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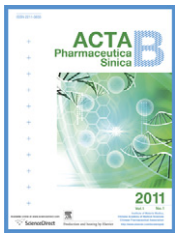

REVIEW

Protein target discovery of drug and its reactive intermediate metabolite by using proteomic strategy

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Abstract Identifying protein targets of bioactive compounds is an effective approach to discover unknown protein functions, identify molecular mechanisms of drug action, and obtain information for optimization of lead compounds. At the same time, metabolic activation of a drug can lead to cytotoxicities. Therefore, it is very important to systemically characterize the drug and its reactive intermediate. Mass spectrometry-based proteomic approach has emerged as the most efficient to study protein functions and modifications. This review will discuss method development for the drug target discovery and the application in different fields including the drug toxicity mechanism caused by reactive metabolites.

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1. Introduction

The process of high-throughput drug discovery typically involves screening protein targets against existing compounds to identify those which bind with high specificity and affinity[1,2]. Unfortunately, this strategy can result in unexpected or undetected off-target effects that lead to high attrition rates in the later stages of drug development. More appealing would be the unbiased identification of proteins and associated complexes to which a drug or drug candidate binds since it would enable direct evaluation and provide valuable insights into target cellular functions. One of the most widely applied methodologies to characterize proteins that bind specifically to candidate compounds is based on affinity chromatography combined with mass spectrometry-based quantitative proteomics. Small molecule-based affinity chromatography was first used for the purification of protein targets as early as the 1960s[3]. Since then, in combination with mass spectrometry-based quantitative proteomics, it has become one of the most widely applied techniques to characterize proteins that bind specifically to drug candidates[4].

In affinity chromatography, a small bioactive molecule is immobilized onto a solid phase support and then incubated with a protein extract. After incubation, the affinity resin is washed extensively with an aqueous buffer to elute any non-binding proteins from the resin. Bound proteins are then eluted from the affinity matrix under denaturing conditions or by incubation with the free ligand and resolved by SDS-PAGE[5]. Finally, the proteins are identified by mass spectrometric analysis. Once a putative target protein has been identified, several follow-up experiments can be carried out to confirm the interaction with purified protein. Direct binding can be analyzed by fluorescence anisotropy, isothermal titration calorimetry, or surface plasmon resonance. If the target is an enzyme, the compound or its analogs can be tested as potential inhibitors. A number of protein targets of biologically important natural products and small molecules have been discovered using this approach.

2. Synthesis of affinity matrix

2.1. Solid phase support

The first step is to immobilize the small bioactive molecule onto a solid phase resin. The latter is typically an agarose or sepharose-based polymer that is functionalized with reactive functional groups such as amines, thiols, or carboxylic acids[6]. Drawbacks of affinity chromatography with such conventional matrices include non-specific binding of proteins, chemical instability and incompatibility with some chemical modification procedures. To solve these problems, Handa and coworkers[7–9] developed a matrix composed of a styrene and glycidyl methacrylate copolymer core and a glycidyl methacrylate polymer surface in the form of a non-porous bead (called SG beads). The extremely small size of the beads (0.2 μm on average) provides a large surface area to increase the capture efficiency of target proteins and their non-porous nature allows easy removal of non-specifically bound proteins. This matrix also has good chemical and physical stability, high capacity for ligand attachment and high purification efficiency. Other matrices like poly(methacrylate)[10–12], poly(acrylamide) polymers[13] and monolithic materials[14–18] have also been used to produce affinity resins with reduced non-specific protein absorption and increased chemical stability.

Using a conventional support, it is always difficult to perform multiple parallel experiments due to the need to remove background proteins and separate resin by filtration or centrifugation. One way to overcome this is to utilize a magnetic support. These are now being widely used for bioseparations because they can be easily separated using a permanent magnet[19–21]. This improves the efficiency of the binding, washing and elution steps in the affinity enrichment process. Due to their low toxicity and stable magnetic properties, magnetite (Fe_3O_4) nanoparticles have been investigated for potential application in bioseparation, biosensing, drug delivery, magnetic fluid hyperthermia, and magnetic resonance imaging contrast enhancement[22]. Such nanobeads are also useful as a matrix material because they offer a large surface area for binding and are easy to resuspend and recover.

For the efficient capture of drug targets which are often expressed in very low abundance, carriers of nanometer size that are highly dispersible are desirable. It is also important that carriers retain their dispersibility and polymer coating in organic solvents since most drugs are water insoluble and require dissolving in organic solvents when synthesizing affinity beads. Furthermore, carrier surfaces must exhibit low non-specific binding of proteins because a large non-specific background signal can interfere with the identification of low-abundance targets.

