Trends in Analytical Chemistry 124 (2020) 115574

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Modification-free approaches to screen drug targets at proteome level☆

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ARTICLE INFO

Article history:
Available online 27 June 2019

Keywords:
Drug target screening
Drug-protein interaction
Modification-free approaches
Quantitative proteomics
Mass spectrometry

ABSTRACT

It is of great importance to explore the on- and off-target of existing drugs and discover novel druggable targets for potential therapeutic compounds. Although the hypothesis driven biological assays based approaches have made significant contribution to the drug target discovery, recent development of mass spectrometry and high-throughput proteomics have brought the drug target screening into a new era. Chemical proteomics approaches have great potential to reveal the drug-protein interaction at proteome level. However, these methods suffer from drawbacks in the chemical derivatization of drugs. Hence, there is a demand to develop novel approaches without the covalent modification. In the last decades, a series of modification-free approaches have been emerged, coupled with the cutting-edge quantitative proteomics, enabling the unbiased large-scale drug target screening. This review will introduce two types of modification-free approaches, including stability shift based methods (CETSA, TPP, SPROX), and proteolytic digestion based methods (DARTS, pulse proteolysis, LiP).

1. Introduction

During the action to cure a disease by the administration of a drug, the drug would bind to the target protein (s), which alters the activities of the target protein (s) and the down-stream proteins, and finally shuts off the abnormal biological process that leads to disease. The knowledge of the protein target (s) binding with the drug would facilitate the understanding of the mechanism of drug action [1]. Target screening is helpful to evaluate drug side effect and toxicity [2,3], as well as drug polypharmacology [4,5]. Up to now, several methods were developed to study the drug-target interaction. Genetic approaches are well suited to identify the proteins and genes involved in the drug action [6–8]. In these methods, drug response is investigated by overexpression or knocking down/out of interested gene which revealed its influence on the phenotype of organism rather than direct evidence of drug-protein interaction [9]. Alternatively, measurement of proteins at proteome level will be an information-rich approach to unravel the drug-target interaction [10]. For example, several chemical proteomics methods have been developed for the screening of drug target proteins [11]. Affinity chromatography is a classical chemical proteomics method. In this method, the immobilized or tagged small molecule was incubated with cell lysates for capturing interacting proteins [12,13]. It was successfully applied to screen the target kinases by using the immobilized nonselective kinase inhibitors (kinobeads) [14,15]. Another widely used method is affinity-based protein profiling (ABPP), which exploits the chemical probes to interrogate a distinct class of proteins [16,17]. The ABPP approach was firstly used to profile enzymes, and was later introduced into the field of drug target screening [18]. The special designed chemical probes typically include three functional moieties: the binding moiety interacts with the drug target proteins, the reactive moiety enables the covalently linking of the probe to

Abbreviations: MS, mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; ABPP, affinity based protein profiling; TSA, thermal shift assay; CETSA, cellular thermal shift assay; TPP, thermal proteome profiling; SPROX, stability of proteins from rates of oxidation; DARTS, drug affinity responsive target stability; LiP, limited proteolysis; SILAC, stable isotope labeling with amino acids in cell culture; SRM, selected reaction monitoring; iTRAQ, isobaric tags for relative and absolute quantitation; TMT, tandem mass tag.

☆ Dedicated to the 70th anniversary of Dalian Institute of Chemical Physics, Chinese Academy of Sciences.

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https://doi.org/10.1016/j.trac.2019.06.024
0165-9936/

the proteins, and the analytical tag allows the quantitative readout or enrichment of the proteins [19,20]. These activity-based probes allow chemical 'freezing' of the interaction between affinity probe and target protein(s) [21], which is extremely advantageous for the screening of target proteins with low drug affinity. Strategies of affinity chromatography and ABPP are powerful tools for the high-throughput target screening thanks to the technology advance in proteomic and mass spectrometry [22–25]. However, it should be noted that both approaches require chemical derivatization of the small molecule drugs, which leads to several drawbacks in target discovery. For instance, some small molecule compounds lack suitable site and space to be chemically modified, making the design of detectable affinity probes or immobilized drugs impossible [26]. Besides, high false positive identification rate is another issue for this method as the chemical derivation may affect the bioactivity/binding specificity of the drug [27,28]. The affinity chromatography based method exploits the affinity interaction between immobilized small molecule drug and target protein. Thus, the capture of target is affinity-dependent, implying that it has difficulty to discover the low-abundance targets and targets weakly interacted with the immobilized drug. ABPP alleviates this problem by employing chemical probes to directly label, enrich and identify the target proteins with covalent modification. Nevertheless, some probes labeled on residues may affect digestion efficiency of trypsin that cannot generate peptide with appropriate length for bottom-up MS analysis. Some probes are too large or labile, leading to the inefficient fragmentation of labeled peptides and the difficulty in assignment of labeling sites [29]. Consequently, alternative drug target screening approach free of chemical modification is in urgent need. In the last decades, several energetics-based methods for drug targets profiling has been developed [9,22,26,30]. In these methods, synthetic probes or chemically modified drugs are not required. Instead, the drug target proteins could be identified by directly measuring the change of particular protein properties, such as thermal stability and proteolysis susceptibility, after the binding of drug. These changes in protein properties are due to the change of energy status of the target protein after drug binding. In thermodynamics, the free energy of binding can be described by the equilibrium constant of drug-target binding as in Equation (1):

$$\Delta G = -\mathrm{RTln}K_{eq} \quad (1)$$

For therapeutic drugs, drug-target complexes typically have the dissociation constants ($K_D = 1/K_{eq}$) less than 1 μM to ensure efficient binding [31]. Thus, the useful drug has the equilibrium constant (K_{eq}) > 1×10^6 M^{-1}. According to Equation (1), the free energy of binding (ΔG) is always negative, indicating the process of drug bound to its target is a spontaneous process. In the free energy landscape model, native protein is at the bottom of energy funnel, while the unfolded protein is at the highest energy state [32]. As the drug bound protein has a lower energy state than the native protein, the binding complex will overcome a higher energy barrier to achieve unfolding than the native protein (Fig. 1A). Thus, the drug bound target protein is more stable than native protein. In a drug target screening experiment, a drug is incubated with a cell lysate and stabilizes its target protein by forming a drug-protein complex. Some energetic properties of the target protein will be changed due to the drug-induced stabilization. Those changes include the increase in thermodynamic stability and the decrease in protein unfolding rate, etc. In practical, a control experiment without the addition of the drug is also performed. Consequently, the target protein can be identified by comparing the changes of energetic properties between the drug bound protein and the free protein from the two experiments (Fig. 1B). These changes of energetic properties are due to the altered free energy which can be ascribed to the changes of enthalpy and entropy. The enthalpy of binding is a quantitative indicator of intermolecular bond energies (hydrogen bonding and van der Waals interaction), while the entropy of binding is more likely an indication of rearrangement undergone by the solvent molecules [33]. Thus, the changes of enthalpy and entropy come from the conformational changes of protein during the drug-protein binding process. Correspondingly, the target protein can also be identified by comparing these conformational changes between the drug bound protein and the free protein in the two experiments. Therefore, recently developed modification-free approaches for drug target screening can be divided into two types, i.e. stability shift based methods and proteolytic digestion based methods. Stability shift based methods identify drug targets by detecting the stability shift between drug-target complex and free target protein with the increased denaturing strength such as elevated temperature or denaturant concentration (Fig. 1B). The proteolytic digestion based methods utilize hydrolytic enzymes to probe the shift in proteolytic susceptibilities, reflecting the conformational changes due to the binding of drug (Fig. 1C). This method is also an energetic based method because the proteolysis susceptibility of a protein is related to its energy landscape [34,35]. Proteolysis of proteins requires access to high-energy cleavable states, where cleavage sites are exposed to proteases through local or global unfolding [36]. The low-energy compactly folded proteins, i.e. drug bound proteins, are poor substrates for proteolysis accordingly. These modification-free approaches could be applied to a wide range of biological specimens at proteome level and have the advantages to bypass the problems in chemical modification approaches, including difficulty in synthesis, non-specific absorption, requirement of strong affinity, etc. The development of these modification-free methods has gone over two decades, from low-throughput purified proteins to proteome level. This review will introduce several popular modification-free approaches for drug target screening and their pros and cons in applications. Those methods include the stability shift based methods: Cellular Thermal Shift Assay (CETSA), Thermal Proteome Profiling (TPP), Stability of Proteins from Rates of Oxidation (SPROX), and the proteolytic digestion based methods: Drug Affinity Responsive Target Stability (DARTS), pulse proteolysis, and two-step digestion based Limited Proteolysis (LiP).

