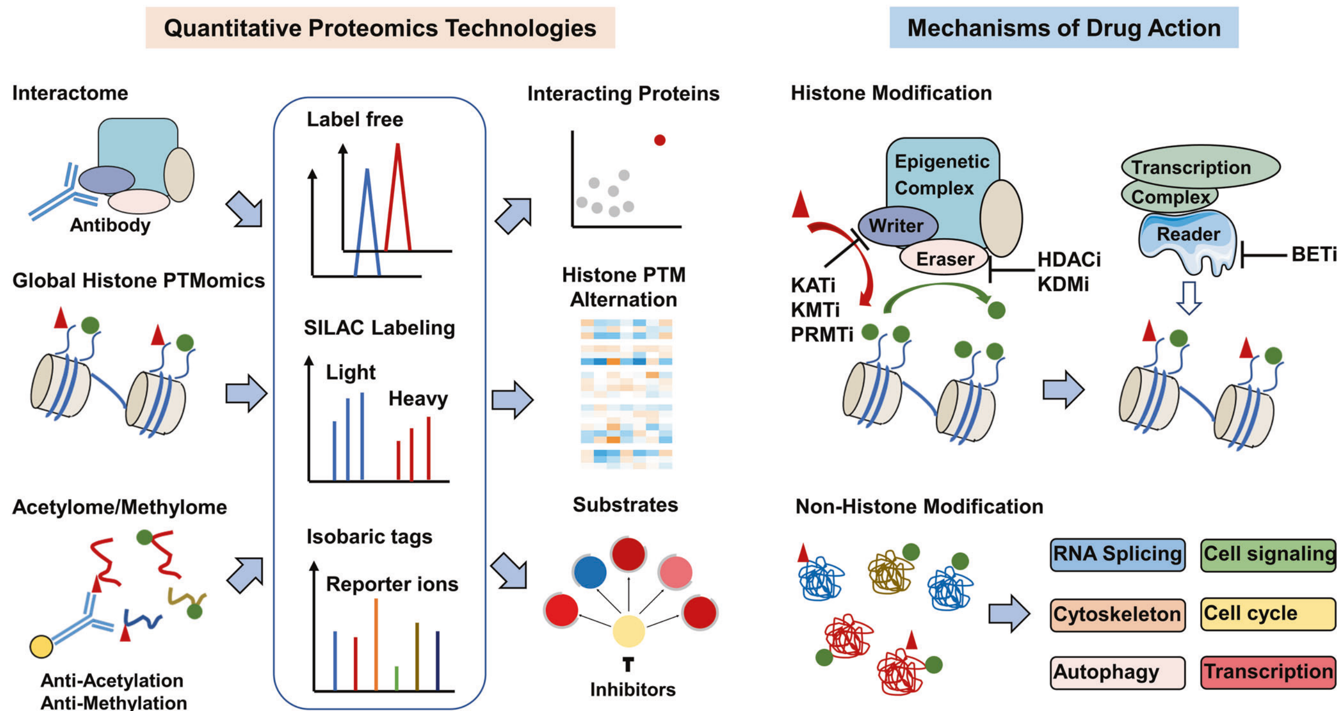

Fig. 3 Schematic diagram of proteomics strategies in studying drugs targeting acetylation and methylation. Acetylation and methylation take place on both histones and non-histone proteins, and are regulated by three classes of enzymes: writers that add the modification, erasers that remove the modification and readers that recognize the modification. For histone substrates, regulators function in complex with other proteins, regulating gene transcription through histone markers. For non-histone substrates, they play a role in various cellular processes by regulating DNA, RNA binding ability, stability, and activity of substrates. Those three classes are all potential drug targets and several proteomics strategies are of benefit to studying mechanisms of drugs targeting acetylation and methylation. Interactome profiling can identify partners in protein complexes. Histone PTM profiling can reveal global histone marker changes. Acetylome and methylome profiling can uncover those non-histone modifications.

Selective HDAC inhibitors. The different HDAC classes exhibit distinct functions. In class I, HDAC1, 2, and 3 predominantly exercise histone deacetylation as constituents of transcriptional repressor complexes, while HDAC8 acts independently and its substrates remain largely unknown. In class II, HDAC6 is the main cytoplasmic isoform, while HDAC4, 5, 7, and 9 exhibit weak catalytic activity, and may serve as scaffolding proteins in vivo. The primary role of class IV HDAC is deacylation of long-chain acyllysine [88]. Because of the distinct functions of HDACs, great efforts have been devoted to developing class/isoform-selective HDAC inhibitors.

The class I HDAC (HDAC1, 2, 3) inhibitors entinostat and tacedinaline are currently under phase III clinical development for cancer treatment. However, entinostat, despite higher selectivity, exhibited a lower therapeutic index than pan-HDACi vorinostat [89]. Recently, a quantitative chemical proteomics analysis established a target landscape for current HDAC inhibitors, demonstrating that despite stronger selectivity, the potency of class I HDACi decreased when HDACs were assembled into repressor complexes; these findings provide an explanation for the observed clinical responses [90].

Beyond the core histone deacetylase, other HDACs are also potential targets for cancer therapy. Substrates of HDAC8 and HDAC6 were studied by a quantitative acetylome strategy. HDAC8 regulates the acetylation of ARID1A, RAI1, MLL2, SMC3, and is involved in transcription and RNA splicing [91], while HDAC6 regulates cytoskeletal (tubulin, cortactin) acetylation and increases cell proliferation, migration, and invasion [92]. Recent acetylome and interactome analyses found that enzymes in the glycolysis pathway were also regulated under HDAC6 inhibition in TNBC, highlighting the therapeutic potential of selective HDAC6i in TNBC treatment [93].

Acetyl lysine reader inhibitors

BET proteins are a group of acetyl-lysine readers containing BRD2, BRD3, BRD4, and BRDT. The BET family is recruited by acetylation on histone, and functions as a scaffold protein to assemble transcriptional initiation and elongation complexes. Dysregulation of BET serves as a cancer driver, as in nut midline carcinoma (NMC) [94, 95]. Therefore, BETs are promising targets in cancer therapy and numerous BET inhibitors have been designed to disrupt the interaction between BET and histone acetylation [96].