2.2. Cell-permeable support

In a typical affinity chromatographic purification, a cell lysate or tissue homogenate is incubated with the affinity matrix under conditions designed to minimize protein degradation, typically 4 °C in the presence of protease inhibitors. Since the protein is not in its native physiological state, such studies remain prone to detecting false positives[23] and may also involve loss of some target proteins during sample preparation. To probe potential protein targets in living systems, the activity based protein profiling (ABPP) strategy has been successfully introduced for the study of enzyme families *in vivo*[24]. ABPP probes function on the basis of either a covalent reaction with the target protein or photoaffinity labeling by the incorporation of photoreactive groups. One important issue to consider is that many important ligands are either hydrophobic or negatively charged and are unable to penetrate the cell membrane of living cells[23]. The use of cell-penetrating peptides may facilitate drug penetration into living cells[25–27] but their use requires design of a conjugate to which it may be hard to attach other groups like a fluorescent tag. Accordingly, the establishment of a general *in situ* approach to probe intracellular protein targets is highly desirable.

Hu et al.[28] introduced soluble nanopolymers (i.e., dendrimers) as matrices for the immobilization of bioactive small molecules. Dendrimers are highly branched nanomolecules with attractive properties as drug delivery vehicles and imaging contrast agents. They have excellent solubility, high structural homogeneity, controlled surface functionalities, cell permeation ability, and low cytotoxicity[29,30]. The soluble dendrimer is multifunctionalized with drug candidates intended to promote specific interactions with protein targets, and with "handle" groups that facilitate final isolation through a highly efficient conjugation (Fig. 1). In a proof-of-concept study, anticancer drug methotrexate (MTX) was used as a model drug. Two proteins known to interact with MTX, dihydrofolate reductase (DHFR) and deoxycytidine kinase (dCK), were identified by this approach as intracellular drug targets. This strategy based on multifunctionalized soluble nanopolymers

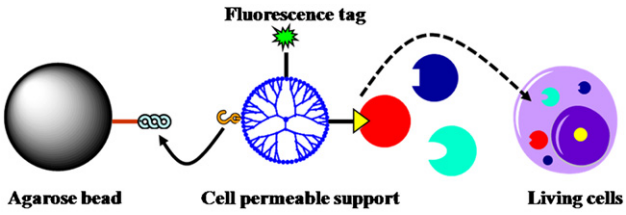

Figure 1 Schematic representation of the soluble nanopolymer-based approach to identify drug targets.

demonstrates their potential to successfully probe drug-target proteins *in vitro* and in living cells. It has a number of advantages over existing methods including the presence of multiple sites of attachment to facilitate the synthesis of intracellular probes and, in combination with mass spectrometry, the ability to provide sensitive, fast identification of proteins of interest in the most physiologically relevant environments. More importantly, a lot of hydrophobic or negatively charged drugs or prodrugs can be immobilized on dendrimers to improve their bioavailability. If at the same time they retain their bioactivity, it will broaden the application of this new strategy to many important biological systems.

2.3. *Spacer arm*

The procedure for immobilization of a compound on a solid support often involves a spacer arm to improve the efficiency of the interaction with target proteins in a cell lysate or tissue extract. Compounds are typically attached to the support through a long, hydrophilic linker such as a polyethylene glycol (PEG) chain[31]. Biological activity of the linker-functionalized compound is then determined to confirm that it interacts with the same proteins as the parent molecule. Besides minimizing non-specific binding, the hydrophilic nature of the linker separates the probe compound from the surface of the resin and gives it greater conformational flexibility so that it can assume a favorable binding orientation and allow efficient target protein interaction.

Sato et al.[32] designed a rod-like polyproline helix linker instead of the more commonly used PEG group (Fig. 2). The chain of nine L-prolines formed a stable left-handed helix of length 27 Å as measured by fluorescence resonance energy transfer experiments. The rigidity of the polyproline helix probably prevents its folding to permit better interaction with target proteins. In comparing the polyproline linker with a PEG linker of length 32 Å, Sato et al. found they could purify $<25\%$ of the target protein obtained using the polyproline linker. Furthermore, they isolated glyoxalase 1 as a new target of indomethacin, a clinically used nonsteroidal antiinflammatory drug that is known to inhibit cyclooxygenases. This further confirmed the ability of a rod-like linker to increase the capacity of affinity purification and the efficiency of separating low-abundance or low-affinity proteins.

3. Challenges for affinity enrichment

3.1. *Non-specific interaction*

In characterizing the specific binding partner(s) of a bioactive small molecule, a major challenge is to eliminate non-specific