2. Stability shift based methods

2.1. Screening of drug targets by thermal stability shift

Over the last twenty years, thermal shift assay (TSA) has become one of the most widely used modification-free method for drug target discovery [37]. It determines a shift in melting temperature typically by measuring changes in light scattering [38] or in fluorescence [39]. Take the fluorescence-based thermal shift assay, a simple and commonly used TSA, as an example. It uses a probe with a low fluorescence signal in a polar environment but with high fluorescence in a nonpolar environment. The probe is added to the protein solution with or without the presence of drug. And then, the fluorescence of the protein solutions is monitored when the solutions are heated. With the increase of temperature, protein chain gradually unfolds to expose hydrophobic core which leads to the increasing of fluorescence signal. In this way, the thermal melting curves for the drug bound protein and the free protein could be plotted, which enables the determination of the shift in midpoints of the curve (ΔT_m). ΔT_m is proportional to the ligand binding affinity [40] and is used to distinguish the drug target. This method is simple and straight forward. However, the probe cannot distinguish different proteins and so the commonly used TSA is

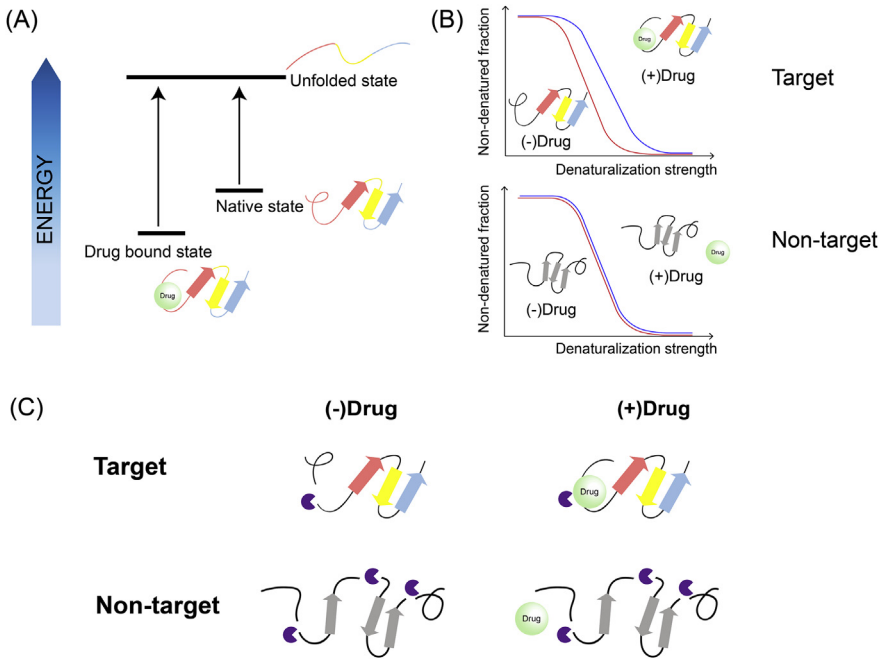

Fig. 1. The principles of modification-free approaches for drug target Screening. (A) Energy states of drug-bound, native, and unfolded protein. Drug binding onto a protein is a spontaneously occurred reaction. Therefore, the protein binding with drug has lower energy state and is more stable compared with the free proteins. (B) Methods exploiting the difference in resistance to denaturalization. (C) Methods exploiting the difference in sensitivity to proteolysis.

only applicable to the experiment with purified protein. To circumvent this problem, a conceptually similar technology named cellular thermal shift assay (CETSA) was developed by Molina et al., for directly investigating drug-target interaction in cellular context [41]. In CETSA method, multiple aliquots of cell lysate or intact cells were treated with drug or vehicle, heated to different temperatures and cooled, followed by centrifugation to separate soluble fractions from precipitated proteins (Fig. 2A). Proteins are gradually unfolded to expose the hydrophobic core with the increasing of temperature, resulting in the precipitation of the proteins in high temperature. The more stable the protein is, the higher the protein resistant to the heat induced precipitation. Thus, the stability of the proteins could be measured by determining the fractions of soluble proteins with the increasing of the temperature. In CETSA, the proteins in the soluble fractions from different temperature points are separately quantified by Western blotting. The thermal melting curves of the detected proteins are then drawn according to the intensity changes along with the elevated temperature. The drug target proteins are then distinguished according to the shifts of ΔT_m between the drug bound proteins and the free proteins [42]. This method was validated by using several well-known drug target engagements, including antifolate cancer drug methotrexate binding to dihydrofolate reductase (DHFR), raltitrexed binding to thymidylate synthase (TS). Obvious stability shifts were observed in melting curves for these target proteins after the drugs were added to cell lysates or intact cells. Furthermore, an isothermal dose-response procedure was established to investigate the relationship between drug-dose and target engagement [41]. In this isothermal dose-response fingerprint approach ($ITDRF_{CETSA}$), lysate aliquots were exposed to different concentrations of drug while time of heating and temperature were kept constant. The $ITDRF_{CETSA}$ has been validated by the revealing of known target engagements, including the selectivity of PD0332991 for CDK4 and CDK6, the target engagement of PLX4032 in lysate and intact cells. Due to the easy operation and good compatibility with intact cell, CETSA has been widely used in laboratories all over the world and now is considered as one of routine methods in drug discovery. CETSA is the first method that allowed study of target engagement in live cells. For some interested target proteins, for example, promising anticancer targets, CETSA combined with small molecule library can be utilized to screen potential inhibitors, evaluate target engagement efficiency, and monitor target specificity [43–46]. In addition, CETSA can also be applied to screen targets for novel drugs and phenotypic compounds, to address problems including off-target proteins, binding mechanism, drug efficacy and resistance in intact cells, etc. [21,47]. Beside qualitatively identifying drug-target interaction, $ITDRF_{CETSA}$ was also used to explore the dose-related target engagement in animals, assisting in determining appropriate drug usage and dosage, and monitoring the acquirement of drug resistance [41].