However, single-agent treatment by BETi has not shown a durable response, largely due to acquired resistance, and considerable effort has been made to uncover the mechanism for this. Chemical proteomics-based kinome analysis revealed that reprograming of the kinase network drove BETi resistance. Moreover, in acquired resistant ovarian carcinoma (OC) cells, PI3K/ERK signaling was activated and inhibition of this kinase signaling re-sensitized OC to BET inhibition [97]. In TNBC, interactome analysis found that decreased activity of PP2A, a BRD4 phosphatase, led to acquired resistance. Moreover, decreased activity of PP2A also resulted in hyper-phosphorylation of BRD4, which enhanced the BRD4 interaction with MED1, a component of the mediator complex [98]. A recent study profiled the interactome of the BET family under BET inhibition and showed that interactions between BRD4 and transcriptional repression complexes were decreased under BET inhibition, providing a mechanism for gene upregulation by BRD4 inhibitor JQ1 treatment. This research also uncovered a negative function of BRD3 in cell proliferation and suggested that the relatively higher expression level of BRD3 compared to other BETs might serve as a marker for therapeutic response [99]. These proteomics studies uncovered the mechanism of BET inhibition and drug resistance and provided potential combination strategies.

SPRINGER NATURE

Table 2. The list of HDAC, BET, KAT inhibitors[a].

Targets	Inhibitors
HDAC	
pan-HDAC	Tucidinostat, Panobinostat, Belinostat, Romidepsin, Vorinostat, Abexinostat
Class I HDAC	Entinostat
BET	
BRD4	CPI-0610, INCB-057643, PLX-2853, GS-5829, CC-90010, BI-894999, ABBV-075, SYHA-1801
Pan-BET	ODM-207, BMS-986158, INCB-54329, BPI-23314, ABBV-744
KAT	
CBP/p300	CCS-1477, FT-7051
MOZ	PF-07248144

[a]The inhibitor information was obtained from cortellis drug discovery intelligence (https://www.cortellis.com/drugdiscovery/home?locale=en-US). Data are to June 2022.

Lysine acetyltransferase inhibitors

Lysine acetyltransferases (KATs) transfer acetyl groups from acetyl-coenzyme A to the ε-nitrogen of lysine. There are three families of KATs, including the GNAT family, MYST family, and P300/CBP family. P300/CBP is a unique family with widespread acetylation substrates ranging from histone to non-histone proteins. P300/CBP acts as a transcriptional coactivator in oncogenic signaling [100]. Targeting P300/CBP is a promising strategy for cancer therapy, especially with the recent reports of inhibitors such as the catalytic inhibitor A485 and the bromodomain inhibitors CCS1477 and FT-7051 [101, 102] (Table 2, Supplemental Table S2). Pioneers are under clinical trials for the treatment of castration-resistant prostate cancer and hematological malignancy [103].

In 2018, an ultra-deep time-resolved acetylome analysis was performed to study altered substrates under P300/CBP inhibition. The results showed that catalytic inhibitors caused extensive downregulation of acetylation sites, while bromodomain inhibitors only exhibited moderate suppression. Both histone and non-histone acetylation were regulated by p300, including key signaling effectors and enhancer-associated regulators. The study also provided the first proteome-level kinetic analysis of acetylation. This P300/CBP acetylome resource supported further mechanistic study of P300/CBP inhibitors [12]. In 2021, multiomics profiling was applied to study the mechanism of P300/CBP inhibition in cancer therapy. RNA-seq analysis revealed that catalytic P300/CBP inhibition disrupted cell-specific core transcriptional programs, which was consistent with its coactivator role. Moreover, the results of CHIP-seq analysis found that P300/CBPi decreased H3K27ac, and suppressed recruitment of the acetyl-lysine reader BRD4 and RNA polymerase II. Furthermore, CRISPR-Cas9 screening identified HDAC3 as a P300/CBP antagonist and the acetylome revealed that co-inhibition of HDAC3 attenuated the acetylation suppression effect of P300/CBPi, especially on histones. Research also uncovered that the crosstalk between H3K27ac and H3K27me contributed to stable transcriptional suppression under P300/CBPi administration [104].

METHYL-PROTEOMICS IN DRUG RESEARCH

Lysine methyltransferases inhibitors

The lysine methyltransferase (KMTs) family transfers methyl groups from S-adenosyl methionine (SAM) to the ε-nitrogen of lysine. Dysregulation of KMTs is widely reported in many diseases, including cancer; therefore, significant effort has been devoted to developing inhibitors against KMTs [105]. Among them, inhibitors targeting enhancer of zeste homolog 2 (EZH2) and disruptor of telomeric silencing 1-like protein (DOT1L) are well studied, with pioneers approved by the FDA or under clinical trial [106].

EZH2 inhibitors. EZH2 is the catalytic subunit of PRC2, which catalyzes mono-, di-, or tri-methylation of H3K27 and leads to transcriptional repression. A series of EZH2 inhibitors (EZH2i) have been reported, and pioneers such as EPZ-6438 have already been approved by the FDA for clinical administration in epithelioid sarcoma and follicular lymphoma, with several others undergoing clinical trials [107, 108].

As epigenetic enzyme inhibitors, early studies used quantitative proteomic strategies to globally profile protein expression alteration as a consequence of EZH2 inhibition. In EZH2i-sensitive tumors (i.e., EZH2 mutant diffuse large B cell lymphoma and follicular lymphoma), proteomic data showed that inhibition caused cell cycle arrest by upregulating cell cycle inhibitors and downregulating CDKs [109–111]. However, other than in EZH2 mutant cancers, EZH2 is also overexpressed in most wild-type solid tumors; however, whether EZH2i treatment is beneficial to such tumors remains to be explored. Researchers have combined histone PTM profiling with proteomic and phosphoproteomic analyses to systematically compare the EZH2 inhibition effect in solid cancer cell lines with different sensitivities to develop precision medicine strategies [112].