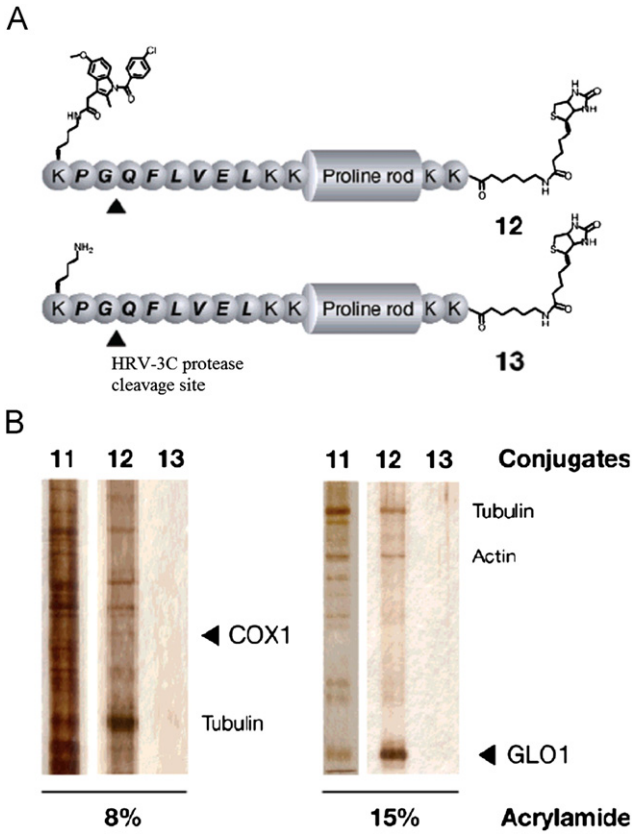

Figure 2 (A) Model structures of the polyproline linker (Cleavage site of HRVC3 protease is shown in bold italic letters and conjugate 11 has no cleavage site.) and (B) its application as a probe to isolate the target proteins, COX1 and GLO1[32].

binding proteins. This is because (1) the abundance of target proteins is often very low and their affinity and specificity for the probe is unknown and (2) many drugs bind to carrier proteins such as serum albumin or other highly abundant proteins like tubulin. Various approaches have been evaluated to remove non-specific binding proteins. For instance, Handa and co-workers[7,9] developed latex beads which considerably reduced non-specific interactions between sticky proteins and the solid support. The inclusion of detergents, salts or denaturing agents is another way to increase elution of non-specific proteins. Although more stringent washing may be used, it increases the risk of losing weakly binding target proteins. Introduction of a specific cleavage linker (as in tandem affinity purification[33]) is another way to reduce contamination with non-specific binding proteins. As an example, Mano et al.[34] immobilized small molecules on an agarose gel through a disulfide linker that is cleavable by mild reduction. This system allowed specific and non-covalent complex formation between the small molecule and the target protein whilst reducing binding of non-specific proteins to both the linker and gel surface to a minimum. Using deoxycholate as a model compound, Mano et al. captured two deoxycholate-binding proteins from mouse ascites with no accompanying non-specific binding proteins. More specific elution can be achieved by competition with free compounds[8,35] but many compounds do not dissolve in aqueous buffer solutions at high concentration.

In using affinity beads, it must be recognized that some proteins are present in very high abundance (up to several

orders of magnitude greater than the target of interest) and some are prone to interacting generically with their hydrophobic or charged surfaces[36]. In fact, even with all the approaches available, it is typical to identify several hundred proteins in an affinity-based chemical proteomics study[37]. One strategy to reduce this problem is to introduce a control experiment using a blank resin or an inactive compound with similar structure to that of the target molecule[38].

3.2. *Quantitation by stable isotope labeling*

Stable isotope labeling has proven to be particularly advantageous in discriminating true interactors from non-specific binders[39]. As an example, Oda et al.[40] used a protein isotopic labeling strategy involving isotope-coded affinity tags (ICATs) to compare the amounts of protein present in two different cell states. To do this, they labeled one state (the reference) with a light isotope label and the other (the test) with a heavy isotope label. The two samples were then mixed and analyzed by MS to determine the ratio of the two isotopic distributions and subsequently the relative amounts of protein. This quantitative approach has proven particularly advantageous for the discrimination of target proteins from non-specifically co-purified contaminants.

More recently, Ong et al.[41] described a proteome-wide quantitative chemical proteomics method. In this work, they combined stable isotope labeling with amino acids in cell culture (SILAC) with MS to analyze two different cell culture samples. The cells were grown in the presence of either heavy or light isotope-labeled arginine/lysine and were either untreated or pretreated with the drug of interest (Fig. 3). The labeled proteomes were then applied to drug affinity matrices and specific interactors (which were reduced or missing in the pretreated sample) were identified and quantified. An example of the use of free ligand and SILAC for quantitative analysis is shown in Fig. 4. By Western blotting, the signal of the target protein can be seen to decrease continuously with increasing free drug concentration. In addition, most proteins showing a 1:1 ratio with and without