The antibody-based readout enables CETSA to measure the stability shifts of different target proteins in a protein mixture, while this method requires prior knowledge of interested targets. Thus, it is not suitable for unbiased drug target discovery and also cannot be conducted at proteome scale. To solve these problems, CETSA was extended to an unbiased proteome-wide screening. This new method, termed as thermal proteome profiling (TPP), combined the CETSA approach with multiplexed quantitative mass spectrometry for drug target and off-target identification (Fig. 2B) [48]. Leveraging the high-throughput identification and quantification of TMT-10 labeling, TPP enables the monitoring of 10 temperature points simultaneously to draw a complete melting curve and thereby allows the determination of the thermal shift for each protein. Like CETSA, TPP can be applied to intact cell as well as cellular context, and the combination of them can distinguish thermal shifts induced by ligand binding from those induced by downstream modification [49]. Proteins with affected thermal stability in TPP experiments in living cells but not in cell extracts are likely to be indirect targets [50].

With the benefit of high-throughput MS analysis, TPP method is more widely used in assaying the drug or other small molecule induced protein structure changes. Azimi et al. utilized TPP

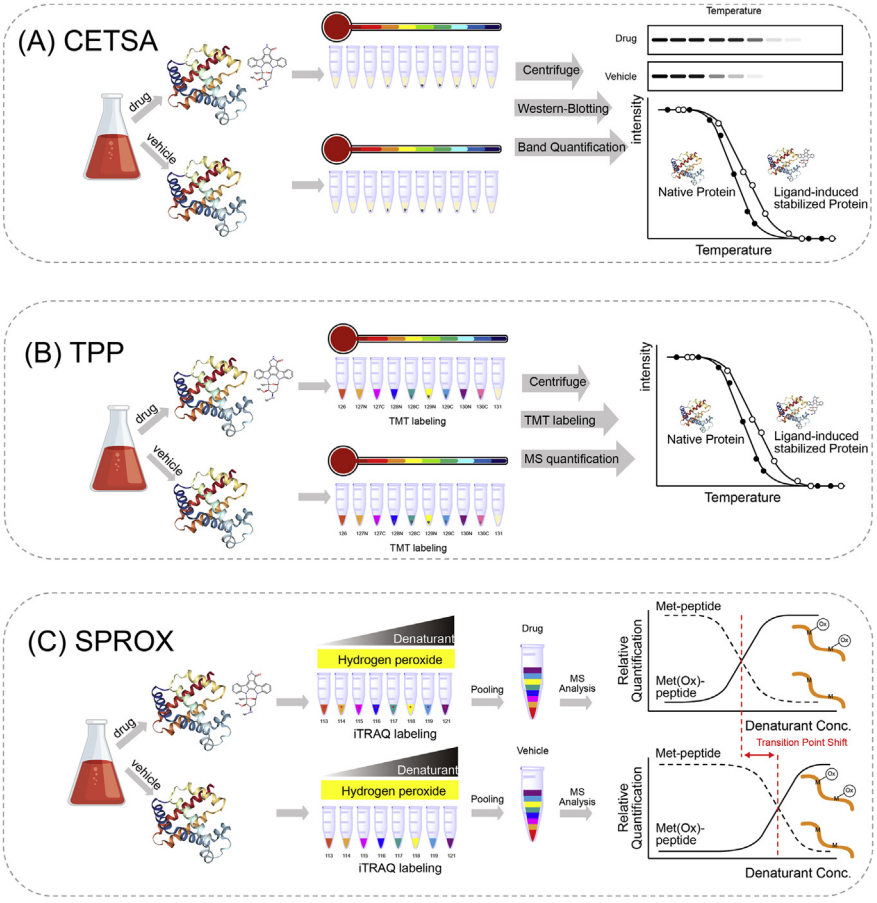

Fig. 2. Screening of drug targets by stability shift. The following approaches distinguish the drug target by the thermodynamic stability changes of target protein in the presence or absence of drug. Due to the lower energy state, the drug bound protein will be more stable than the free protein, requiring more denaturalization strength to reach the unfolded state. (A) Cellular Thermal Shift Assay (CETSA), in which a temperature gradient is used to profile the thermal stability shift between proteins with and without the presence of drug. Western blotting is applied as a quantitative readout of interested proteins. (B) Thermal Proteome Profiling (TPP), the schematics of which are similar to CETSA, while protein stability is profiled through high-throughput multiplex-labeling quantification instead of the western blotting in CETSA. The above two approaches, CETSA and TPP, both provide drug target information at the level of protein. (C) Stability of Proteins from Rates of Oxidation (SPROX), in which a denaturant gradient is used to profile the chemical stability shift of proteins after adding drug. Particularly, this approach can provide an insight view of drug-target interaction at the level of protein domain.

approach to identify the protein targets of HSP90 and BRAF inhibitor, in a pair of sensitive and unresponsive melanoma cell lines [51]. Through TPP, several protein kinases were identified as potential targets of XL888, a HSP90 inhibitor. CDK2 was revealed as a driver of resistance to both BRAF and Hsp90 inhibitors. Miettinen et al. applied TPP approach to breast cancer cells, revealing the mechanism of proteasomal activation by CDK4/6 inhibitor palbociclib [52]. Utilizing the multiplexed liquid chromatography-tandem MS, Becher et al. designed a two-dimensional thermal proteome profiling (2D-TPP) strategy to study the dose-dependent effects of small molecule inhibitors on their targets [53]. Proteome-wide protein thermal stabilities were measured at 12 temperatures and 5 different compound concentrations of panobinostat, a histone deacetylase (HDAC) inhibitor. Aside from known HDAC targets, TTC38 and PAH also exhibited increased thermal stabilities at low panobinostat concentrations. These two off-target proteins were verified by chemo-proteomics method. The panobinostat-induced inhibition of phenylalanine hydroxylase leads to increases in phenylalanine and decreases in tyrosine levels, suggesting a repurposing of the drug for treatment of tyrosinemia. Recently, TPP method was applied to interrogate ATP-mediated regulation of protein thermal stability and protein solubility in proteome-wide [54]. It was found that the biological roles of ATP depend on its concentration. When the concentration of ATP is lower than 500 μM, it was observed that ATP specially interact with proteins and use the proteins as substrates or allosteric modulator. While, with a wild concentration (between 1 and 2 mM), ATP will affect protein-protein interactions of protein complexes. With a higher ATP concentration (>2 mM), at least 25% of the insoluble proteome exhibit an ATP-dependent increase in solubility. This is because ATP can work as the biological hydrotrope to increase the solubility of positively charged, intrinsically disordered proteins. Another very similar method named Target identification by ligand stabilization (TILS), utilizes the precipitate rather than the soluble protein fraction, demonstrates itself to be a complementary method for TPP in drug target discovery [55].

