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Review

Chemical proteomics for drug discovery based on compound-immobilized affinity chromatography☆

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Abstract

Chemical proteomics is an effective approach to focused proteomics, having the potential to find specific interactors in moderate-scale comprehensive analysis. Unlike chemical genetics, chemical proteomics directly and comprehensively identifies proteins that bind specifically to candidate compounds by means of affinity chromatographic purification using the immobilized candidate, combined with mass spectrometric identification of interacting proteins. This is an effective approach for discovering unknown protein functions, identifying the molecular mechanisms of drug action, and obtaining information for optimization of lead compounds. However, immobilized-small molecule affinity chromatography always suffers from the problem of non-specific binders. Although several approaches were reported to reduce non-specific binding proteins, these are mainly focused on the use of low-binding-affinity beads or insertion of a spacer between the bead and the compound. Stable isotope labeling strategies have proven particularly advantageous for the discrimination of true interactors from many non-specific binders, including carrier proteins, such as serum albumin, and are expected to be valuable for drug discovery.

Keywords: Affinity chromatography; Drug discovery; Proteomics

Contents

1. Introduction

After the completion of the human genome project in 2001, the focus of research has shifted to establishing the functions of the numerous gene products, i.e., proteins. This is a complex task, because the functions of most proteins are tightly regulated by a variety of post-DNA/RNA processes, including control of expression levels, localization to different subcellular organelles, and post-transcriptional and post-translational modifications [1]. The major aim of proteomics is thus to identify, characterize, and assign molecular and cellular functions for the tens of thousands of proteins encoded by eukaryotic and prokaryotic genomes [2]. To accomplish this goal, high-throughput methods have been utilized for large-scale studies of proteins, including their expression levels, cellular functions, localization, and interaction networks [3–6].

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How should we apply human proteome information to understand biological systems and disease mechanisms, so as to contribute to human health care? The National Institutes of Health (NIH) in the US published a roadmap in October, 2003 and has already begun to grapple with this important issue (http://nihroadmap.nih.gov). As a part of this project, the NIH Molecular Libraries Initiative (MLI) is focused on small molecules that are instrumental in the treatment of diseases. In fact, most medicines currently on the market are small molecules. Although classical chemical biology involved the exploration of physiologically active compounds one by one, the purpose of chemical genomics in MLI is to expand chemical biology to proteome-wide scale. In order to identify those small molecules that will have the greatest effect on a disease or biological process, MLI utilizes robotic systems to screen molecular targets that are provided by 25,000 human proteins against hundreds of thousands of chemicals.

There are two categories of small-molecular compound libraries, one being a diversity-oriented library based on diversity-oriented synthesis (DOS) [7] and the other being a focused library [8] based on target-oriented synthesis [9] (TOS). In DOS, the synthetic effort aims to create broad representations of compounds in chemical space, including currently poorly populated regions. MLI has given priority to the maintenance of this diversity-oriented library. On the other hand, a focused library is constructed with concentration on a specific chemical motif (pharmacophore) and related compounds. From a cost point of view, a focused library is more attractive for small or medium-size laboratories/companies, because their limited resources can be focused on a potentially interesting pharmacophore. Although a focused library covers only a narrow range of compound structures, bioactive compounds usually interact with several molecules, generally proteins, in cells. For drug discovery, an understanding of both protein function and drug selectivity is highly important. The elucidation of the targets would thus contribute to effective clinical application and the prediction of unexpected side effects, as well as possibly highlighting the compound as a potential lead compound for other projects. Therefore, chemical proteomics using compound-immobilized columns (compounds covalently bound to columns) is a promising and powerful strategy for drug discovery independent of resource size of research facilities, because the elucidation of drug–protein complexes is a direct approach, as distinct from indirect analysis, such as 2-dimensional electrophoresis or the use of DNA chips to monitor changes of protein/mRNA expression (Fig. 1). In addition, identification of drug-interacting proteins may provide clues to the functions of these proteins. Although protein arrays represent an important proteomic tool, and allow the global observation of biochemical activities on an unprecedented scale [10–12], proteins must be on an array chip and keep physiological states including modifications and complex formation. Therefore, protein arrays seem have limitation for unbiased evaluations for small molecules. Also compound-immobilized surface plasmon resonance (SPR) technologies are interesting tools to study protein–drug interactions [13], however the identification of enriched binding proteins on a SPR chip might be not easy due to limited amount of loading samples. Small molecule microarrays have already been successfully applied in pharmaceutical area [14], but in this review, we focus on recent advances in chemical proteomic methods based on compound-immobilized affinity chromatography to evaluate drug-candidate selectivity in an unbiased, comprehensive way, followed by mass spectrometric based protein identification.