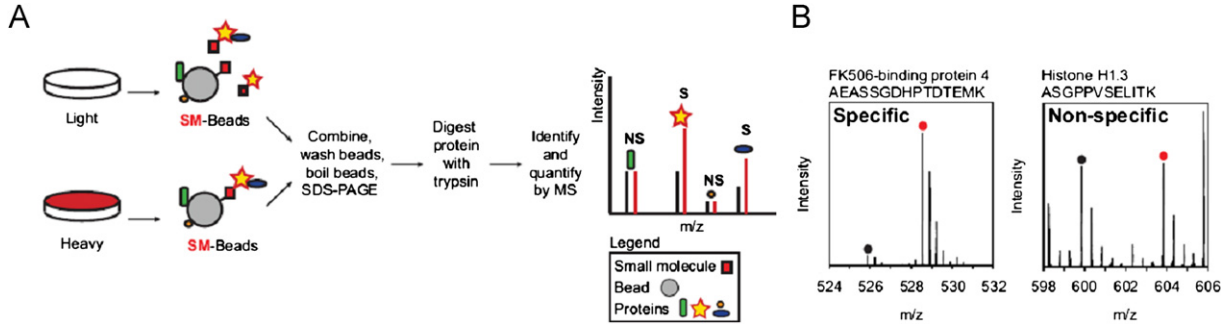

Figure 3 Identification of specific protein-small molecule interactions with quantitative proteomics. (A) SILAC identifies specific protein interactions using small molecule baits. (B) Experimental mass spectra showing specific and non-specific protein interactions with the immobilized ligand[41].

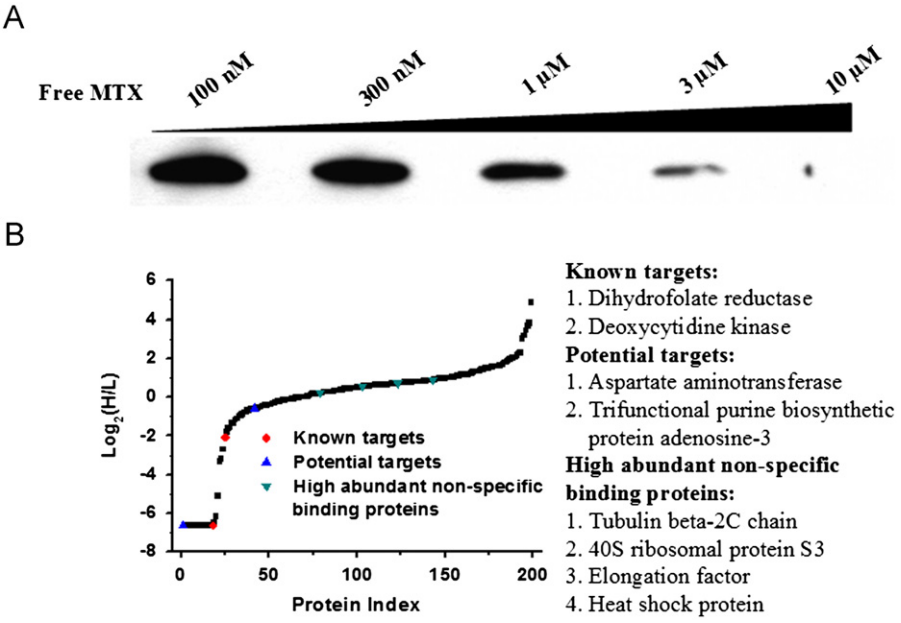

Figure 4 (A) Western-blotting analysis of MTX targets with free MTX of different concentrations as the competitive binding agent. (B) Profiling of proteins identified in the SILAC experiment against their $\log_2$(H/L) value (H and L are the peak areas of the "heavy" and "light" peptides)[28].

addition of free drug can be assigned as non-specific binding proteins. Only proteins for which the compound competes are considered as real target candidates worthy of further investigation.

Another way to differentiate the binding affinity of interacting proteins is through the use of a coupling competitive binding assay with stable isotope labeling. A recent report describes the successful combination of a mixed broad specificity kinase inhibitor matrix and free kinase inhibitor drug elution with quantitation of drug-protein binding parameters using isobaric tags for relative and absolute quantitation (iTRAQs)[42,43]. By measuring the competition with the affinity matrix, the binding curve of drugs to their targets in cell lysates can be obtained and the binding affinities of different proteins differentiated with a high throughput MS-based assay. Using this approach, Daub and coworkers[44,45] applied SILAC to characterize changes in the kinome during the cell cycle. To achieve this, they captured a large proportion of the kinome by serial drug affinity chromatography with different nonspecific kinase inhibitors, allowing them to compare the expressed kinome at two cell cycle stages. This work represents an important milestone in the study of kinases and kinase inhibitors since it successfully combines chemical, phospho- and quantitative proteomics methods into a powerful platform that provides a cellular understanding of kinase- and kinase inhibitor-related mechanisms.

4. Application to drug target discovery

4.1. Kinase inhibitors

Protein kinases are currently one of the most investigated classes of drug targets[46] as demonstrated by the dozens of kinase inhibitors that have entered clinical trials in recent years. Imatinib (Gleevec), a tyrosine kinase inhibitor, is such a drug that is now FDA-approved to treat certain types of cancer[47]. As there are more than 500 known human protein kinases and most of them engage the ATP binding pocket which is highly conserved, selectivity is a critical issue.

