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A brief introduction to chemical proteomics for target deconvolution

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Abstract. – OBJECTIVE: Drug-target relationships provide the basis for network-based polypharmacology, and target deconvolution is a key step in phenotypic-screening based drug discovery. Due to the complexity of the mammalian proteomics and the often-limited affinity of the lead compound, it is challenging to identify the drug targets, especially when the goal is to identify all targets. This paper attempts to provide a brief and comprehensive introduction to the various methods in chemical proteomics for target deconvolution by categorizing them into two groups: the biochemical enrichment and the proteomics-screening methods. Moreover, a brief introduction of related Mass Spectrometry techniques is also provided, together with recent progress.

MATERIALS AND METHODS: The data for this review were queried from Web of Science and PubMed, the keywords used were Drug targets, Target deconvolution, and Chemical Proteomics. A total of over 500 relevant articles, with a time limit from 1953 to 2022, were identified according to search strategy. Duplicate records and review articles were excluded by their titles and abstracts. Finally, we found about 120 articles matching our inclusion criteria, which covered representative research and reviews of various target discovery methods.

RESULTS: Existing target discovery methods can be grouped into either biochemical enrichment or the proteomics-screening methods, with the recent emergence of a hybrid method combining these two such as lysine reactivity profiling. The advantage of the biochemical enrichment method is the ease of operation and the comprehensive target coverage. However, most biochemical enrichment methods require a high-affinity binding of the drug to the target proteins and cannot differentiate direct/indirect targets. The proteomics-screening methods do not require drug modification but have limited protein coverage, and most of them cannot differentiate direct/indirect targets.

CONCLUSIONS: Although existing target discovery methods have greatly facilitated pharmacological research, each of these methods has advantages and disadvantages. New strategies/methods are needed to further improve both the coverage of the proteosome and the specificity.

Key Words:
Drug targets, Chemical proteomics, Target deconvolution, Biochemical enrichment, Proteomics-screening, Mass spectrometry.

Introduction

More than 200 years ago, Sertürner[1] successfully extracted morphine from poppies, opening a new era of new drug discovery from plants. Currently, there are two main strategies for discovering small molecule drugs: target-based drug discovery and phenotypic-screening based drug discovery[2]. Target-based drug discoveries firstly identify a protein target or a group of targets critical for etiology, then screen compound libraries for hits. Phenotypic-screening based drug discoveries firstly screen compound libraries for hits that induce certain phenotypes, then identify the drug targets and further optimize the hits[3-5]. Historically most first-in-class drugs are discovered through phenotypic-screening[6], while the target-based drug discovery pathway has been rising along with a deeper understanding of disease etiology. With the development of multi-omics technologies, phenotypic-screening has been re-surging[7].

Drug-target relationships provide essential information for both phenotypic-screening based drug discovery, and the construction of pharmacology networks. Moreover, the combination of phenotypic screening with network pharmacology provides a viable option for discovering drugs with multiple targets, which could be advantageous for treating diseases with complex etiology[8]. Finally, the molecular action mechanism of some clinical drugs remains unknown[9], and the discoveries of the drug targets will pave the way for drug repurposing[10-12].

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A variety of methods have been developed for target discovery, including but not limited to chemical proteomics, CRISPR/RNAi libraries, other "omics" technologies, and computational predictions, or the combinations of them, for example, network pharmacology and molecular docking are helpful in exploring the effective components and mechanisms of traditional Chinese medicine[13,14]. The main goal of this paper is to give a brief but relatively comprehensive review of chemical proteomics methods. Chemical proteomics target finding methods can be classified into two categories: one is a biochemical enrichment strategy, i.e., firstly enrich the protein targets using drug-affinity capture, then identify the enriched proteins by MS; the other is a proteomics screening strategy, i.e., firstly subject proteosome samples to denaturing/digestion conditions with/without the drugs, then identify the protein targets based on stability changes induced by the drug via proteosome analyses. Reviews have been published on chemical proteomics for target identifications[15-17]. This paper briefly summarized these two categories of methods, emphasizing the method comparisons and recent developments, followed by a brief introduction to MS techniques involved.