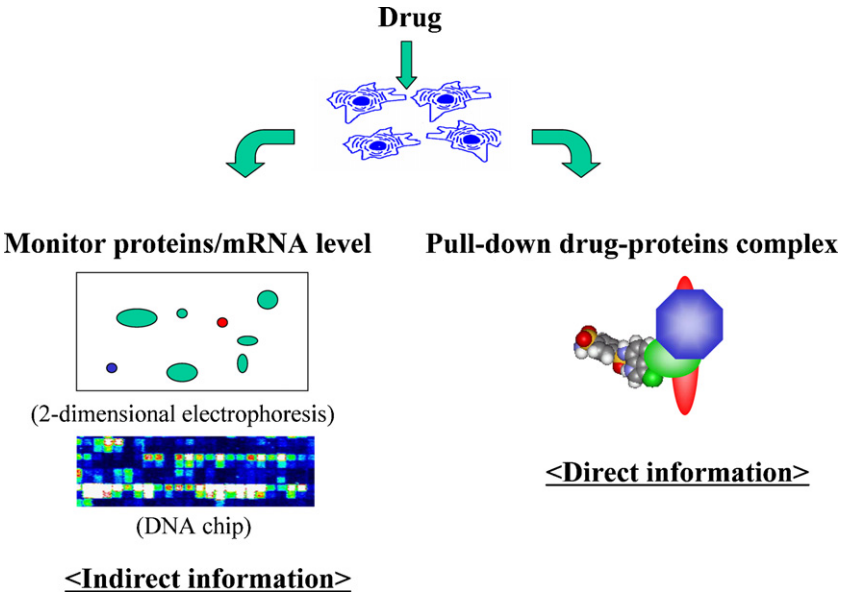

Fig. 1. Drug-related proteomic/transcriptomic researches. After drug administration to cells/animals, comprehensive protein/mRNA level quantitation provides a tremendous amount of information, such as drug–response pathways, though the information is generally indirect. On the other hand, drugs interact with their primary targets, so the elucidation of drug–protein complexes provides direct evidence about the mode of action of a drug.

2. Studies on molecular mechanisms of drug candidates

Cell-based phenotypic and pathway-specific screening of natural products and synthetic compounds within living cells, which is a chemical–genetic approach, has historically contributed to the discovery of promising drug candidates. This kind of unbiased drug screening is generally followed by research efforts to identify the protein targets and molecular mechanisms involved in the compound-induced phenotypes. Recent advances in combinatorial synthesis in combination with prediction of novel proteins encoding putative drug targets have provided unprecedented access to large compound libraries of considerable structural complexity and diversity, shifting the bottleneck in drug discovery to the development of efficient screening methods for protein targets. The selectivity of drug candidates found by high-throughput target-oriented techniques can be overestimated, because counter-screening is conducted against only a modest number of related proteins available in pure form, and alternative protein targets, such as different types of enzymes, are usually excluded altogether. In fact, candidate compounds are sometimes found to induce unexpected phenotypes, not necessarily toxic, in cultured cells or animal experiments during drug development. It is therefore very important to identify both the primary and other targets of drug candidates in order to accelerate the discovery of small molecules that selectively modulate protein activity.

One of the most versatile methods to profile cellular targets of selected drug candidates is compound-immobilized affinity chromatography. The procedure involves immobilization of a compound on a solid support through a spacer arm and the application of this matrix to fish for interacting proteins in a cellular lysate or tissue extract. For example, Schreiber and coworkers reported the identification of FK506-binding protein [15] and mammalian histone deacetylase 1 using affinity matrices [16]. Fukuda et al. found that leptomycin B was a potent and specific inhibitor of the nuclear export signal-dependent protein, CRM1 [17]. Now, the power of affinity chromatography combined with advances in protein identification by sensitive and high-throughput MS analysis offers huge potential for finding previously unrecognized activities and potential therapeutic applications. Nevertheless, optimization of the affinity approach is still urgently required to gain new insights into the cellular modes of action of small molecules (Fig. 1).