Brehmer et al.[48] immobilized analogs of bisindolylmaleimide, a potent protein kinase C (PKC) inhibitor, to profile kinase classes. They were able to identify several known and previously unknown enzyme targets in total cell extracts of HeLa cells. Subsequently, *in vitro* binding and activity assays confirmed the presence of the protein kinases, Ste20-related kinase and cyclin-dependent kinase 2 (Cdk2), as well as the non-protein kinases, adenosine kinase and quinone reductase type 2, as novel targets of bisindolylmaleimide inhibitors. This demonstrates selectivity profiling of closely related kinase inhibitors within a cellular proteome. Rix et al.[49] used three BCR-ABL inhibitors, imatinib, nilotinib and dasatinib, as affinity matrices to probe the expressed kinomes/proteomes of K562 and primary CML cells. Imatinib and nilotinib displayed greater target profile specificity than dasatinib which targeted a large number of Tyr and Ser/Thr kinases. Lolli et al.[50] immobilized cyclopropylpyrazole, a Cdk2 inhibitor, to selectively enrich Cdk2 and other kinases.

Recently, ''kinobeads'' have been developed displaying the non-selective kinase inhibitors, bosutinib, imatinib and dasatinib[42], and preferentially binding kinases and other purine-binding proteins in cell lysates. Using iTRAQ quantitation, the dose-response binding profiles of more than 500 proteins was determined. The combination of the mixed-affinity matrix with quantitative MS provides a versatile tool to map the direct and indirect targets of a drug in a single set of experiments. This approach should prove valuable at various stages of drug discovery as well as in translational studies of drug action in patient tissues. By mapping drug-induced changes in the phosphorylation state of the captured proteome, it was also possible to analyze signaling pathways downstream of the target kinases. This technology was further applied to selective histone deacetylase (HDAC) inhibitors with anti-cancer and antiinflammatory properties[43]. In this study, 16 inhibitors were clustered in terms of their effects on 1,251 proteins that specifically interacted with the probe matrix[43].

Due to the highly conserved ATP binding site, off-target effects have been reported for several drugs. For example, Missner et al.[51] reported off-target decoding for the multi-target protein kinase inhibitor, C1, by chemical proteomics. Besides several kinases known to bind to the compound, the pyridoxal kinase PDXK, known to interact with the CDK inhibitor, (R)-roscovitine, was captured. These workers later demonstrated that the PDXK–C1 interaction occurs at the substrate binding site rather than at the ATP binding site. These results greatly improve our knowledge of the off-target profile of the inhibitor. By using the ATP-competitive broad-specificity kinase inhibitor, bosutinib, Fernbach et al.[52] identified 70 individual targets in a K562 cell extract of which 19 had not been previously reported.

4.2. Natural products

Natural products have shown very promising resources for drug discovery with high structural complexity and diversity[53,54]. However, the majority of newly discovered natural products are only limited to biological activity with unknown targets. It is believed that natural products are more like to have multiple targets. Therefore, it would be essential to study the mechanisms for the multiple component interactions. Unlike synthesized combinatorial chemical libraries, most natural products lack the functional groups required to attach them to solid surfaces. It is also very difficult to predict the binding domain of small molecules that interact with unknown proteins. Osada and coworkers[55,56] developed a non-selective universal coupling method based on a photo-affinity reaction which enabled the introduction to a solid phase surface of a variety of small molecules such as rapamycin, cyclosporin A, digoxigenin, digoxin, digitoxin, and FK506. The only limitation of this approach is that it cannot be applied to small molecules that degrade under UV irradiation.

While chemical proteomics can identify protein targets of small molecules, subsequent analysis of changes in the genome-wide expression pattern on exposure to the compound can provide insight into the broader cellular context and indicate downstream effectors of the compound-target interaction. Wang et al.[57] immobilized levotetrahydropalmatine (L-THP), one of the main active ingredients in the traditional Chinese medicine, *Corydalis yanhusuo*, to probe the L-THP interactome. Furthermore, they compared L-THP interaction proteins with the proteins induced by L-THP treatment. The combined results indicated that proteins associated with signal

transduction, vesicular trafficking, neurotransmitter release, energy metabolism and ion transport play important roles in L-THP-induced antinociception. Suzuki et al.[58] prepared affinity beads bearing conophylline, a vinca alkaloid extracted from the tropical plant *Ervatamia microphylla* previously shown to induce differentiation of insulin-producing β-cells in cultured cells and animals. Unfortunately, its mechanism of action and molecular target remain unclear. Using conophylline-linked affinity beads, it was shown that conophylline directly interacted with ARL6IP (ADP-ribosylation factor-like 6 interacting protein) suggesting a possible mechanism of the drug action at the molecular level. Covalently linking the antiinflammatory marine natural product, petrosaspongiolide M (PM), to solid beads led to identification of PM interaction proteins and evaluation of PM bioactivity both *in vitro* and in living cells[59].