Enrich Drug Targets Using Probes

This strategy uses a drug-based probe to enrich the protein targets, and a typical process is composed of the following steps: connecting the drug to a tag to form a probe, probe binding to the protein targets, "fishing" out the drug-target complexes using the tag and determine the protein targets identities by MS[18]. Chen et al[19] published a recent review on this path. For this strategy to work well, probes ideally shall have a high affinity to the protein targets, which often is not the case with the un-optimized lead compounds, so strategies have been improved to solve this problem.

Direct Enrichment

An early strategy directly fixes drugs to solid carriers (usually agarose or magnetic beads) for affinity chromatography[20-22] (Figure 1). The advantage of this method is the ease of use; however, protein target binding could be compromised due to the steric hindrance created by the anchoring to the solid carrier[23]. Furthermore, non-specific bindings, either to the probe or to the beads are inevitable, which requires a drug with sufficient affinity[24-26]. With the emergence of new methods, this method is less applied, and new research along this path is focused on improving solid-phase materials, such as using nanoparticle Fe_3O_4 as a carrier[27]. In addition, there is a Unique Polymer Technology (UPT) method that uses weak interactions to adsorb small molecules on polymers, and then uses polymers to catch the protein targets[28].

Enrichment With a Tag

To solve the steric hindrance introduced by the first method, a "tag" for later enrichment is added to the drug by covalent attachment *via* a linker (Figure 1). Biotin is often adopted as the tag, together with a solid carrier coated with streptavidin for enrichment[29]. The advantage of this method over the first one is that it allows the drug to contact the sample more completely and has been successfully applied to identify the protein targets of withaferin A, adenanthin, and berberine[30-32].

However, this method still requires a drug with high affinity. When the affinity is not sufficient, choosing the strength of the washes after the pro-

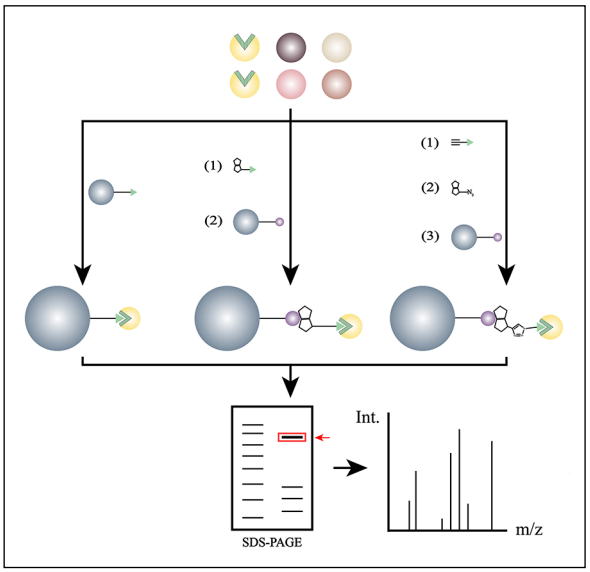

Figure 1. Enrichment of protein targets by drug-based affinity chromatography. The drug is directly immobilized on a solid phase, then incubated with the protein lysate to capture the targets (*left*); A probe of the drug and a biotin tag mixed with the protein lysate, and the target proteins enriched by biotin-streptavidin (*middle*); A probe of the drug and an alkyne tag mixed with the protein lysate, and the target proteins enriched via click chemistry (right). The enriched target proteins are identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and LC-MS/MS analysis.

tein-probe binding becomes a catch-22 scenario: less stringent washes could lead to the identification of non-specific proteins as targets, while stringent washes could lead to the loss of the target proteins. Moreover, the design of the linker between biotin and the drug might need optimization: the linker length shall be long enough to prevent steric repulsion, while it does not bind to proteins nor change the probe solubility[33-37].