3. Characterization of nucleotide-mimetic compounds, i.e., kinase inhibitors

Most current drugs are antagonists for receptors or inhibitors of metabolic enzymes, so cellular signaling pathways, including protein kinases, are an attractive area for the pharmaceutical industry [18]. Gleevec, developed for cancer therapy, is an example of a successful kinase inhibitor [19]. Pharmacological inhibition of protein kinases can be achieved with small-molecular inhibitors, which block the catalytic activity of kinases by interfering with the relatively well-conserved ATP-binding site. Most of these inhibitors are thought to be highly specific, based on parallel enzymatic assays with sets of recombinant protein kinases, though only small subsets of the more than 500 human protein kinases can currently be used to test the selectivity of drug candidates. Therefore, efficient proteome-wide methods to assess kinase inhibitor selectivity are important for both signal transduction research and drug development.

In studies on comprehensive assessment of kinase inhibitor selectivity, affinity purification methods combined with MS have revealed the relevant cellular target kinases in cases where suitable compound derivatives can be immobilized on chromatography beads [20,21]. SB 203580 is an anti-inflammatory drug, which belongs to the pyridinyl imidazole class of compounds and was originally designed as an inhibitor of mitogen-activated protein kinase p38 [22]. SB 203580 was thought to be relatively specific for p38, based on protein kinase inhibition in vitro. However, in addition to p38, SB 203580 also inhibits cytochrome P450 enzymes, cyclooxygenase and thromboxane synthase, although only at higher concentrations. Subsequently, the immobilization of a suitable analogue of SB 203580 on chromatography beads led to the identification of several protein kinases as previously unknown high-affinity targets of SB 203580, and these results imply a far more complicated cellular mode of action of this inhibitor than had previously been assumed.

The indolinone compound SU6668 is an ATP-competitive inhibitor that was originally designed as a selective inhibitor of receptor tyrosine kinases, such as PDGFR, VEGFR2, and FGFR1, involved in tumor vascularization [23]. Although SU6668 monotherapy was effective in clinical studies, the achievable plasma concentration was insufficient to inhibit the target kinases. The use of immobilized SU6668 analogues showed that SU6668 had previously unknown targets, including Aurora kinases and TANK-binding kinase 1. Thus, SU6668 appears to exert pharmacologically relevant cellular effects via inhibition of previously unknown Ser/Thr kinase targets.

Some quinoline compounds, such as the 4-aminoquinoline chloroquine and the quinolinemethanol mefloquine, were used as anti-malarial drugs until the emergence of drug-resistant parasites, though their modes of action were not fully understood. Graves et al. developed an effective approach to elucidate the primary targets of these quinolines. They used gamma-phosphate-linked ATP-Sepharose to isolate purine-binding proteins, including several kinases, and then they selectively identified targets of quinoline compounds, which were aldehyde dehydrogenase 1 and quinine reductase 2, from the purine-binding subset proteome [24].

The selectivity of protein kinase inhibitors remains problematic, and moreover, alternative protein targets, such as different types of enzymes, are not routinely analyzed. This is a very important issue in drug development programs. The affinity purification methods established for the SB 203580 derivative and SU6668 derivative should work equally well with other kinase inhibitor scaffolds, and should therefore be of general utility to define the selectivity and molecular modes of action of small-molecular kinase inhibitors.

The cyclic nucleotide monophosphates cAMP and cGMP act as second messenger molecules in many signal transduction pathways. Scholten et al. immobilized cAMP/cGMP onto

agarose beads via flexible linkers at either the 2- or the 8-position of the nucleotide moiety [25]. They also developed a sequential elution protocol from the beads using solutions containing ADP, GDP, cGMP and/or cAMP to selectively elute ADP-, GDP-, and DNA-binding proteins. They did not use ATP as a competing elution buffer, because ATP activates many proteins, such as kinases, which may cause dramatic changes in the protein complexes. They found that cAMP/cGMP-binding proteins, such as PKA/PKG, strongly bound to the beads, and could be only eluted after boiling the beads with SDS. This last fraction contained sphingosine kinase type 1-interacting proteins, which are potential AKAPs.