4.3. Other biological ligands

Other important bioactive molecules (enzyme inhibitors[60], metals[61–63] etc.) have been examined in chemical proteomic studies. Tian et al.[64] immobilized p-aminobenzamidine (ABA), an inhibitor of trypsin-like serine proteases, to characterize its interacting proteins in human plasma. By proteomic analysis, 214 proteins with affinity for immobilized ABA were identified. Based on Gene Ontology annotation, the identified proteases were shown to have catalytic activity and to bind calcium and to be mainly involved in blood coagulation. In another study, Tan et al.[65] used heparin affinity chromatography to analyze butyrate-treated HCT-116 colorectal cancer cells in an attempt to better understand butyrate's chemopreventive role. These workers detected 46 differentially expressed spots by 2-dimensional difference gel electrophoresis of which 24 were identified by MS analyses.

Dadvar et al.[66] used the novel PDE5 inhibitor, PF-4540124, as bait to probe its interactome in mouse lung tissue (Fig. 5). Initial affinity enrichment revealed the binding of hundreds of proteins to the immobilized compound after which pre-clearing and elution protocols in combination with stable-isotope labeling were used to elimninate non-specific binding proteins. Different isoforms of PDE5 were identified and, in addition, a novel interactor of PF-4540124, the prenyl binding protein PrBP, was also identified and verified by *in vitro* binding assays. In another study, ampicillin was immobilized onto agarose-based Affi-Gel to target ampicillin and penicillin binding proteins in extracts of *Escherichia coli* membranes. The aim was to illuminate the mechanism of antibiotic side effects through the identification of non-target drug targets[67].

Heck and coworkers[68,69] developed an affinity matrix by immobilizing cAMP and cGMP onto agarose beads. Through MS analysis, they identified many of the highly abundant AMP/ADP/ATP, GMP/GDP/GTP and general DNA/RNA binding proteins as well as cAMP/cGMP binding proteins. Subsequently, they used solutions containing ADP, GDP, cGMP, and/or cAMP for sequential elution and were able to sequentially and selectively elute ADP, GDP and DNA binding proteins. Further analysis of the cGMP/cAMP "interactome" in rat heart ventricular tissue enabled the specific pull-down of known cAMP/cGMP binding proteins such as the cAMP and cGMP dependent protein kinases, PKA and PKG, several phosphodiesterases and 6 AKAPs which interact with PKA. In the same way, Wong et al.[70] applied affinity chromatography using immobilized ATP, cAMP and cGMP to differentially profile the nucleotide-binding proteome of active and resting platelets. The specificity of the immobilized nucleotides was demonstrated by competitive assays and by immunoblotting. In another report, a synthetic ATP analog, 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) was used as an affinity probe of nucleotide-binding proteins[71,72]. Because adenine nucleotides are small, abundant molecules that bind numerous proteins involved in pivotal cellular processes and are co-factors or substrates for enzymes, regulators of protein function, or structural binding motifs, some 185 different labeled sites were identified, several of which were verified using three dimensional structures of the affected or related proteins. These workers also showed that FSBA labeled known *in vivo* tyrosine phosphorylation sites. Affinity matrices derivatized with synthetic phosphoinositides enabled the capture of several multiple phosphoinositide binding proteins in cell and tissue extracts and the identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases[73].

5. Protein target of reactive intermediate metabolites

Lots of drugs will generate the reactive metabolites, which are the short-lived intermediates and will covalently bind to protein or DNA to cause the toxicity. The covalent binding between drug metabolite and macromolecules was first found in the 1940s[74]. Most reactive metabolites will have low electron densities of high actitivies and target proteins usually contain the nucleophilic sites such as thiol and amine groups. Now a large number of drugs were found to be able to modify proteins leading to cell damage. Therefore, it is very important to characterize the binding of the intermediates and the corresponding macromolecules. To understand how the drug induces toxicity we will not only identify the target proteins and also need to identify the binding site of the reactive metabolite. For example, Gardner et al.[75] compared the covalent binding of clozapine and olanzapine to human neutrophils *in vitro* and *in vivo*. A major 58-kDa clozapine-modified protein was detected in neutrophils of patients treated by clozapine while only unmodified polypeptides can be detected in neutrophils from patients taking olanzapine. The differences in covalent binding exhibited by the two compounds may help to explain the reason that the olanzapine reactive metabolite did not cause toxicity toward human neutrophils at concentrations 10 times higher than clozapine[76].

Proteomics has provided an alternative approach for the study of drug-protein interaction and the possibility of identification of the modification sites. Traditionally a radiolabeled drug can be used to treat the cells or animals and then the proteins can be extracted and separated by two-dimensional gel electrophoresis (2D-Gel)[77]. The radioactive protein spot can be cut and analyzed by mass spectrometry[78,79]. Several protein targets have been identified by this approach. For example, Isbell et al.[80] used real sample of mouse liver microsomal fractions and ^{14}C-naphthalene for *in vitro* experiment. After 2D-Gel separation and MALDI-TOF fingerprinting analysis, 18 adducted proteins were identified to be modified by naphthalene, which needs to be further confirmed by *in-vivo* experiment and the modification sites also need to be identified by tandem mass spectrometry. Also this