Since biotin could still create steric hindrance and reduce the activity of the drug, smaller groups like the alkyne groups for click chemistry have been adopted as the tag in the probes[38-41] (Figure 1). After the probes form complexes with protein targets, azide-functionalized solid carriers are applied so that click chemistry reaction occurs to connect the probes to the solid carriers, which are then enriched for identification of the protein[42,43]. Alkyne or azide are smaller in size and have less impact on the activity of the drug, and the discovery of drug targets is likely to be successful[44].

It is worth noting that the above strategy still requires the drug to have a sufficient affinity to the protein targets. The introduction of a covalent bond between the protein targets and the drug could efficiently stabilize the interaction, hence the "enrichment with covalent linkage" strategy is developed.

Enrichment with Covalent Linkage

The "enrichment with covalent linkage" strategy enables the formation of a covalent bond between the probe and protein targets. For a subclass of the drug targets, the enzymes, there is a well-established activity-based protein profiling (ABPP) method that uses probes containing electrophilic groups to react covalently with enzyme active site nucleophiles, which stabilize the drug-target interactions[45,46]. A set of methods has been developed based on ABPP, including the fluorescence polarization (fluopol)-ABPP, isoTOP-ABPP, the reductive dimethyl tandem orthogonal proteolysis (rdTOP)-ABPP, the quenched near-infrared fluorescent (qNIRF)-ABPP, and the quantitative acid-cleavable (QA)-ABPP, etc. A recent review[47] has provided a succinct summary of these methods.

For another subclass of the drug targets, the membrane receptors, a method called "GLiCo-Click" uses a click reaction between a glycan on the target proteins, and a clickable group attached to the lead compound, forming a covalent link between the target protein and the lead compound[48].

A method with wider application scope is the photoaffinity labeling technology (PAL), theoretically applicable for all protein targets. PAL is centered on a photo-affinity probe (PAP)[49], which is composed of three parts: the drug, the photo-active group, and a tag for later enrichment[50,51] (Figure 2). The photo-active group forms a highly reactive intermediate under the irradiation of light of a suitable wavelength, which reacts with the amino acids of nearby proteins, thereby stabilizing the drug-target interaction[52,53]. Protein enrichment, mass spectrometry, or microscopic imaging experiments can be performed to identify, quantify, or visualize the target protein[54-60].

In the early studies, benzophenone was used as the photoactive group due to its stability and ease of synthesis[61]. The disadvantage is that the steric hindrance is large, and the ultraviolet irradiation time is long, which may cause protein damages[62-65]. In recent years, the more efficient and smaller bisaziridine groups have been applied[66]. In 2016, Li et al[67] developed a new type of tetrazole-based photo-crosslinking

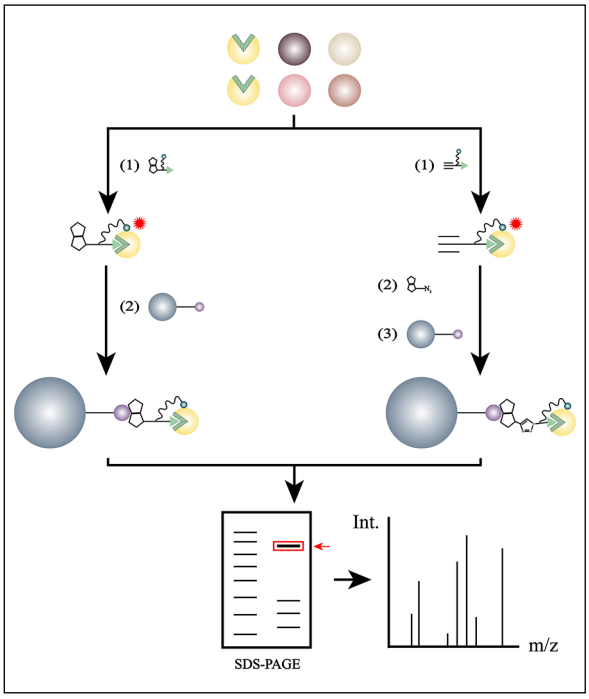

Figure 2. The process of enrichment with covalent linkage using photoaffinity technology. The Photoaffinity probe is composed of the drug, a photo-active group, and a tag for enrichment. After the probe binds to the target protein, the photo-active group reacts with the protein upon activation with the light of a specific wavelength. Then enriched and purified by biotin-streptavidin (*left*) or click chemistry (right), depending on the choice of tag. Then target proteins can be identified and analyzed by SDS-PAGE and LC-MS/MS.