CETSA and TPP have been increasingly popular for the study of drug targets [51–53,56], however there are some limitations behind their principles (Table 1). Firstly, because denaturing reagents are typically not added in the extraction solution to preserve the proteins' activity, these thermal shift assays are only applicable to screen soluble target proteins. Thus, the hydrophobic proteins like membrane proteins will be escaped from the detection. Whereas, it was reported that adding mild detergent will not affect the thermal shift behaviors of proteins but help the dissolving of membrane proteins and make them amenable for subsequent thermal shift analysis [57]. Furthermore, these methods are not likely to work for proteins in which unfolding of the ligand-binding

Table 1
Strengths and limitations of modification-free approaches for drug target screening.

Modification-free methods	Biological Specimens	Detection methods	Quantitative Strategies	Biophysical information	Limitations	Main References
CETSA	◆ Intact cells ◆ Cell lysates	◆ Western-Blotting	◆ Protein-centric	◆ Thermal melting curves	◆ Low throughput ◆ Requires prior knowledge and antibody of interested targets ◆ Cannot be applied at proteome scale ◆ Not applicable to thermal insensitive proteins ◆ Cannot reveal binding sites	[30,31]
TPP	◆ Intact cells ◆ Cell lysates	◆ Multiplexed quantitative mass spectrometry	◆ Protein-centric	◆ Thermal melting curves	◆ Incompatible with membrane proteins ◆ Not applicable to thermal insensitive proteins ◆ Cannot reveal binding sites	[38,44]
SPROX	◆ Cell lysates	◆ Multiplexed quantitative mass spectrometry	◆ Peptide-centric (revealing binding sites)	◆ Protein folding energy (ΔG_f) ◆ Binding free energy ($\Delta\Delta G_f$) ◆ Disassociation constant (K_d)	◆ The drug target screening relies on the exposure and distribution of reactive amino acid residues. ◆ Chemical modifications are limited to several amino acid residues: Met, Trp, Lys.	[45–47]
DARTS	◆ Cell lysates	◆ Western-Blotting ◆ In-gel digestion and mass spectrometry	◆ Protein-centric	◆ Protease susceptibility	◆ Requires prior knowledge and antibody of interested targets (Western-Blotting based readout) ◆ Cannot elucidate some low-abundance proteins and co-migrating proteins (MS based readout) ◆ Cannot reveal binding sites ◆ Not applicable to the proteins that resist proteolysis under native condition	[60,61]
Pulse Proteolysis	◆ Cell lysates	◆ 2D gel electrophoresis and mass spectrometry	◆ Protein-centric	◆ Protein folding energy (ΔG_f) ◆ Binding free energy ($\Delta\Delta G_f$) ◆ Disassociation constant (K_d)	◆ Cannot elucidate some low-abundance proteins and co-migrating proteins ◆ Cannot reveal binding sites	[63,64]
Two-step digestion based LiP	◆ Cell lysates	◆ Mass spectrometry	◆ Peptide-centric (revealing binding sites)	◆ Protease susceptibility	◆ Requires accurate quantification ◆ Incompatible with membrane proteins ◆ Extremely complex sample ◆ Not applicable to the proteins that resist proteolysis under native condition	[78,80]

domain does not promote aggregation [41]. In principle, the thermal stability of protein will be affected by ligand binding to some extent. However, there are some proteins that do not show statistically significant shifts in apparent melting temperature [49]. For multi-domain proteins or huge proteins, the ligand-induced structural changes may only happen in a small area, which are not sufficient to enable the aggregation and precipitation of the protein [42,49]. In some particular protein structure, the structural change in the drug-protein interaction area will not affect the general hydrophobicity of the whole protein, and so these proteins will not precipitate with the increasing of the temperature. Clearly, these protein targets cannot be revealed by these thermal shifts based methods. According to the relationship between ΔG and equilibrium constant in equation (1), the protein will be always stabilized after the binding of a drug. However, reduced stabilization was also observed for some proteins by CETSA [58]. One explanation is that these proteins are not the direct target proteins but the interacting proteins of the true target proteins. Once the drug binds with the true target protein, the interacting proteins will dissociate from the protein complex and the free energy of these proteins will accordingly be increased, leading the decreasing of stability. Finally, these thermal stability-based methods detect the change of general protein property, and therefore cannot reveal the detail information on ligand binding domain.

2.2. Screening of drug targets by chemical stability shift

Stability of proteins from rates of oxidation (SPROX), another stability based method, was proposed by West and Fitzgerald et al. [59]. In SPROX, the stabilities of proteins are assessed by oxidation rates of methionine residues as a function of the chemical denaturant (e.g., guanidine hydrochloride or urea) concentration. In each analysis, aliquots of the protein mixture with or without the presence of drug are diluted into a series of buffers containing increasing concentrations of a chemical denaturant, e.g., guanidinium hydrochloride (GdmCl). The protein samples in each GdmCl-containing buffer are reacted with the same amount of hydrogen peroxide for the same amount of time. The protein oxidation reaction is quenched and the oxidized protein samples are subjected to quantitative proteomic analysis and the nonoxidized and oxidized methionine-containing peptides are quantified as a function of the SPROX buffer denaturant concentration. The drug bound proteins, interacting with drug either direct or indirect, are those identified with methionine-containing oxidized and non-oxidized peptides that show transition midpoint shifts between conditions with or without presence of drug (Fig. 2C). The transition midpoint shifts are used to calculate the protein folding free energy (ΔG_f), binding free energy ($\Delta\Delta G_f$) and disassociation constant (K_d) as described [59]. In this way, the ligand-induced stabilization ($\Delta\Delta G_f$) and binding affinities (K_d values) could be obtained [60]. As CETSA method, SPROX was firstly developed for intact protein readout [59] but subsequently facilitated by the development of quantitative bottom-up shotgun proteomics, which enables simultaneously assessing protein folding and ligand-binding properties in complex biological mixtures [61]. As proof-of-principle experiments, the protein-folding and ligand-binding properties of 327 proteins were measured by SPROX in a yeast cell lysate in the presence and absence of cyclosporin A (CsA). Ten targets of CsA in yeast proteome were identified with SPROX, two of which, cyclophilin A (CypA) and UDP-glucose-4-epimerase, were known to interact with CsA. CypA, known to directly bind CsA (K_d 30−200 nM), was detected transition midpoint shifts of approximately 1.5 M and 1.3 M for nonoxidized and oxidized peptides, respectively. These shifts can be used to calculate K_d values of 26 and 100 nM for the CsA-CypA complex, which are close to the earlier SPROX result of 86 nM by using purified CypA binding to CsA [59]. Another known CsA related protein, UDP-glucose-4-epimerase, is indirectly modulated by CsA as its refolding reaction is reported to be regulated by CypA. The CsA-induced stabilization of UDP-glucose-4-epimerase detected in SPROX experiments is most likely due to a thermodynamic consequence of CsA inhibition of CypA's kinetic effect on the UDP-glucose-4-epimerase folding reaction. The other eight protein targets discovered in SPROX, including several proteins involved in glucose metabolism, can be used to create a new framework to investigate the molecular basis of CsA side effects in humans, e.g., posttransplant diabetes mellitus (PTDM).