EZH2 also plays a role in cancer drug resistance. Chemotherapy-induced senescence (CIS) is a tumor suppressive mechanism that leads to cell cycle arrest; however, some cells can survive chemotherapy by escaping CIS. Indeed, researchers have found that EZH2 mediated CIS escape, with proteomics data showing that EZH2 inhibition altered proteins such as AP2M1, GAK, and AAK1 to mediate CIS escape. This research proposed targeting EZH2 as a potential strategy for chemotherapy-resistant cancers [113]. For cancer-targeted therapy, EGFR inhibitors are pioneers that have been applied to NSCLC. However, EGFR inhibition also leads to drug resistance, and epigenetic regulation by EZH2 has been shown to be involved in establishing a drug-resistant state. Methylome analysis revealed that EZH2 inhibition not only downregulated H3K27 methylation, but also non-histone methylation, such as G9a, which catalyzes H3K9 methylation and leads to transcriptional repression. EZH2 inhibition has also been shown to alter G9a methylation and regulate chromatin recruitment. Further histone PTM analysis demonstrated that EZH2 inhibitors regulated H3K9 methylation in resistant cells. These results show that crosstalk between EZH2 and G9a contributes to EGFRi resistance and reveals the therapeutic potential of the combination of EZH2 inhibitors and EGFR inhibitors [114].

The combination of EZH2i and immunotherapy has attracted attention in recent years. It has been reported that EZH2 inhibition enhances antigen presentation and T cell infiltration in tumors [115]. However, a recent study found EZH2 inhibition resulted in metabolic exhaustion in tumor-infiltrating T cells. Moreover, multiomics analysis revealed that crosstalk between H3K27me and H3K36me led to mitochondrial dysfunction. The toxicity of EZH2 inhibitors to T cells suggests that the use of these drugs in combination is a challenge [116].

DOT1L inhibitors. DOT1L catalyzes H3K79 methylation, primarily di-methylation, which is a transcription activation signal. DOT1L is involved in mixed lineage leukemia (MLL)-rearranged leukemia, where chromosome translocations cause fusion proteins between MLL and partners that interact with DOT1L. The inappropriate recruitment of DOT1L leads to the upregulation of the *HOXA* cluster and the development of MLL-r leukemia [117].

As a promising therapeutic target, inhibitors against DOT1L have been tested for therapy of MLL-r leukemia. Pioneers such as EPZ-5676 have been proven safe in a phase I clinical trial, but mono-administration only caused a limited response in a limited

number of patients [118]. Therefore, researchers have focused on developing combined strategies for DOT1Li. DOT1L and BRD4 both function as epigenetic regulators in MLL-r leukemia, and combined quantitative proteomic and chemoproteomic data showed they had interdependence. The results of CHIP-seq and RNA-seq analyses showed that H3K79me facilitated H4K5ac and BRD4 binding. Taken together, these data show the synergistic efficacy of DOT1Li and BRD4i [119].

Efforts have been made to expand the application of DOT1Li to cancers beyond MLL-r leukemia. Indeed, in AR-positive prostate cancer, co-immunoprecipitation–mass spectrometry data showed that DOT1L inhibition increased the interaction between AR and the E3 ubiquitin ligases HECTD4 and MYCBP2, leading to degradation of AR and suppression of downstream MYC signaling [120]. In ER-positive breast cancer, CHIP-MS analysis identified DOT1L as a component of the ER complex [121]. These results indicate the potential benefit of DOT1L inhibition in hormone-dependent cancers.

Other KMTs inhibitors. Beyond EZH2i and DOT1Li, other KMT inhibitors at the pre-clinical stage have also shown potential as cancer therapeutics. For instance, G9a/GLP, which catalyzes the transcriptional suppression marker H3K9me, has attracted increasing attention in recent years due to its oncogenic role, with gain-of-function (GOF) mutations, gene amplification, and overexpression being reported in several cancer types. RNA-seq and proteomics analyses showed that the application of a G9a/GLP inhibitor regulated stress response genes and mTOR signaling in hepatic cell carcinoma, highlighting its therapeutic potential [122]. However, the clinical application of G9a/GLP inhibitors is hampered by their wide methylation substrates. Global detection of lysine methylation revealed non-histone substrates of G9a/GLP, including LIG1, p53, and WIZ; therefore, it is important to clarify the effect of G9a/GLP inhibitors in such substrates before clinical use [123]. A similar challenge is also faced by SMYD2 inhibitors. SMYD2 is a cytoplasmic KMT that catalyzes mono-methylation (MMA). Quantitative lysine methylome analysis revealed that non-histone lysine methylation sites regulated by SMYD2 inhibition, including AHNAK and AHNAK2, mediated migration and invasion [124]. Moreover, in recent years the non-histone methylation activity of SMYD2 has been reported to play an oncogenic role in cancer, although the detailed mechanism underlying the effects of SMYD2 inhibitors remains to be explored.

Lysine demethylases inhibitors

Lysine demethylases (KDMs) are divided into two classes according to their mechanism of action: FAD-dependent KDM1 and Fe (II), 2-oxoglutarate (2OG)-dependent JMJC demethylases [125]. KDM1A (also named LSD1) demethylates mono- and di-methylated H3K4 and assembles into a transcriptional repressor CoREST complex, which acts as an oncogene by inhibiting the differentiation of AML and Merkel cell carcinoma (MCC). Owing to the structural similarity to well-studied drug target monoaminoxidases (MAO), many MAO inhibitor derivatives have been developed to target KDM1, with pioneers currently in clinical trials [126].

Mono-administration of LSD1i has shown a variable response in AML, and a combination strategy such as LSDi combined with retinoic acid (RA) restores sensitivity. RA is a differentiation stimulus for the treatment of acute promyelocytic leukemia [127] - a subset of AML that exhibits resistance to LSD1i. The combination of LSD1i and RA showed a synergistic effect in both APL and non-APL LSD1i-resistant AML. Interestingly, the catalytic activity of LSD1 is dispensable in this synergistic effect. Interactome analysis showed that LSD1i dissociated LSD1 with the transcription factor GFI1, which acts as a brake to differentiation stimuli. This study emphasized the scaffolding role of LSD1 and suggested the combined administration of LSD1i and RA in resistant AMLs [128].