agent, which can effectively reduce non-specific marks and show excellent photo-crosslinking efficiency. However, PAL has its limitations: the efficiency of the photoreaction is not very high, and some natural products lack suitable sites that can be used to introduce tags[68]; furthermore, the photo-active group can react with any protein in the vicinity. The success rate of PAL is not high enough. Recently a cleavable tag has been added to the PAL method for further improvement of the success rate[69].

Enrichment from Display Libraries

Targets are enriched from display libraries, including phage, yeast, and other display libraries with probes (Figure 3). Moreover, a three-hybrid system based on the yeast two-hybrid system has been developed[70,71]. There are several advantages of using a display library instead of cell lysates: (1) Retrieval and identification of protein targets are easy; (2) The expression level of the displayed peptides will be less variable compared to the whole-cell lysates, where there is a bias toward the identification of highly expressed proteins as drug targets; (3) Due to the limited displayed peptide length, only direct targets that bind to the leads will be identified, while indirect targets won't. To take full advantage of the robustness of the display platform, Wu et al[72] have combined the compounds library with the phage-displayed proteosome and achieved rapid discovery of the leads.

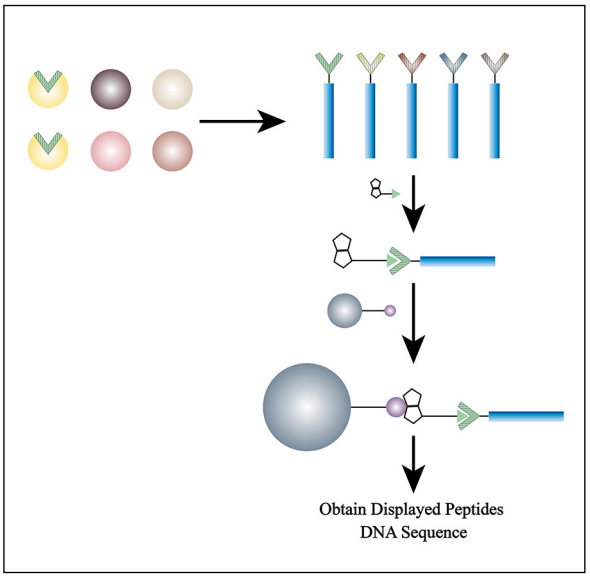

Figure 3. Using display libraries to identify small molecule targets. Small molecule probes are co-incubated with phage or yeast display libraries for enrichment, then the protein targets are identified by the DNA sequences encoding the displayed peptides.

However, the limited display of sequences also means that real targets will be missed in several scenarios: the displayed peptides from the protein targets could have lost the 3D structure and failed to bind to the drug; post-translational modifications can't be displayed in phage libraries, etc.

Proteosome Screening Using Stability Changes

This group of methods relies on the protein stability changes associated with drug binding to distinguish the target proteins from the other proteins[73,74]. A big advantage of these methods is they utilize the drug-induced changes in the energetics or biophysical properties of proteins and do not require any chemical modification of the drug[75-77]. Three aspects of protein stability have been utilized including chemical, thermal, and proteolysis stability, by subjecting the proteins to chemical denaturant, heating, or protease. Methods including Stability of Proteins from Rates of Oxidation (SPROX), Thermal Proteome Profiling (TPP), Drug Affinity Responsive Target Stability (DARTS), Limited Proteolysis (LiP), Chemical denaturation and Protein Precipitation (CPP), and Pulse Proteolysis (PP) have been reviewed in several papers[78,79]. Here, we briefly introduce these methods, highlighting several recent developments.