Theoretically, any quantitative approaches can be utilized in SPROX to quantify the nonoxidized and oxidized methionine-containing peptides. However, considering the multiple denaturation conditions in SPROX, the proteome-wide SPROX prefers multiplex quantitative proteomics technologies, including tandem mass tag (TMT), Isobaric tags for relative and absolute quantitation (iTRAQ), and stable Isotope labeling with amino acids in cell culture (SILAC) [62,63]. A recent study using SPROX approach together with multiplexed tandem mass tagging (TMT), termed as high-resolution SPROX (HR-SPROX), was reported to quantify the folding stabilities of ~10000 unique regions within ~3000 proteins in human cell extracts [64]. The lysosomal and extracellular proteins are classified as the most stable ontological subsets of the proteome. It is also found that the stability of proteins impacts their tendency to become oxidized and is globally altered by the chemical chaperone trimethylamine N-oxide (TMAO). This strategy was applied to study target engagement for several drugs, for instance, exploring potential targets of anticancer natural product Manassantin A [65], and explaining slow-tight binding behavior of the geldanamycin-Hsp90 complex [66], discovery of Tamoxifen and N-Desmethyl Tamoxifen protein targets [67], etc. While the SPROX methodology was initially designed for the investigation of protein-drug affinity, nowadays it has also been extended to study protein interaction with other small molecule ligands [62,68], protein structural changes induced by disease and PTMs [63,69].

A limitation of SPROX is that it can be only applicable to the proteins containing buried methionine residues, but does not work for the methionine-absence protein domains (Table 1). To overcome this drawback, methods by chemical modification of other amino acid residues were proposed. The amine reactive reagent, s-methyl thioacetimidate (SMTA), is used to probe the protein folding and ligand binding in the lysine-rich area [70]. Conceptually like SPROX, SMTA reagent probes the globally protected amine groups in proteins along with the increasing of chemical denaturant concentration. Another similar strategy with covalent labeling of tryptophan residues by tryptophan-selective reagent dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide (HNSB) was reported to probe the thermal stability of proteins [71]. As different types of residues are targeted, combining SMTA and HNSB protocols with SPROX would increase the coverage and depth for investigating protein folding and stability. The SPROX technique is closely related to a previous amide H/D exchange strategy, termed as stability of unpurified proteins from rates of H/D exchange (SUPREX), analyzing the protein stability by probing the globally protected amide groups on proteins [72]. Within a polypeptide, certain labile hydrogen atoms can exchange freely with the surrounding solvent. The stability of a protein can be analyzed by monitoring the exchange rates of these 'globally protected' hydrogens with D_2O. In principle, SUPREX and SPROX both measure the unfolding/refolding equilibrium of proteins. The major advantage of SPROX over SUPREX is the irreversible nature of the chemical reaction compared with the reversible issue of H/D exchange [59]. Like CETSA and other thermal stability based methods, SPROX is mainly

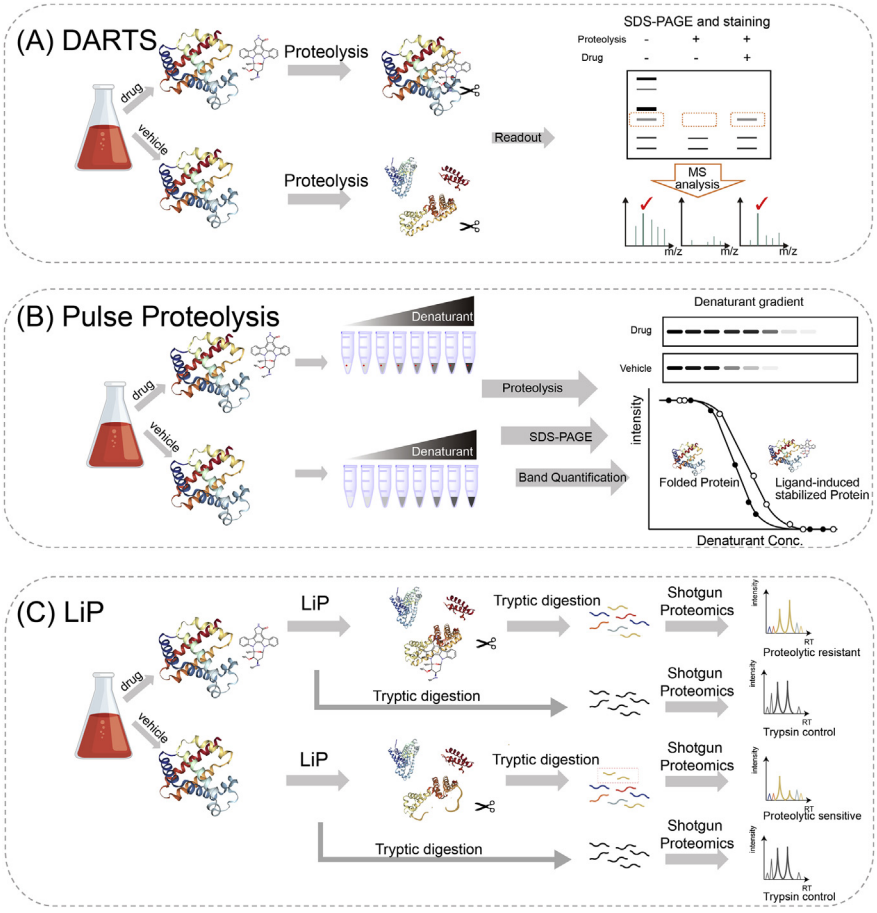

Fig. 3. Screening of drug targets by difference in sensitivity to proteolysis. The following approaches distinguish the drug target by the shift of proteolytic susceptibility due to the presence or absence of drug. (A) Drug Affinity Responsive Target Stability (DARTS), in which proteins are limited digested and the remaining proteins are separated by SDS-PAGE. The drug bound proteins are determined by drug protected bands and identified by MS analysis or western blotting. (B) Pulse Proteolysis, in which a denaturant gradient is applied and stability shift curves is available. The above two approaches, DARTS and pulse proteolysis, both determine the remaining protein after limited digestion. (C) Two-step digestion based Limited Proteolysis (LiP), in which a two-step proteolysis is applied to determine the conformational changes on target protein after drug-protein binding. The first-step digestion with unspecific protease is for probing the structural changes on protein, and the second-step digestion is for cutting the protein into peptides of suitable length for MS analysis. Particularly, this approach can detect proteolytic resistance and sensitivity at the level of protein domain. For the control samples, the same protein extracts are directly denatured and subjected to one-step trypsin-only digestion as in conventional shot-gun proteomics. These trypsin-only controls are used for the normalization of protein abundance across different samples.

applicable to probe the interaction of drug with soluble proteins. Unlike CETSA, SPROX can profile the oxidized and nonoxidized methionine at peptide level, indicating that it has the potential to elucidate the localized stability of each unique protein domain [64].