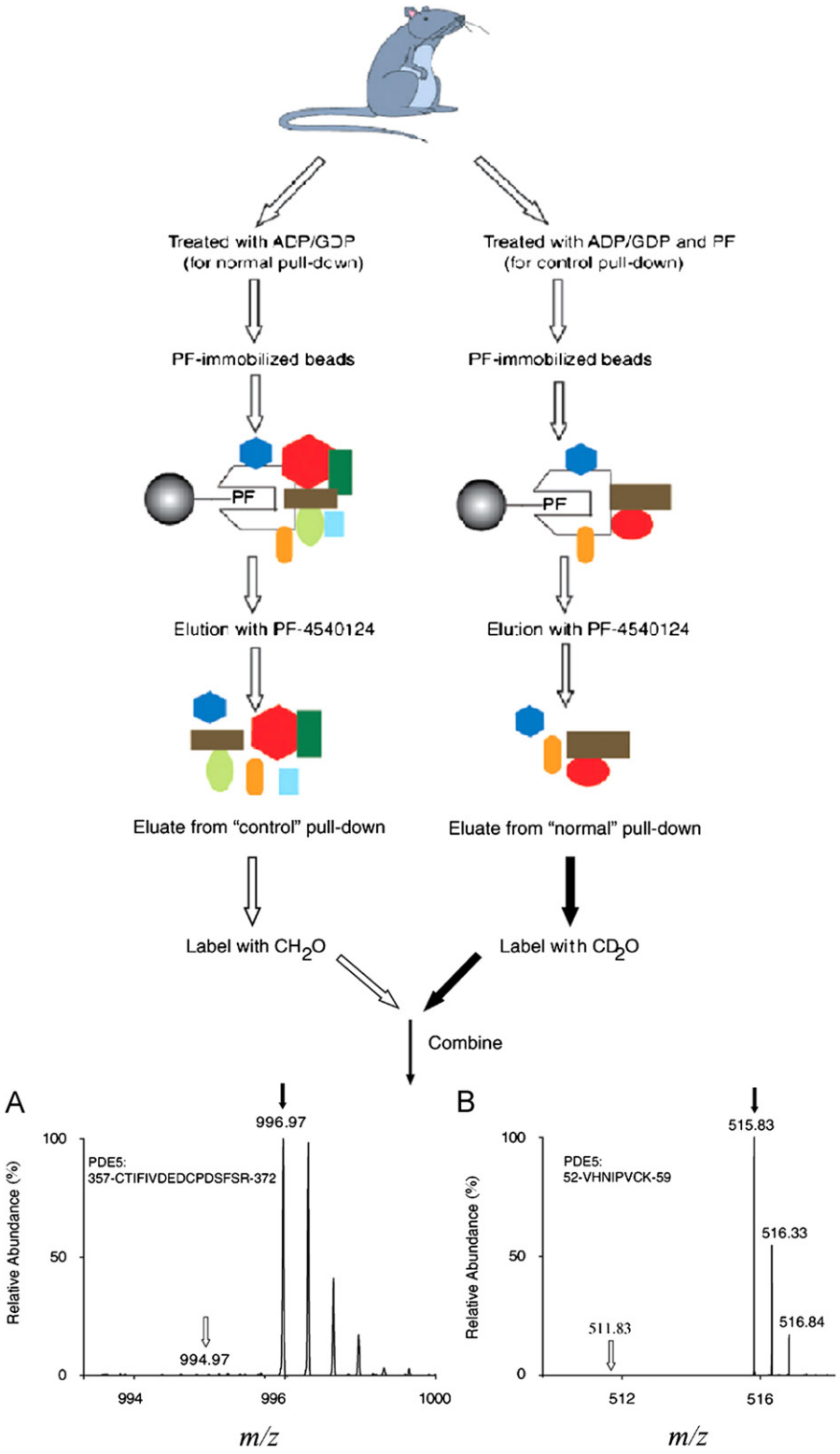

Figure 5 Schematic representation of the protocols for selective enrichment of the PF-4540124 "interactome" and quantitative analysis by stable isotope dimethyl-labeling[66].

method is limited by the radioisotope and the low throughput. Advanced mass spectrometry-based proteomics has accelerated this process with the powerful ability of mass spectrometry for the identification of number of proteins without prior knowledge of the protein sequence[81]. To know the toxic mechanism of naphthalene, Zhang et al.[82] extended tandem mass spectrometry and bioinformatics analysis to identify the binding site of the target proteins. As shown in Fig. 6, naphthalene is metabolized