Stability Against Proteolysis

This aspect of stability has been creatively utilized in DARTS[80,81] (Figure 4a). DARTS firstly digests the protein samples with/without the lead compound with non-specific protease, then analyzes the samples by electrophoresis and MS[82,83]. DARTS has been used for the identification/verification of a variety of drugs including FK506, rapamycin, terpenoid Laurifolioside[84], etc.

LC-MS/MS has been adopted to perform gel-free DARTS. The advantage of DARTS technology is its simple operation, the experiment process does not include washing steps, so it applies to low-affinity drugs[85-87]. Furthermore, indirect protein targets are digested, while direct targets are identified. However, some proteins in cell lysates are resistant to hydrolytic enzymes and may interfere with the determination of the results[88]. Moreover, methods utilizing stability against proteolysis are not fitful when the binding of the drug does not protect the protein targets from protease digestion.

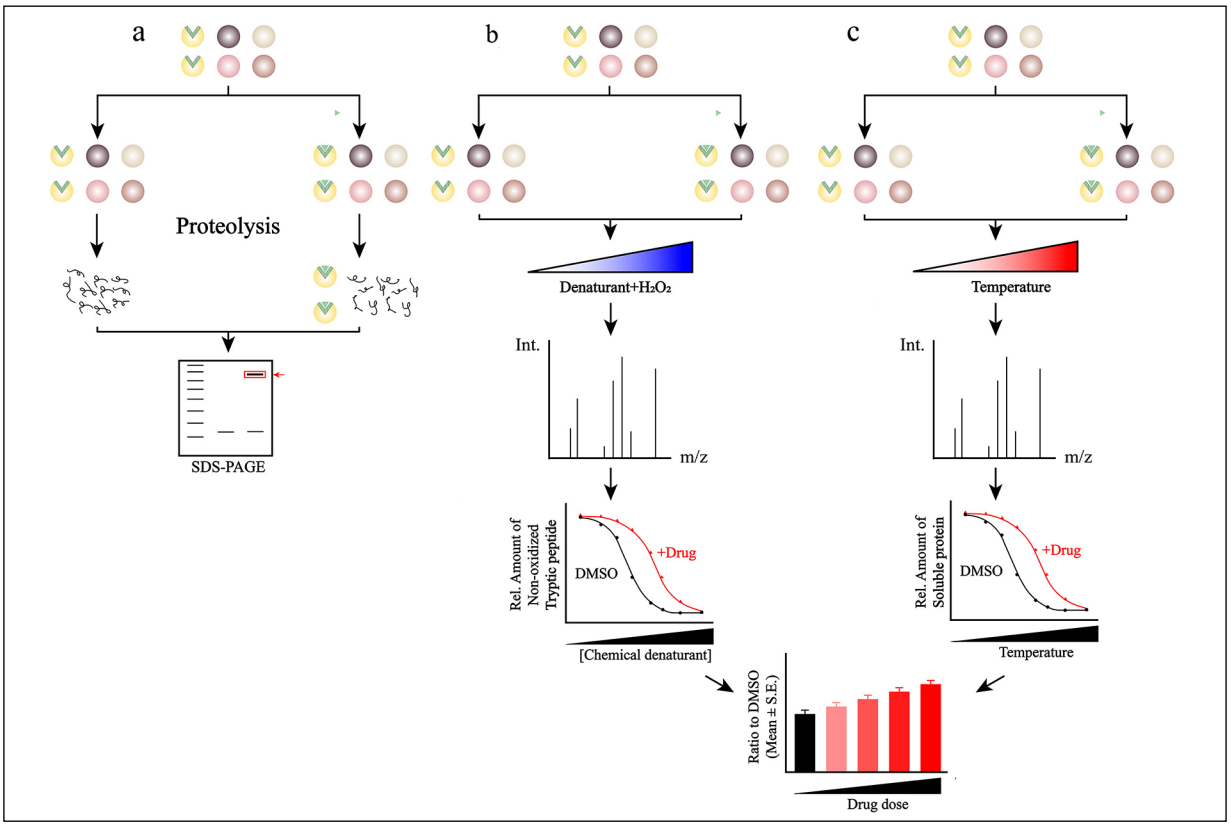

Figure 4. Proteomic screening strategies for target identification based on protein stability changes. **a**, DARTS firstly digests the proteins samples with/without the lead compound with non-specific protease, then analyzes the samples by electrophoresis and MS. **b**, SPROX firstly subjects proteins with or without the drug to a series of chemical denaturant concentrations to cause protein unfolding and the exposure of buried methionine, then use H_2O_2 to oxidized the methionine, and quantified the oxidized or the non-oxidized methionine-containing peptides by digestion and MS. **c**, CETSA. Protein lysates samples are subject to a set of heat denaturation at different temperatures, with/without drugs. Then the samples are centrifuged, the proteins in the solution analyzed by Western blot, and the drug targets identified by their shifted denaturation curves.

Limited proteolysis (LiP) is similar to DARTS. In that protein samples with or without drugs are treated with non-specific protease, quenched, and the cleaved peptides are measured by LC-MS/MS[89]. LiP is different from DARTS in their detection objects: LiP is focused on the detection of peptides while DARTS is focused on the detection of proteins[90]. So, LiP has a peptide-level resolution of the ligand-binding sequences. A succinct summary of LiP described its advantages and disadvantages[91].

Stability against Chemical Denaturant

This aspect of stability is utilized by subjecting the proteosome to a series of chemical denaturant concentrations, with or without the drug, and measuring the stability of proteins[92]. Proteosome of the drug-treated group is compared to that of the non-treated control, to identify the proteins with altered stability curves. These are essentially chemical denaturant-induced unfolding experiments where the folded and unfolded proteins are measured.