3. Proteolytic digestion based methods

3.1. Screening of drug targets by the shift of proteolytic susceptibility

It was known that drug-bound protein is less susceptible to proteolysis than the drug-free protein [73,74]. Proteolytic susceptibility of proteins is determined by the accessibility of these cleavable states. Once the small molecule drug binds to the target proteins, the drug bound proteins are thermodynamically stabilized according to equation (1). This stabilization of drug bound proteins results in changes of energetic properties, i.e. the decrease of protein unfolding rates. As a result, the cleavage sites on drug bound proteins are more likely inaccessible to proteases. Therefore, drug binding enhances the stability of the target protein, causing an increase in resistance of proteolysis [75]. Take advantage of this feature, drug affinity responsive target stability (DARTS) approach was developed for drug target screening [76]. DARTS is performed by treating aliquots of protein sample with the drug of interest and vehicle control, followed by limited digestion of the proteins in the cell lysate with proteases (Fig. 3A). And then, the samples are separated by SDS-PAGE. Compared with the vehicle control, the protein bands that are protected from proteolysis by the small molecule binding could be determined [77]. The protein sample could be purified protein or complex protein mixture such as total cell lysate and the readout of SDS-PAGE could be western blotting or MS analysis depends on different purposes. Total cell lysate coupled with MS analysis is usually applied in the experiment for drug target screening. For example, DARTS was successfully applied to identify the target proteins of resveratrol, a small molecular natural product that has not been demonstrated targets before because of its modest structure, weak affinity, and poor potency of chemical modification [76]. In the study, the yeast cell lysates were treated with/without resveratrol and then were subjected to SDS-PAGE separation. Two silver-stained bands were found more intense in the resveratrol-treated lysate postproteolysis. Mass spectrometry analysis of both bands showed that eIF4A, along with several ribosomal proteins, were enriched in the resveratrol-treated sample. These proteins were the potential targets of

resveratrol. Their further biological study indicated that eIF4A could be a previously uncharacterized druggable target for anti-aging therapy. DARTS was also used to identify the targets of disulfiram (DSF) [78]. DSF is an FDA-approved drug used to treat alcoholism. However, it was found that DSF was one of the most potent growth inhibitors following high-throughput screens of 3185 compounds against multiple triple-negative breast cancer cell lines. To identify its targets, DARTS was performed by incubating the cell lysates with varying concentrations of DSF before digestion with pronase. After the separation of the samples by SDS-PAGE, the DSF-protected bands were excised and subjected to mass spectrometry analysis. Two proteins, IQGAP1 and myosin heavy-chain 9 (MYH9), were finally identified to be the direct binding targets of DSF. The identification of these targets facilitated the understanding the inhibition mechanisms. This study suggested that DSF may be repurposed to treat triple-negative breast cancer in combination with doxorubicin.

In DARTS, the proteins are limited digested typically under native condition. It was reported that some proteins are kept intact even after several days of digestion [36]. This is likely because these proteins are compactly folded under native conditions and the cleavage sites are inaccessible to proteases. Thus, DARTS is normally unable to identify these proteins as the drug targets. Furthermore, DARTS is unable to reveal the folding free energy as only the folded proteins are subjected to digestion and analyzed. Instead, pulse proteolysis approach is a promising approach to address above issues [79]. Unlike in DATRS, the proteins in pulse proteolysis approach are prepared in a series of different denaturant concentrations (Fig. 3B). In this way, the proteins are denatured and unfolded in different degrees. Therefore, even the most compactly folded proteins could be completely unfolded in high denaturant concentration such like 8M urea. Protease is then added to above solutions for pulse proteolysis, i.e. digestion for a short period of time to selective digestion of unfolded proteins but keep the folded proteins intact [79]. The resulting samples were then separated by SDS-PAGE and the remaining intact protein could be quantified by staining, e.g. coomassie brilliant blue staining for purified protein or by Western Blotting for a protein in a complex protein mixture. Thereby, the fraction of folded protein, i.e. the remaining protein, could be determined and a curve for dependence of this fraction on denaturant concentration could be plotted. This curve is finally used to calculate the global stability, i.e., the free energy for the unfolding of the protein. It was found the global stabilities determined by pulse proteolysis for RNase H* and its variants, I53A and I53D were highly consistent with those determined by circular dichroism. The pulse proteolysis can be used to monitor the ligand binding induced stability change. For example, it was applied to determine the stability of maltose-binding protein (MBP) with and without the presence of maltose. And higher stability was indeed observed for the ligand bound protein. Specifically, when MBP was incubated in 4.5 M urea for 1 h with or without ligand and then digested with 0.20 mg/ml thermolysin for 1 min. Without ligand, MBP was fully digested under this experimental condition. In contrast, 100 mM maltose successfully protected MBP against proteolysis.

In DARTS and pulse proteolysis methods, SDS PAGE is usually used to separate the digest and the intensity-changed lanes are then subjected to subsequent MS analysis. However, it might be difficult to find the target lanes in some complex samples as many proteins comigrate together and the target protein maybe suppressed by high abundant non-target proteins [80]. To overcome this difficulty, better separation technique, 2D gel electrophoresis, was also applied in pulse proteolysis approach [34]. In addition to determine the protein amount by gel spot/band intensity, LC-MS/MS-based quantitative proteomics platforms using either isobaric mass tags [81] or stable isotope labeling with amino acids in cell culture (SILAC) [82] were also used to quantify the relative amount of intact protein in each protein sample. The application of these new techniques would certainly improve the sensitivity of these drug target discovery methods. The principle for DARTS and pulse proteolysis approach are both based on the ligand-induced stabilization. The drug binding could protect the target protein from digestion in the absence of denaturant (DARTS) or in the presence of denaturant (pulse proteolysis). As both of the methods determine the remaining intact protein rather than the digested peptides, these methods cannot reveal the binding sites of drug targets.

3.2. Screening of drug targets based on the change of protein conformation

In shotgun proteomics, proteins in complex mixture are always denatured to unfold them, which ensure all cleavage sites accessible to the protease and got complete proteolysis. This complete proteolysis approach cannot provide the information on three dimensional structure of the proteins. While, the limited proteolysis, digestion of proteins under native condition with short period of time, could provide. A native-unfolded equilibrium theory was proposed by Fontana et al. to explain the process of limited proteolysis [83]. In the theory, the partly unfolded region on the protein, like flexible loop, exhibits higher segmental mobility so that provides more accessibility for binding at the active site of protease. Leveraging the different susceptibility of protein structure upon exogenous broad-specificity protease, limited proteolysis method provided a simple strategy without tedious and expensive structural biological experiment [83,84]. Most proteolytic reactions are often monitored by SDS-PAGE. To determine the exact sites of proteolysis, mass spectrometric methods are also used. DARTS and pulse proteolysis are both based on limited proteolysis strategy. However, both of them only detect the general proteolysis susceptibility of the intact protein, without consideration of structural features provided by proteolysis sites. Limited proteolysis was applied to elucidate the protein structure for a long history, however it is limited to purified protein due to the difficulty to identify the cleavage sites in complex sample [85]. Shot-gun proteomics is a powerful tool to identify and quantify proteins in complex mixture. It depends on the LC-MS/MS analysis of small digested peptides with molecule weight of 600−3000 Da. However, limited proteolysis yields larger protein fragments which are not applicable for shot-gun proteomics analysis. To circumvent this problem, Picotti et al. presented a two-digestion step based limited proteolysis approach termed as LiP [85,86]. In this approach, the proteins extracted from different biological states under nondenaturing condition are subjected to two-step digestion. The first step, limited digestion, is conducted using a broad-specificity protease (e.g., proteinase K, thermolysin) at a low enzyme to substrate ratio and for a short time as in the conventional limited proteolysis. Under these conditions, the sites of initial proteolysis are dictated by the structural properties of the proteins, generating large protein fragments. In the second digestion step, the resulting digest is then shifted to denaturing conditions to quench the first-step proteolysis and fully digested by trypsin to generate peptides amenable to shot-gun proteomics analysis. For the control samples, aliquot of the same protein extracts is directly denatured and subjected to one-step trypsin-only digestion as in conventional shot-gun proteomics. Finally, all the above samples are subjected to quantification by MS analysis (Fig. 3C). The proteolytic patterns of different biological samples subjected to the two-step digestion are compared, after correction for protein abundance changes using trypsin-only controls. With this two-step digestion strategy, the peptide mixture includes canonical fully tryptic peptides, half-