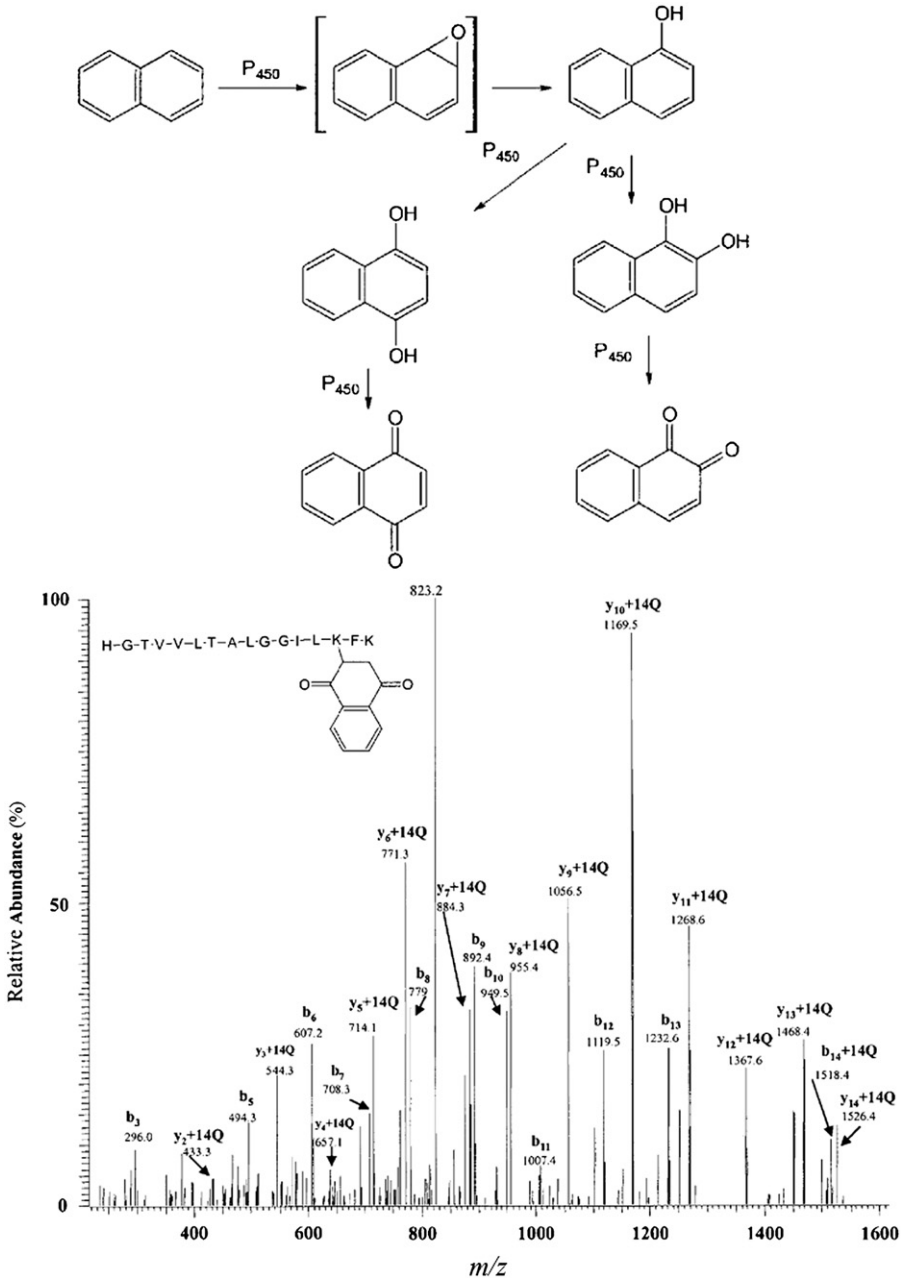

Figure 6 Scheme of naphthalene quinone metabolites and MS/MS spectrum for peptide of HGTVVLTALGGILKK modified by 1,4-naphthoquinone[82].

to naphthalene-1,2-oxide (NPO) and further converted into 1,4-naphthalenediol and 1,2-naphthalenediol by cytochrome P450. The diols can be oxidized to the corresponding 1,4-naphthoquinone and 1,2-naphthoquinone, which are good electrophiles and can covalently bind proteins leading to the toxic effects of naphthalene. An *in-vitro* experiment was performed by incubation with hemoglobin and then the protein was digested for mass spectrometry analysis. The MS/MS spectrum of one peptide is shown in Fig. 6. The modification site can be determined by SALSA searches of the tryptic peptide. Bateman et al.[83] reported the detection of covalent adducts to cytochrome P450 3A4 with its two known inhibitors L-754,394 and 6′,7′-dihydroxybergamottin by analysis of the intact protein adduct using top-down proteomic strategy. The information may provide new source for understanding the mechanism of covalent protein modifications in drug discovery and development

6. Prospective

As proteomic technology matures, chemical proteomics will be more widely applied. It will provide unbiased fingerprint profiling of protein targets on a proteomic scale which will substantially assist in drug optimization. More unanticipated off-target information will also be obtained to improve the process of drug design[84]. Although a number of reports have already demonstrated the potential of affinity chromatography

for drug discovery, its routine use remains to be adopted. One issue is the high-cost of mass spectrometric analysis compared to conventional biological assays. Another is the problem of non-specific binding and recognition specificity. The novel design of affinity enrichment and increasing bioinformatics analysis of the output data will be essential to the understanding of protein function. Last but not the least understanding the toxicity of the reactive metabolite is no less important than the drug target discovery, which will greatly decrease the chemical toxicities of a potential drug at an early stage.

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