SPROX firstly subject proteins with or without the drug to a series of chemical denaturant concentrations to cause protein unfolding and the exposure of buried methionine, then uses H_2O_2 to oxidize the methionine, and quantified the oxidized or the non-oxidized methionine-containing peptides by digestion and MS[93] (Figure 4b). When drug binding protects the target proteins from the chemical denaturant, the methionine-peptide oxidation rate is reduced, and the oxidized /non-oxidized ratio changes. The detection of methionine-peptide with conventional bottom-up shotgun proteomics platforms, such as LC-MS/MS has limited proteosome coverage, probably because methionine is the second rarest

amino acid. Alternatively, Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) is adopted to expand the proteosome coverage (SILAC-SPROX)[94].

Both CPP and PP share with SPROX similar denaturant treatment schemes. After the denaturation, CPP dilutes and centrifuges the treated samples, then quantifies the soluble or precipitated proteins by MS[95]. PP adds additional proteolysis treatment and measures the intact or cleaved peptides after chemical denaturation. A comparison of these methods reveals that PP has a similar proteosome coverage as SPROX, while CPP provides affinity estimation (Kd) and better proteosome coverage.

Recently Zhang et al[96] have developed a proteosome screening method named "solvent-induced protein precipitation, SIP" that measures protein stability against chemical denaturants (organic solvents). SIP subject the drug-treated or untreated proteosomes to a mixture of organic solvent, then detect the non-denatured proteins in the solution[97]. SIP is further combined with quantitative proteosome technology and area under the curve analysis to improve the proteosome coverage.

Thermal Stability

The binding of a drug may increase or decrease protein targets' thermal stability. Martinez et al[98] utilized this property and developed the CETSA technology (Figure 4c). Protein lysates samples are subject to a set of heat denaturation at different temperatures, with/without drugs. Then, the samples are centrifuged, the proteins in the solution analyzed by Western blot, and the drug targets identified by their shifted denaturation curves. The throughput of the original CETSA is low, then the proteomics techniques have been combined with CETSA, including 2D gel electrophoresis[99], isobaric tandem mass tag (TMT)-based quantitative proteomics[100], etc. Thermal Proteome Profiling (TPP) is the combination of CETSA with proteosome analysis, enabling high-throughput screening of target proteins. Reviews on TPP[101,102] and high throughput CETSA[103] provide more details. As a successful example of multiple-target identification, Savitski et al[100] have used TPP to identify more than 50 Staurosporine targets[100]. Another example of CETSA combined with "one-pot" approach is the determination of the effects of the binding of methotrexate to dihydrofolate reductase[104].