Cell differentiation status altered by LSD1i also sensitizes AML to kinase inhibition. The sequential application of epigenetic intervention and kinase inhibitors has been shown to increase the AML response; among those combination strategies, LSD1i + MEKi exhibited the strongest effect. Genetic, proteomics, and phosphoproteomics analyses showed that LSD1i reshaped the kinase network by switching PI3K signaling to MAPK signaling and suppressing the negative feedback that compensated for MEK inhibition. The research revealed crosstalk between epigenetic regulators and the kinase network, suggesting the potential of LSD1i to overcome resistance to MEKi [129].

In addition to AML, LSD1i also benefits therapy of other cancer types. For instance, in Merkel cell carcinoma (MCC), which is driven by Merkel cell polyomavirus integration and oncogenic T antigen expression, CHIP-seq and IP-MS analyses revealed that the T antigen transactivated the LSD1 complex to suppress differentiation-associated genes, such as bone morphogenetic protein (BMP) signaling factor. Moreover, combined RNA-seq and quantitative proteomics data showed that LSD1i upregulated proteins associated with cell adhesion and neuron differentiation, clarifying the detailed mechanism of LSD1 in MCC development and highlighting the potential of LSD1i in MCC treatment [130]. In breast cancer, LSD1 and KDM6A co-localized with ER. Interactome and CHIP-seq analyses showed that treatment with the LSD1-KDM6A dual inhibitor modulated the ER complex and hormone signaling pathway, suggesting that the application of combined inhibition of LSD1 and KDM6A may be useful in treating breast cancer [131].

Arginine methyltransferases inhibitors

Arginine can also be methylated in the form of monomethylation (MMA), symmetrical dimethylation (SDMA), or asymmetrical dimethylation(ADMA) [132]. Arginine methylation occurs on more than 4000 proteins and functions in epigenetic regulation, RNA processing, and translation. Protein arginine methyltransferases (PRMTs) transfer methyl groups to arginine and are divided into three families: type I (PRMT1, 2, 3, 4, 6, 8), which catalyzes ADMA; type II (PRMT5, 9), which catalyzes SDMA; and type III (PRMT7), which catalyzes MMA. PRMTs are targetable and this strategy is beneficial in cancer therapy [133, 134]. However, the lack of a holistic view of different substrates of PRMTs makes it difficult to fully elucidate the mechanism of PRMT inhibitors, which hampers their clinical application. Thanks to the development of efficient enrichment strategies, the establishment of confident site identification methods, and the discovery of potent and selective PRMT inhibitors, mass spectrometry-based analysis of PRMT substrates has recently become possible.

Type II PRMT inhibitors. PRMT5 is a predominant type II PRMT that is closely related to multiple cancers. In 2019, proteomics profiling of PRMT5 substrates was accomplished using pan-SDMA and -MMA antibodies to enrich modified peptides. Heavy methyl SILAC was used for highly confident site localization of the methyl group, while manual inspection of neutral loss on SDMA in MS2 was used to discriminate SDMA from ADMA. A group of altered methylation sites with high confidence were identified upon PRMT5 inhibition, with the majority shown to be involved in RNA processing. This study highlighted RNA processing as a downstream process of PRMT5 inhibition [135]. In the same year, similar work was carried out by another group; beyond the identification of altered methylation sites, this later study confirmed that SRSF1, a serine/arginine-rich splicing factor, is a direct PRMT5 target. PRMT5 regulates SRSF1 methylation to mediate its interaction with mRNA and proteins. PRMT5 inhibition disrupts the SRSF1 interaction network to induce alternative splicing of essential genes and further cell death in AML [136]. PRMT5 inhibitors have also been shown to suppress the proliferation of glioblastoma (GBM) cells by disrupting splicing and affecting cell cycle gene

products. In GBM cell lines with variable responses to PRMT5i, combined whole-genome seq, RNA-seq, proteomics, and metabolomics data showed that the previously reported genetic mutation markers (e.g., *MTAP*) did not contribute to sensitivity variance in GBN, nor did the key metabolite methylthioadenosine (MTA) level. However, splicing event analysis showed distinct splicing patterns between good and bad responders. These data indicate the potential of targeting PRMT5 in GBM and identified the markers of a therapeutic response [137].

Type I PRMT inhibitors. PRMT1 is a predominant type I PRMT and an emerging drug target (Table 3, Supplementary Table S3). Since discovery of efficient inhibitors, targeting PRMT1 has been widely studied. The type I PRMT Inhibitor GSK3368715 was discovered in 2019, with pronounced anti-tumor activity among AML, lymphoma, and subsets of NSCLC and pancreatic cancer. Affinity-enriched proteomics analysis revealed that type I PRMT inhibition altered methylation of proteins involved in RNA splicing, ribosomal activity, and MYC signaling, and further induced splicing alternation. Due to shared substrates with PRMT5, type I PRMTi was tested along with PRMT5i, and the results showed significant MMA, SDMA, and ADMA suppression, as well as splicing alternation. Moreover, in *MTAP*-deficient cancer, increased levels of its metabolite MTA inhibited PRMT5 and showed a synergetic effect with type I PRMTi [138]. In the same year, researchers found that spliceosomal mutant leukemias are preferentially sensitive to inhibition of PRMTs, with PRMTi + PRMT5i exhibiting synergetic effects. With high confident site localization of heavy methyl SILAC, data showed that two PRMT families catalyzed distinct substrates in the synergetic regulation of RNA splicing [139]. In 2021, proteomics profiling of arginine methylation and RNA splicing analysis was applied to study PRMT1 sensitivity in pancreatic ductal adenocarcinoma (PDAC). The results showed that PRMT1 inhibition impaired RNA processing and triggered the downregulation of DNA damage response pathways, indicating the therapeutic potential of PRMT1i in cancer with enhanced genomic instability, as well as its synergetic effects with DDR inhibitors [140].