4. Compounds covalently binding to enzymes

Although many drug candidates bind non-covalently to proteins, several enzyme inhibitors have played a role by providing new reagents to characterize protein function on a global scale based on activity rather than abundance. Recent developments in chemical biology have made it possible to analyze protein functions by the design of active site-directed probes that measure enzyme activity in complex biological samples, even in vivo. The conceptual and experimental foundation for this approach, referred to as activity-based protein profiling (ABPP), has been reviewed elsewhere [26–29]. The general strategy in activity-based profiling typically involves a small molecule-based, active-site-directed probe which targets a specific class of enzymes based on their enzymatic activity. The design template requires a reactive group, a linker and a tag. Upon interaction of the inhibitor with the target enzymes, the reactive group reacts with the active site in a mechanism-based manner to generate a covalently bound tag. The resulting probe–enzyme adducts are easily distinguishable from unmodified proteins. Functional immobilization of suitable enzyme inhibitor analogues in combination with the specific purification of cellular binding proteins by affinity chromatography can lead to the identification of both known and previously unknown enzyme targets. A number of activity-based chemical probes have been reported, and some have been successfully used for proteomic profiling of different enzyme classes in complex proteomes. ABPP is very powerful technique to elucidate protein functions globally. However, most current drugs interact non-covalently with target proteins, so the purpose of ABPP is distinct from that of drug discovery research in the pharmaceutical industry.