The CETSA/TPP methods have a similar or slightly larger proteosome coverage (5000-8000) compared to LiP (5000-6000), while both are larger than SPROX or PP (~1000)[79]. Due to the relative ease of operation and larger proteosome coverage, TPP has been gaining popularity in target identification. It is worth noting that larger proteosome coverage does not necessarily mean better target identification. A direct comparison of TPP and SPROX using a one-pot 2D platform has found that although TPP coverage is ~1.5 fold of SPROX, SPROX offers protein domain-level information, identifies comparable numbers of kinase hits, has a higher signal (R-value), and requires ~3× less MS time[105].

One disadvantage of CETSA/TPP though, is that indirect targets are identified along with direct targets, due to a phenomenon called thermal proximity coaggregation[106,107]. Although sometimes these indirect targets provide clues for pinpointing the direct targets, in many cases, they make it harder to discern the direct targets. In contrast, DARTS or LiP can discern the direct target more easily, and LIP can provide domain-level information[108]. Furthermore, regular CETSA/TPP requires the protein targets to be soluble. Insoluble membrane proteins can be studied using other methods such as PP or by adding non-ionic detergent to increase the solubility[109,110].

Drugs themselves may also affect proteins' solubility. Sridharan et al[111] have used multiplexed MS to quantify the soluble and insoluble populations of individual proteins to assess ATP's effect on solubility, a strategy they dubbed "Solubility Proteome Profiling (SPP)". SPP has been combined with TPP for the identification of ATP-binding proteins.

Separation and Proteomics Techniques

For biochemical enrichment methods, usually after gel separation, both bottom-up and top-down MS can be applied to analyze the enriched samples. For proteosome screening methods, three stable isotope-labeling techniques have been applied, in combination with various stability-changing strategies mentioned above. A review devoted to drug target finding related MS techniques provided the details[110].

When using the enrichment strategy, 1D or 2D electrophoresis is often used to separate the enriched proteins, then the interested portion (usually judged by comparing to a control group), will be cut out and analyzed by MS. Electropho-

resis is also combined with some stability-based target identification strategies such as DARTS or CETSA.

Without the gel, the bottom-up proteomics technologies like LC-MS/MS are applied most widely to identify the enriched proteins, by firstly digesting, then identifying the peptides. Top-down approaches are also adopted in certain cases, for example in the DARTS method to detect the whole proteins instead of the peptides.

To better compare the probe-mediated pulldown to the relevant control samples, stable isotope labeling technologies have been adopted. Early studies used isotope-coded affinity tag (ICAT) technology[112,113]. Later on, isobaric tags for relative and absolute quantification (iTRAQ) have been adopted to compare the probe-mediated pulldown without or with different concentrations of free lead as competitors[114], which enables the estimation of the binding parameters.

The combination of the SILAC with pulldown assays in the presence of free drug competition has proved to be a useful target finding method (Figure 5). To quantify the kinome changes during the cell cycle, Daub et al[115] applied either heavy or light stably isotope-labeled amino acid in different cell cycle stages, then captured kinases using probes containing multiple unspecific kinases inhibitors and compared the kinome of different stages. Although the purpose of this study was not to identify drug targets, it provided an early example of combining SILAC and drug affinity chromatography. Ong et al[116] collected heavy or light stably isotope-labeled cell culture proteins, incubating one of them with probes only, another with probes in presence of free drugs. The protein targets in the pulldown mixture will be reduced with free drug competition, while the non-specific binders remain unchanged. However, the indirect protein targets share a similar pattern as the direct ones in this assay and will be identified, too. SILAC technology has also been combined with SPROX and PP[117].