UBIQUITIN-PROTEOMICS IN DRUG RESEARCH

Protein ubiquitination is orchestrated by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases; these catalyze the covalent binding of ubiquitins to the lysine residue or the N-terminus of substrate proteins, which can be reversibly regulated by deubiquinases (DUBs). The ubiquitin-proteasome system (UPS) is a ubiquitin-dependent protein degradation pathway mediated by proteasomes. The UPS maintains cellular homeostasis, and its dysregulation is closely related to tumorigenesis and development. Targeting the regulatory enzymes in the UPS is a powerful strategy, and several drugs have been approved by the FDA or are currently in clinical trials for tumor therapy (Table 4, Supplementary Table S4).

E1 enzymes

UAE (ubiquitin activating enzyme), one of two E1 enzymes, modulates the formation of the majority of protein ubiquitination. MLN7243 (TAK-243), a first-in-class inhibitor of UAE, inhibits the combination of UAE and ubiquitin by forming a TAK243-ubiquitin adduct, which ultimately induces cancer cell death by suppressing the ubiquitination-dependent protein turnover, impairing the cell cycle and DNA repair, and increasing proteotoxic stress [141]. MLN7243 has anti-tumor effects on small cell lung cancer, AML, and MM both in vitro and in vivo [142]. However, the clinical study of MLN7243 on solid tumors has been terminated due to serious adverse events. Nevertheless, MLN7243 still shows significant antitumor effects on hematologic malignancies.

NAE (NEDD8 activating enzyme) E1 enzyme activates a ubiquitin-like (UBL) protein NEDD8 and initiates the protein neddylation pathway. MLN4924 is a first-in-class inhibitor of NAE by forming a covalent adduct with NEDD8 [143], which has been in clinical trials for various cancers. Repression of the NEDD8 pathway regulated by MLN4924 leads to cell cycle arrest, DNA damage, and apoptosis. Cullin proteins are the core scaffold of the cullin-RING ligases (CRLs), and are the best-characterized

Table 3. The list of KMT, KDM, PRMT inhibitors[a].

Targets	Inhibitors
KMT	
EZH2	EPZ-6438(tazemetostat), CPI-0209, SHR-2554, MAK-683, CPI-1205, FTX-6058, PF-06821497
DOT1L	EPZ-5676
MLL1	DS-1594, SNDX-5613, KO-539, JNJ-75276617, BMF-219
SMYD2	EF-009
KDM	
LSD1	CC-90011, IMG-7289, GSK-2879552, ORY-1001, ORY-2001, HCI-2577, INCB-059872, TAS-1440, SYHA-1807, [18F]MNI-1054, ET-1002
KDM5B	GS-5801
PRMT	
PRMT5	AMG-193, MRTX-1719, PRT-811, PRT-543, JNJ-64619178, SCR-6277, SKL-27969, TNG-908

[a]The inhibitor information was obtained from cortellis drug discovery intelligence (https://www.cortellis.com/drugdiscovery/home?locale=en-US). Data are to June 2022.

Table 4. The list of E1 ligase, E3 ligase, proteasome inhibitors, and molecular glues, PROTACs[a].

Targets	Compounds
MDM2	RG7112, Idasanutlin, SAR405838, Milademetan, APG-115, AMG 232, NVP-CGM097, MK-8242
NAE	MLN4924
VCP/p97	CB-5339
CRBN	Thalidomide, Lenalidomide, Pomalidomide, CC-90009, CC-220, CC-122
Proteasome	Bortezomib, Carfilzomib, Ixazomib, Oprozomib, Delanzomib, Marizomib
DCAF15	Indisulam, CQS, Tasisulam
Heterobifunctional degraders	
Androgen receptor degrader	ARV-110, ARV-766, AR-LDD
Estrogen receptor degrader	ARV-471
BCL-XL degrader	DT2216
IRAK4 degrader	KT-474, KT-413
STAT3 degrader	KT-333
BTK degrader	NX-2127, NX-5948
TRK degrader	CG001419
BRD9 degrader	CFT8634, FHD-609
Molecular glue degraders	
IKZF2 degrader	DKY709
IKZF1/3 degrader	CC-92480, CC-99282, CFT7455

[a]The drug information was obtained from database (https://www.clinicaltrials.gov). Data are to June 2022.

substrates of the NEDD8 pathway [144, 145]. Given that MLN4924 is a specific inhibitor of NAE, non-cullin neddylation substrates regulated by NAE were system-wide profiled by the identification of the NEDDylome, which further revealed a new neddylation-mediated function in actin organization [146].

E3 ligases
E3 ubiquitin ligases confer the specificity of ubiquitinated substrates, which regulate the stability of several tumor promoters and suppressors. Numerous E3 ligases are considered as important targets for tumor therapy, and several E3 modulators have been approved or are currently in clinical trials for treating various cancers.

MDM2 (mouse double minute 2) is the primary negative regulator of the tumor suppressor p53. The overexpression of MDM2 in many human tumors impairs the function of p53 [147]. Accordingly, the inhibition of MDM2 activity can lead to p53 activation and apoptosis. Since the first MDM2-p53 antagonist was reported, eight MDM2 inhibitors have been evaluated in the clinic [148].

CRBN (cereblon) is a subunit of CRL4^{CRBN} (CUL4–DDB1–RBX1–CRBN) E3 ubiquitin ligase, which is the specific target of immunomodulatory imide drugs (IMiDs), such as thalidomide and its analogs. Thalidomide was initially used as a sedative to treat the morning sickness experienced by pregnant women, but it led to the birth of more than 10,000 teratogenic children [149]. Nevertheless, IMiDs exert potent effects as tumor therapies. Currently, lenalidomide and pomalidomide have been approved for the treatment of MM and myelodysplastic syndromes, while other thalidomide analogs, such as CC-122, CC-220, and CC-90009, are used clinically [150].