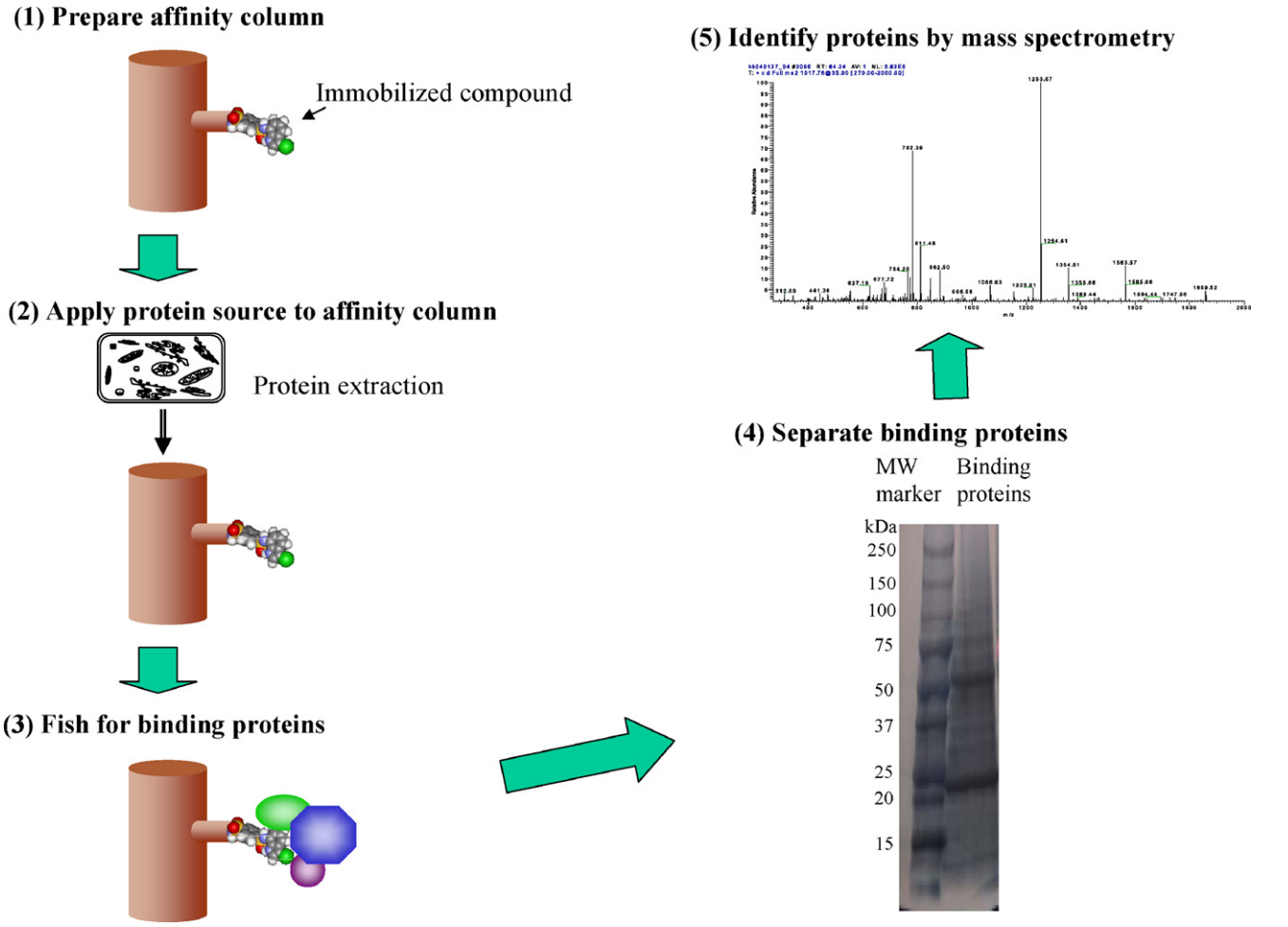

Fig. 2. Schematic illustration of chemical proteomics. To identify compound-binding proteins, the compound needs to be immobilized on a solid-support (1) before or after complex formation (2). Washing the support enriches binding proteins (3), which are then separated, e.g., by SDS-PAGE (4). Finally, binding proteins are identified by mass spectrometry (5).

5. Discrimination of specific interactions from non-specific binders

Chemical compounds may interact with proteins to form transient or stable complexes which mediate biological activities. Coupled with an affinity technique to purify a specific protein binder of interest, MS can rapidly and reliably identify the components of such complexes, placing MS at the forefront of technologies for studying small-molecular compound–protein interactions. In general, appropriate negative affinity purifications are conducted in parallel to discriminate between bona fide components of the complex and background contaminants. Purified protein complex components are separated using techniques such as SDS-PAGE. Individual proteins may then be visualized by staining or western blotting and can be identified by MS (Fig. 2).

In characterizing binding partners for a small molecule by MS, the major challenge is to identify bona fide interacting partners, because, compared with natural products, the affinity and specificity of synthetic small molecules for their protein targets are often low. For instance, many drugs bind to carrier proteins such as serum albumin. The affinity purification method thus plays a critical role. Moreover, the very high sensitivity of MS analysis can permit identification of almost all proteins, even contaminants present at very low levels in the sample. Although more stringent washing may be used to reduce levels of contaminating proteins, this increases the risk of losing true – albeit weakly binding – partners. In addition, highly abundant proteins which bind extremely weakly to a compound can be detected by MS, and the existence of proteins that associate with specific/non-specific binders can make the situation even more complicated. Thus, non-specific interactions between a synthetic compound and binding proteins often lead to difficulty in specifying the primary binding partner(s).

Various approaches have been tried to overcome the contamination problems inherent in protein interaction studies. Tanaka and co-workers noted that the hydrophobicity of the spacer between the resin and the compound affects non-specific interaction [30]. They optimized the structure of the spacer, and showed that introduction of a hydrophilic spacer, such as tartaric acid derivatives, dramatically decreased non-specific binders of abundant proteins, such as actin and tubulin. Introduction of a dual purification strategy, called tandem affinity purification (TAP) of tagged proteins of interest can dramatically improve the signal-to-noise ratio via the generation of cleaner samples [31–33]. For identification of compound-binding proteins, competitive elution from affinity columns with free compounds is generally used. However, many compounds do not dissolve in aqueous buffer solution at high concentration, and carrier proteins such as albumin cannot be discriminated by competitive elution with free compounds, so this approach has limitations. If targets are predictable, such as kinase inhibitors, endogenous substrates, such as ATP, can be used as competitors to immobilized compounds, but the affinity of these native ligands is usually low, and strong binders to the affinity column may not be eluted. State-of-the-art surface chemistry for affinity beads provides ways to achieve ultra-low background levels in enrichment fractions. Von Rechenberg et al. investigated a drug-target