tryptic peptides and some non-specific peptides. Among these peptides, fully tryptic peptides embedding LiP sites are less abundant in doubly digested samples than in controls, and half-tryptic peptides generated by intra-tryptic peptide cleavage are present only in doubly digested samples. These peptides with altered abundance across differently treated conditions were termed as conformotypic peptides as they are structure-specific for each condition. To obtain more robust and sensitive results, this two-step LiP approach was coupled with targeted proteomics using selected reaction monitoring (SRM) to accurately quantify these conformotypic peptides in complex mixture [86]. In Parkinson's disease patients, unfolded α-Syn switches to a β-sheet rich fold and polymerizes into fibrillar, amyloid aggregates. To validate the method, the α-Syns with these two different conformations were respectively spiked into *Saccharomyces cerevisiae* proteome extracts to mimic the complex samples. The conformotypic peptides specific to the two conformations were successfully identified, indicating that the regions undergoing the most pronounced conformational changes were the N terminus and the amyloid core. This experiment showed that this two-step digestion method can be used to probe conformational transitions of a protein in a complex biological background. This method was further applied to assess the structural features on large scale. Focusing on the metabolic transition from glucose-to ethanol-based growth, 1622 yeast proteins were identified by shotgun proteomics and altered conformations for 283 proteins were validated by SRM. This method was also used to monitor the thermally induced unfolding of proteins recently which enabled the determination of the thermal stability of proteins on a proteome-wide scale and with domain-level resolution [87].

As ligand-protein interactions also lead to structural changes on proteins, limited proteolysis has been applied to probe the ligand-induced conformational changes in purified proteins for many years [88–90]. The two-step digestion based LiP-MS approach allowed the detection of altered conformations of proteins in complex mixture. It certainly can be applied to discover the ligand bound proteins at proteome level. Indeed, this method was applied to yeast cell lysate and demonstrated that the structural changes on pyruvate kinase Cdc19 are due to allosteric regulation and dependent on the concentration of fructose-1,6-bisphosphate, a metabolite directly binding Cdc19 [86]. The method was further modified for systematically detecting proteins that become differentially susceptible to proteolysis upon binding of small molecule metabolite [91]. In this method, two-step digestions were applied to process the experimental sample incubated with interested metabolite and control sample only added with vehicle. After quantitative analysis of these samples, peptides with altered abundance between experimental and control samples are referred to as conformotypic peptides. To validate this method, it has been applied to screen the binding proteins in *E. coli* proteome for three metabolites with different levels of promiscuity, i.e. Adenosine triphosphate (ATP), L-phenylalanine (L-Phe), phosphoenolpyruvate (PEP), and the results were demonstrated to be highly reliable. For example, 231 ATP binding protein were identified, 92 of which are known ATP binding proteins. This method was further validated by screening the target protein of an antifungal drug, cerulenin, which is known to interact with only one protein, Fas2. The quantitative proteomics analysis of yeast cell extracts treated with and without cerulenin identified more than 2500 proteins. Among them, only one protein, Fas2, was observed an altered proteolytic pattern in treated relative to untreated extracts, indicating low false positive identifications of this method. All the proteolysis based methods mentioned above including TPP and DARTS measure the global energetic shift of intact proteins induced by the ligand binding and therefore cannot reveal the binding sites. However, conformotypic peptides can define the structurally changed regions on a protein upon treatment of protein sample with a given ligand. For example, in experiment with cerulenin the only one Fas2 peptide with altered abundance upon drug treatment was found at the known drug binding site. This was further confirmed by the fact that the conformotypic peptides identified for the metabolite binding proteins were most frequently positioned in very close proximity to binding sites [91]. Clearly the determined conformotypic peptides could pinpoint ligand binding sites on a proteome-wide scale. This approach was applied for the systematic identification of metabolite-protein interactions in *Escherichia coli*, revealing 1678 interactions and 7345 putative binding sites.

All the proteolytic digestion based methods use limited proteolysis to probe the shift of proteolytic susceptibility. They are classified into two types based on probing shift of proteolytic susceptibility globally or locally. The binding of drug stabilizes the protein-drug complex, which decrease proteolytic susceptibility globally and so increase the resistance of proteolysis. DARTS and pulse proteolysis exploit this feature, determining the amount of protein remaining after limited proteolysis. These two methods focus on the general proteolytic resistance so that cannot reveal the binding sites. Another type is the two-step digestion based method, which probe locally shifted proteolytic susceptibility. This method determines the cleavage sites for the initial proteolysis, reflecting the locally conformational changes on proteins. Compared with DARTS and pulse proteolysis, two-step digestion based LiP reveals that the locally structural changes on protein are toward either sensitivity or resistance of proteolysis. This is because that the ligands not only affect the binding sites of substrate proteins, but also regulate the formation or dissociation of protein complexes and high-molecular weight protein assemblies [91]. These high-order structural rearrangement can be detected in the two-step digestion based LiP as increase or decrease of proteolytic resistance. Thus, this approach can pinpoint regions in a protein locally or distantly affected by ligand binding [85]. The first type of method was widely used to screen protein drug targets from human proteome samples, while the second method was mainly restricted to probe the ligand-protein interaction in relative simple system such as *E. coli* or yeast, but not in human cell lines. This probably because the resulting digest of human proteome is extremely complex due to the two-step digestion, which leads to difficulty in high throughput quantification by LC-MS/MS.

4. Discussion and future perspective

The research in drug target screening has been developed from hypothesis driven biological assays to systematic analysis via multi-omics technologies. Recent development of mass spectrometer, especially the quadrupole Orbitrap based instruments, have bring the drug target screening to a new era of proteome-wide screening [92]. Chemical proteomics based drug screening methods enable the identification of target engagements in complex biological specimens. However, they suffer a few disadvantages including: (i) the small molecule drug must contain a derivatizable functionality, (ii) the derivatization may affect the bioactivity/binding specificity of the small molecule, and (iii) solid beads and/or linkers may hinder the binding of target protein to drug and may result in capture of non-specific proteins. Clearly there is a demand to develop modification-free approaches for drug target profiling. Indeed, a series of modification-free approaches have emerged during the last two decades. Combining these methods with the cutting-edge quantitative proteomics enables high-throughput, large scale and unbiased screening of drug targets at proteome level.