Recently, the mechanisms of action (MOAs) of IMiDs have been delineated. Affinity purification mass spectrometry (AP-MS) technology first demonstrated that CRBN is the direct target of thalidomide and could form a complex with CUL4 and DDB1 protein to function as an E3 ubiquitin ligase [151]. Since then, further evidence has confirmed that the CRBN protein is necessary for the pharmacological activity of IMiDs. Moreover, structural analysis has shown that IMiDs bind in a hydrophobic pocket on the surface of CRBN by a glutarimide ring, while another moiety interacts with diverse substrates to induce protein degradation via the ubiquitin-proteasome system [152–154]. For example, system-wide quantitative proteomics analysis demonstrated that the key transcriptional factors in hematological differentiation, Ikaros and Aiolos, could be induced for degradation by all thalidomide analogs [155]. Protein kinase casein kinase 1α (CK1α) is only the degradation substrate induced by lenalidomide [156]. Another study systematically identified lenalidomide-induced CRBN-binding proteins in MM, further demonstrating that some of these proteins, such as IKZF1 and KPNA2, were related to response and survival outcomes in patients with MM [127]. In addition, An et al. performed pulse-chase SILAC (pSILAC) mass spectrometry-based proteomics, and found that lenalidomide could also induce the ubiquitination and degradation of ZFP91 [157]. By comparing the proteome changes after thalidomide, lenalidomide, and pomalidomide treatment, the neo-substrate of IMiDs, SALL4, was found, which revealed a new mechanism for the species-specific pathogenesis of IMIDs teratogenicity [158]. Recent studies have demonstrated that other potential proteins containing a Cys2-His2 (C2H2) zinc-finger domain, such as the known substrates Ikaros, Aiolos, ZFP91, and SALL4, could be induced to degrade by IMiDs [159]. Additionally, CC-885, another thalidomide analog, exerts a more potent anti-tumor effect on diverse cancers by inducing GSPT1 for degradation [160]. Our recent study found that BNIP3L, a protein related to mitophagy, was a neo-substrate of CC-885-induced degradation [161]. These studies highlight a novel therapeutic approach of targeting protein degradation for cancer therapy. Currently, a novel GSPT1-selective cereblon E3 ligase modulator CC-90009 has been developed and evaluated in a phase I trial for AML.

IMiDs are the mainstay of MM therapy. Nevertheless, most patients are susceptible to drug resistance and relapse. Previous studies have indicated that mutation and abnormal expression of CRBN, and mutation of substrates and downstream genes are potential mechanisms of IMiD-induced drug resistance [162–164]. A recent study performed integrated global quantitative TMT-based proteomic and phosphoproteomic analyses and RNA sequencing in patients with MM and unveiled a CDK6-governed drug resistance signature in non-genetic resistance mechanisms [25]. These proteomics-based studies provide potential strategies of drug combination therapy for relapsed MM therapy.

DCAF15 can form an E3 ligase complex with CUL4 and DDB1, and specifically ubiquitinate and degrade substrates via the proteasome-mediated pathway. The aryl sulfonamide agents, indisulam, CQS, and tasisulam, function as molecular glues to recruit neo-substrates for ubiquitination and degradation by targeting DCAF15 E3 ligase, which are being evaluated in the clinic. Almost simultaneously, two studies discovered that RBM39 (also known as CAPERα) is recruited to CRL4^{DCAF15} for ubiquitination and degradation by aryl sulfonamide agents in various cancer cell lines, which affects RNA splicing and leads to cancer cell apoptosis [165, 166]. Furthermore, Lu et al. applied proximity labeling and a quantitative proteomics approach to confirm that indisulam could indeed enhance the interaction between RBM39 and DCAF15 [167]. Moreover, a recent study integrated transcriptomics, proteomics, and metabolomics in neuroblastoma models treated with indisulam, demonstrating that indisulam also resulted in cell cycle disruption, metabolome perturbations, and mitochondrial dysfunction, in addition to splicing errors by inducing rapid loss of RBM39 [168]. In addition to the known substrate RBM39, using the pSILAC method, we previously showed that PRPF39 was an indisulam-dependent DCAF15 neo-substrate[169]; this finding indicates that more substrates might be induced for ubiquitination and degradation by aryl sulfonamide agents.

The MOAs of these IMiDs and aryl sulfonamide agents deliver a new concept of a “molecular glue” to induce specific proteins for ubiquitination degradation by targeting E3 ligases, providing a novel therapeutic strategy for tumor treatment [170]. Similarly, PROTACs, a type of heterobifunctional small molecule, can simultaneously bind target protein and E3 ubiquitin ligase and promote the ubiquitination and degradation of undruggable proteins. Currently, more than 100 proteins can be induced to degrade by PROTAC-based therapy, and show more potent anti-tumor effects than traditional inhibitors. Of these compounds, 12 PROTAC degraders have been used clinically by targeting androgen receptor, estrogen receptor, BCL-XL, IRAK4, STAT3, BTK, TRK, or BRD9 for degradation [171]. Molecular glue/PROTAC can lead to downregulation at the protein level, upregulation at the ubiquitination level, and enhance the interaction between the target protein and E3 ubiquitin ligase; therefore, MS-based quantitative proteomics is a powerful approach to globally profile the proteome, ubiquitinome, and interactome. Fig. 4 provides a summary of a multiomics strategy to identify the molecular glue/PROTAC-induced substrates in a system-wide and unbiased manner.