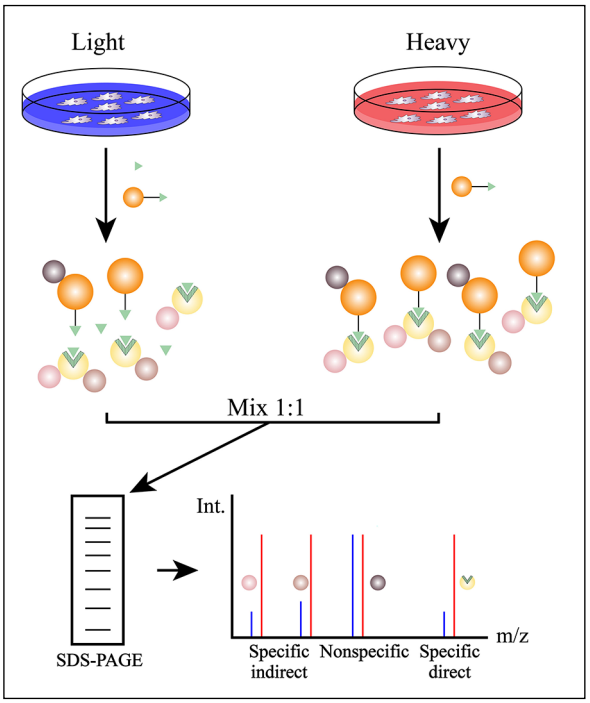

Figure 5. Flowchart of proteomics analysis using SILAC for target deconvolution. Cells are cultured with light or heavy isotope-labeled amino acids, proteomic samples collected, incubated with free small molecules or small molecule probes. Then proteins are analyzed by Electrophoresis and MS.

The adoption of multiplex TMTpro labeling technology has facilitated the detection of multiple samples in "one-pot" and has efficiently reduced the MS time required[118]. The one-pot method has been plugged into various proteosome screening methods including CETSA, CPP, PP, and SPROX[119].

A Hybrid Method- Lysine Reactivity Profiling

Recently lysine reactivity profiling method has been invented and adopted in probing drug-target interaction[120-122] and target identification[123]. This method is a hybrid of the proteosome screening and the enrichment method. A probe containing both the drug and a lysine reactive group has been designed and synthesized, for the enrichment of the protein targets, and simultaneous detection of the specific lysine reactivity at the drug-binding domains, *via* coupling to MS. In searching for targets of staurosporine, this method's sensitivity is found to be comparable to LiP combined with machine learning (LiP-quant) and TPP[123].

Discussion

Current protein target identification strategies can be grouped into two major categories based on their principles: the biochemical enrichment path and the proteosome screening path. To successfully implement the probe enrichment methods, the design of the probe is critical, which sometimes requires prior structure-activity relationship (SAR) information about the drug. To successfully fulfill the proteosome screening methods, it is assumed that drug binding will incur certain stability changes. Some proteosome screening methods have better proteosome cov-

erage than others[79], yet none of them have full coverage. A study that directly compared CETSA, CPP, PP, and SPROX has shown that different methods provide complementary information, so to catch as many drug targets as possible, a combination of methods is recommended[120]. A combination of different techniques has been successfully adopted in several cases, for example, TPP combined with SPP and TPP combined with SPROX[124].

Conclusions

This article summarizes two main strategies for drug target discovery: biochemical enrichment and proteosome screening. Numerous studies have shown that both strategies have been widely adopted for target development and achieved remarkable results. However, they still suffer from over-reliance on affinity, limited protein coverage, and false positives/false negatives to overcome. This field is becoming a more urgent need with the development of AI drug research, and more innovations in this field are expected. For enrichment technologies, bio-orthogonal reactions could perform better than the PAL, if a universal clickable group can be found on protein targets. Such clickable groups could be inserted via post-translational modification, for example, ubiquitination.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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