In this review, we introduced 6 modification free approaches, i.e. CETSA, TPP, SPROX, DARTS, pulse proteolysis and two-step

digestion based LiP, for drug target screening. These methods have their strengths and limitations as listed in Table 1. According to the specimen types, detection methods, quantitative strategies, and biophysical information, an appropriate method can be selected. In the aspect of specimen types, only CETSA and TPP are able to directly study the ligand-target interaction in living system. These two thermal-shift based methods can be applied to intact cells or cell lysates, revealing the different distribution of target proteins with or without drug binding. The experiment conditions in the other methods are not suitable for intact cells, i.e. SPROX and pulse proteolysis require a gradient of GdmCl to force the protein unfolding, while DARTS, pulse proteolysis, and two-step digestion based LiP require enzyme treatment. Without an effective cell lysis, the cytoplasmic target proteins are not accessible to these treatments. In the aspect of detection methods, CETSA, DARTS and pulse proteolysis mainly use western blotting as readout. They are simple, effective and do not need expensive instrument. They have been powerful tools for the validation of protein targets. But since these methods require prior knowledge and the availability of antibody, they are mainly fitted to hypothesis driven study but not discovery driven study. The other methods, TPP, SPROX and two-step digestion based LiP, mainly use MS-based bottom up proteomics as the readout, which allows the identification of drug target at proteome level in an unbiased way. In the aspect of quantitative strategy, these MS-based approaches could be divided into two groups, i.e., protein-centric or peptide-centric. The measurement in TPP is protein centric. The amounts of soluble proteins at different temperatures are quantified by shot-gun proteomics. Each protein could be quantified by multiple peptides which increasing the reliability of the screening. The measurement in SPROX and two-step digestion based LiP are peptide-centric. In other words, specific peptides are quantified to reveal the drug induced property change. For example, the unoxidized and oxidized methionine-containing peptides in SPROX are quantified to reveal the shift of thermodynamic properties in a protein's folding reaction, while the conformotypic peptides in two-step digestion based LiP are quantified to reveal the conformation change in proteins. These methods have the potential to reveal the binding sites. However, it is susceptible to interference that these two peptide-centric methods use the quantitative result of a single specific peptide for hit selection (unoxidized or oxidized methionine-containing peptides in SPROX, conformotypic peptides in two-step digestion based LiP) [93]. The identification for ligand binding site will be more confident with a series of specific peptides identified around the proximity. More biological or technique replicates also help to distinguish the true positive hits. In the aspect of data analysis, these modification-free approaches evaluate the changes of peptide/protein either under single native condition for point-to-point comparison, or among a series of denaturing extent for plotting a curve. The methods using native condition such as DARTS, two-step digestion based LiP can only identify protein targets, while the methods using a serial of different denaturing conditions such as CETSA, TPP, SPROX and pulse proteolysis can generate stability curve. The stability curves in SPROX and pulse proteolysis can, while CETSA and TPP cannot, be used to determine the thermodynamic properties, including protein folding free energy, binding free energy, and disassociation constant. This is mainly because that a statistical relationship is well established between protein folding free energy and chemical denaturant [94]. Besides, these approaches with denaturing gradient are intrinsically more advantageous to characterize the conformational properties of proteins. For some proteins that are too compact to be unfolded or resistant to proteolysis under native condition, adding denaturant could help to extend the protein structure for oxidation (SPROX) or enzyme cleavage (pulse proteolysis). For these methods that cannot provide affinity information directly, a dose-dependent experiment would help to solve the problem. With a series of drug concentrations applied to the experiments of these methods, a dose-dependent curve will be plotted and the half-saturation point is obtained which is directly related to affinity. In addition to drug target screening, modification-free approaches could also be applied to study the biomarker in samples of different biological states, e.g., normal and cancer cells. For example, SPROX coupled with SILAC quantification was used to large-scale analysis of breast cancer-related conformational changes on proteome [63]. These approaches are also suitable for the study of protein-protein interactome. TPP or MS-CETSA was used to profile system-wide protein complex dynamics in cells [95], and the modulation of protein-interaction states through the cell cycle [96].

However, the modification-free methods also suffer from some disadvantages. Firstly, these methods detect the ligand-induced property changes of proteins within proteome context, which may result in the identification of indirect binding proteins. This is because some proteins may present in a protein complex. Ligand-induced change of the target proteins may result in change of other proteins in the complex as they are associated together in some way. Although this promiscuity can be addressed via subsequent validation by using purified proteins, it is time-consuming and expensive to verify candidates one by one. In the contrary, ABPP can directly probe and chemically modify the active center of target protein, providing straightforward evidence for drug-protein interaction. Secondly, a current challenge in target deconvolution is to identify the binding protein from the complex proteomic sample. Like the affinity chromatography based method, the interference of non-specific proteins is also a problem for the modification-free methods. Since some low abundant target proteins will be 'drowned' in the high abundant non-targets, it will be difficult to detect the low abundant targets by quantitative proteomics. Though these issues can be partly alleviated by fractionation and/or multiple experiment replicates [60], the improvement of specificity and sensitivity in these approaches still needs more effort. An evaluation system should be established to balance the sensitivity and confidence for drug target screening. Corresponding work of confidence evaluation and false positive rate determination was reported [97]. Furthermore, the modification-free methods are designed to probe the changes of some properties of target proteins upon drug binding, e.g. the changes of thermodynamic stability and the rearrangement of conformation. However, some properties may not exhibit significant changes as expected, hence cannot be distinguished in the selected method. For instance, dasatinib showing no obvious stabilization effect for its known target BCR-ABL [48]. In this case, the target protein without detectable changes would not be identified. A combination of complementary methods will compensate the above drawbacks to some extent. It can be expected that higher confident drug-protein interactome could be obtained if multiple detectable property changes are used together to profile. For example, by combination of thermal gradient and limited proteolysis, protein thermodynamic stability was profiled on a proteome-wide scale and at domain-level resolution [87]. In addition, some novel modification-free approaches, for instance, chemical denaturation and protein precipitation (CPP) [98], semi-tryptic peptide enrichment strategy for proteolysis procedures (STEPP) [99], were developed to tackle this problem. Although there are some examples of using modification-free approaches to study drug-protein interaction site and stoichiometry, the structure information provided by these approaches are of lower resolution than conventional NMR, X-ray, and cryo-EM. These modification-free approaches are fitted for unbiasedly high-throughput screening drug target proteins on the proteome scale rather than deciphering fine molecular details of proteins'

structure. With all these pros and cons, modification-free approaches will be further evolved along with the future advances in mass spectrometry.

Acknowledgements

The authors acknowledge funds from the National Key R&D Program of China (2016YFA0501402, 2017YFA0505003), the National Natural Science Foundation of China (21535008, 81730016) and the innovation program (DICP TMSR201601, DICP&QIBEBT UN201802) of science and research from the DICP, CAS. MY is a recipient of the National Natural Science Fund of China for Distinguished Young Scholars (21525524).

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