In addition, CRLs are the largest E3 ubiquitin ligase family in eukaryotes. Neddylation of cullin proteins is necessary for CRL activity, and further regulates CRL-dependent protein ubiquitination degradation. As above mentioned, MLN4924 can increase CRL-mediated substrates by inhibiting the activity of cullin proteins. Thus, by applying MLN4924 treatment and quantitative proteomics technology, previous studies have identified the CRL substrates and dynamics of the CRL network [172, 173]. Our recent study systemically unveiled a series of phosphorylation-dependent ubiquitinated substrates regulated by SKP1-CUL1-F-box (SCF) ubiquitin ligases by using this strategy [174], the results

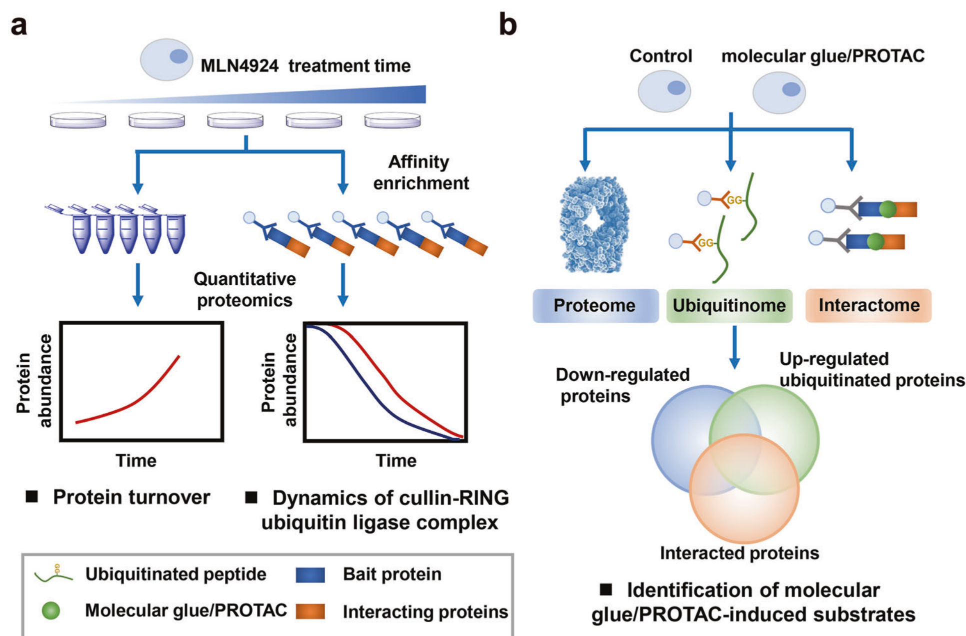

Fig. 4 Proteomics strategies to explore protein turnover, E3 ligase complex dynamics and degraded substrates regulated by drugs targeting ubiquitin-proteasome system. a Cells are treated with MLN4924 for incremental time. Samples from different treatment time are quantified to explore cumulative curve of MLN4924-regulated proteins. In these samples, cullin-interacting proteins were pulled down by bait protein cullin to study the dynamics of CRL E3 ligase complex. **b** Molecular glue/PROTAC-induced degraded substrates were identified by integrating proteome, ubiquitinome, and interactome after drug treatment by quantitative proteomics coupled with affinity enrichment approach.

of which confirmed the effect of PTM crosstalk on protein degradation.

Proteasome

The proteasome is a large and multiunit complex containing two 19S regulatory subunits and a 20S catalytic subunit, which mediates cellular protein degradation [175]. Proteasome inhibition is an important anti-tumor therapy for various cancers, with three drugs (bortezomib, carfilzomib and ixazomib) currently approved by the FDA and three compounds (oprozomib, delanzomib, and marizomib) in the clinical trials for MM and MCL.

Bortezomib is a first-generation proteasome inhibitor that was first approved by the FDA in 2003 [176]. By activity-based protein profiling (ABPP) and LC-MS/MS analysis, known bortezomib-targeting proteasome subunits, such as β5/β5i and β1/β1i subunits, were identified, and the potency and subunit specificity of bortezomib were accurately quantified [177]. During bortezomib-induced apoptosis, the global changes in the transcription, translation, and proteolytic degradation levels were examined, with the results demonstrating that non-caspase proteolytic events were also involved in cellular deconstruction [178]. The second inhibitor, carfilzomib, was approved in 2012 [179], with lower neurotoxicity than bortezomib. A recent study compared the global protein levels in cells treated with bortezomib and carfilzomib, and found that the bortezomib-induced toxicity might be related to cytoskeletal damage, excessive protein carbonylation, and actin filament destabilization [180]. Carfilzomib can covalently bind β5 subunit of the proteasome to block proteasome activity; nonetheless, two carfilzomib-targeting proteins, cytochrome P450 27A1 (CYP27A1) and glutathione S-transferase omega 1 (GSTO1), were identified using click chemistry coupled with streptavidin and the shotgun tandem mass spectrometry approach [181]. The analysis of phosphoproteomics and transcriptomics after carfilzomib treatment uncovered an unrecognized PI mechanism regulated by additional modes of spliceosome modulation, implying that a co-inhibitor of the spliceosome and proteasome might be a more potent anti-tumor activity for MM [182]. Additionally, the mechanisms of PI-induced drug resistance were explored by quantitative proteomics, and the analysis of global protein changes suggested that rewired glucose metabolism [183] and compensation of the autophagy-lysosome system [184] were related to bortezomib-induced drug resistance.

Given that proteosome inhibitors can lead to the accumulation of ubiquitination signaling and proteins by suspending the degradation of ubiquitinated proteins, proteosome inhibitors are often used as tools to measure the protein ubiquitination and proteome dynamics [185, 186]. Using SILAC-based quantitative mass spectrometry, the signature of the unfolded protein response (UPR) and large-scale feedback inhibition of protein synthesis after PI treatment were revealed [187]. Additionally, the global changes in the protein, ubiquitination, phosphorylation, and acetylation levels disrupted by bortezomib were integrated and analyzed via serial enrichment of different post-translational modifications by SILAC-based quantitative proteomics [188]. This study illustrated that the networks of the main cellular processes, such as the cell cycle, replication, transcription, translation, and the proteasome, were co-regulated by changes in protein abundance and PTMs induced by bortezomib.

Deubiquitinase

USP14 is a proteasome-related deubiquitinase, which has a dual role in promoting protein degradation by activating the proteasome and inhibiting protein degradation by removing the ubiquitination chain. USP14 is involved in diverse canonical signaling pathways by mediating the stability of substrates. Our previous system-wide study identified the ubiquitinated substrates of USP14 by proteome, ubiquitinome, and interactome analysis [189], providing a useful resource for elaborating the biological characteristics of USP14.

As USP14 is associated with various cancers, various inhibitors have been developed for cancer therapy [190]. VLX1570 inhibits

the activity of USP14 in a competitive mode, which can lead to the accumulation of ubiquitinated proteins and induce the apoptosis of MM cells. Although the combination of VLX1570 and dexamethasone has entered a phase 1/2 clinical trial for MM, it was terminated because of its severe toxicity.

p97
The p97 (also known as VCP) ATPase is a key component of UPS, which can extract ubiquitinated proteins from different cellular components for degradation with the aid of other co-factors. The mutation and overexpression of p97 protein is related to various cancers, and the inhibition of p97 is an important anti-tumor therapy. CB-5083 is the first ATP-competitive p97 inhibitor in the clinic, but the phase I clinical trials have been terminated due to severe side effects. CB-5339, the second-generation ATP-competitive p97 inhibitor, is currently being evaluated in the clinic for AML.

The cofactors and substrates of p97 have been identified by system-wide proteomic profiling of p97 inhibitor treatment [191–193]. The MOAs of the p97 inhibitors were dissected using a quantitative proteomics approach, with the results indicating that p97 inhibitors could upregulate UPR and factors associated with protein processing in the ER pathway, block E2F1-mediated transcription via downregulation of the CCND-CDK4/6 complex, and promote the downregulation of cell cycle oncoproteins in a p97-dependent manner [194, 195]. In addition, by using chemical proteomics and drug-induced thermal proteomics approach, a previous study found a p97-independent manner for anti-tumor effects of p97 inhibitor [196].

PTM CROSSTALK STUDY-BASED DRUG RESEARCH

PTMs regulate enzymatic activity, protein interactions, and subcellular location, among others. PTMs on protein promote rapid cellular responses to diverse stimuli from both inside and outside the cell. Increasing studies have proved that different protein PTMs often interact with others (known as crosstalk) to form a dynamic regulatory network and finely regulate the whole life phenomenon. For example, PTMs on histone proteins are known as histone codes, and numerous studies have shown the intrinsic interactions between different PTM types or modified sites that jointly regulate DNA transcription. Moreover, it has been reported that the interaction of histone methylation and acetylation regulates gene expression [197]. Histones can also recruit epigenetic regulatory enzymes through specific site ubiquitination to regulate histone methylation/acetylation levels and further affect gene expression [198]. In terms of protein degradation, some proteins require phosphorylation, methylation, or acetylation to be recognized and degraded by the ubiquitination-proteasome system [199]. Our previous study proved the universality and specificity of protein phosphorylation during ubiquitination and degradation [148].

PTM crosstalk studies are common in life science and drug research and provide a global and synergistic perspective for pharmacodynamic marker screening, fine mechanism analyzing, and drug combination application strategy development. The research summarized in this manuscript highlights the overlap between different kinase-mediated signal pathways. Therefore, combinations of different targeting drugs are often used to regulate different modification types or sites to synergistically block disease-related signal pathways, with the end goal to successfully treat disease or overcome resistance.

Our previous study quantitatively analyzed the global histone PTMs in different tumor cells with different sensitivities to the lysine methyltransferase EZH2 inhibitor. We revealed that the interaction between histone H3K27 methylation and acetylation was the key mechanism leading to drug resistance. Therefore, the co-intervention of tazemetosat and JQ1 to inhibit the level of methylation and acetylation modification could significantly increase the efficacy of EZH2 inhibitors in the treatment of solid tumors [112]. We also analyzed nearly 20,000 phosphorylation modification sites of *KRAS* mutant tumor cell lines and developed a novel drug combination scheme prediction strategy (CPBA) based on the complementary correlation between phosphorylation and drug sensitivity. Using this strategy, we found a series of new drug combination stragies for precise treatment of one *KRAS* mutant cancer subtype. We finally verified that the combination of the histone methyltransferase DOT1L inhibitor (SGC0946) and phosphatase SHP2 inhibitor (SHP099) has an effective synergistic effect in treatment of the most maligant *KRAS* subtype at both cellular and animal model levels [200]. Our previous study also revealed that AMPK regulated a wide range of signaling pathways in the DNA damage response by using quantitative phosphorylation proteomics. Moreover, the BRD4 inhibitor JQ-1 has been shown to further enhance AMPK by interfering with histone acetylation levels [201].

CONCLUSIONS AND FUTURE PERSPECTIVES

Proteomics technologies remarkably benefit PTM-associated drug research. For kinase inhibitors, phosphoproteomics provides a landscape of kinase network disturbance. For inhibitors against acetylation and methylation, global PTM analysis reveals a holistic view of altered histone marks, the acetylome and methylome, and systematically uncovers non-histone substrates. For inhibitors against ubiquitination, combined ubiquitinome and proteomics analysis can be used to monitor global protein turnover and identify substrates of molecular glue/PROTAC. These strategies benefit the delineation of drug mechanisms, potential off-target effects, and drug resistance mechanisms, as well as the development of synergistic drug combinations. Moreover, PTM crosstalk commonly exists in cells: cellular signaling pathways in kinase network interplay with each other, histone modifications exhibit meticulous crosstalks for epigenetic regulation, and ubiquitination can be modulated in an acetylation- or a phosphorylation-dependent manner. Therefore, targeting PTM crosstalk has emerged as a powerful strategy in drug research. Due to the ultra-depth landscape profiling and multiomics integration ability, proteomics technologies exhibit outstanding advantages in the study of PTM crosstalks in diseases and for the development of new drug therapies.

ACKNOWLEDGEMENTS

This work was supported by grants from the Basic research projects of Shanghai Science and Technology Commission "science and technology innovation action plan" (No. 19JC1416300), the National Natural Science Foundation of China (No. 32071432; No. 32171434), the Youth Science and Technology Talents in Shanghai Sail Plan of China (No. 21YF1456000), the State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, China (Grant No. KF-202201), the Innovative Research Team of High-Level Local Universities in Shanghai (SHSMU-ZDCX20212700), the Guangdong High-level new R&D institute (2019B090904008), the Guangdong High-level Innovative Research Institute (2021B0909050003), the Program of Shanghai Academic Research Leader (22XD1420900).

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41401-022-01017-y.

Competing interests: The authors declare no competing interests.

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