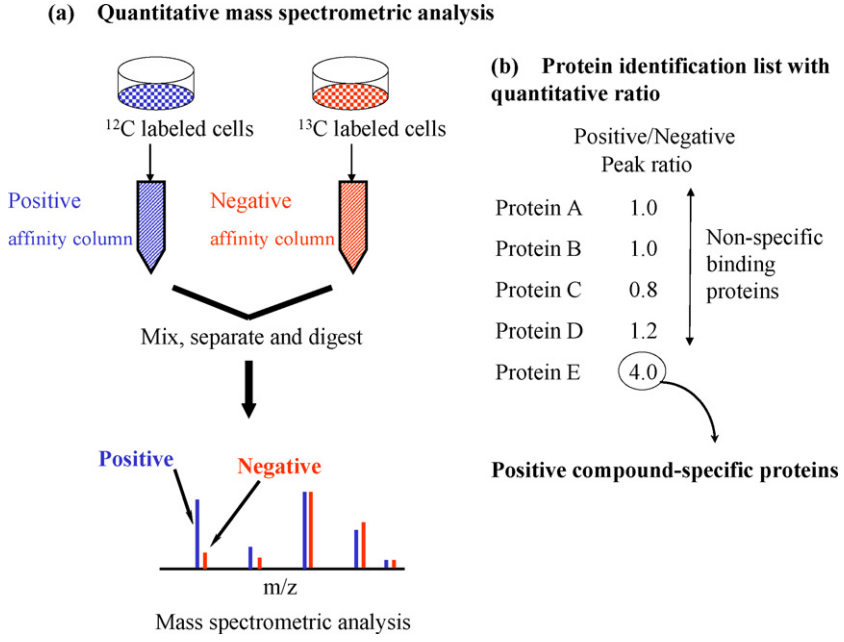

Fig. 3. Strategy for discrimination of specific binding proteins from non-specific binders. (a) First, two cell pools are prepared, but one cell pool is cultured in a stable isotope-enriched medium, so that all the proteins are labeled with stable isotopes in this pool. Second, two affinity columns, bearing an immobilized positive-binding compound and a negative-binding compound, are prepared. The cell lysates from the two cell pools are each loaded onto each type of affinity column, and binding proteins are collected. The two binding protein pools are mixed. Binding proteins can be identified from pairs of peaks observed in the mass spectra, one being derived from the normal medium cell pool and the other from the stable isotope-labeled cell pool. (b) The peak ratios are calculated; a 1:1 ratio means that this protein binds both types of compound equally, but a 10:1 ratio means that this protein binds to one type of compound 10 times more strongly than to the other compound. Thus, specific binding proteins can be quantitatively found from among a huge amount of non-specific binders.

system consisting of ampicillin- and penicillin-binding proteins to evaluate non-specific binders when the compound was immobilized with different amino-reactive beads [34]. They succeeded in identifying a primary target as well as its associated protein complex by using Dynal M-270 magnetic beads to pull down drug-binding proteins. Handa and co-workers reported that the latex beads developed in their laboratory could considerably reduce non-specific interactions between numerous sticky proteins and the solid support [35]. Although their special beads might be useful to decrease non-specific binders, biologically inactive proteins that bind to a compound, such as carrier proteins, cannot be discriminated from specific partners that are responsible for phenotype changes in response to administration of the compound. The subtraction approach using a positive affinity column and a negative affinity column can identify a specific target protein of a drug candidate [36]. This strategy is relatively straightforward, though highly sensitive MS analysis can still detect proteins which may be present at trace levels in both sample fractions.

The distinction between normal states and stimulated/disease states in proteome level are very important to elucidate indicators of drug safety, mechanism of action, efficacy, and disease state progression. These indicators, referred as biomarkers may dramatically improve the efficiency of drug discovery and development. Today, several high-throughput methods are available in quantitative proteomics [37–41]. Seminal work on differential isotopic labeling of proteins by Oda et al. has led to novel strategies for quantitative proteomics [42,43]. The common feature of these techniques is that protein profiling can be performed by comparing the amounts of proteins present in two different cell states by designating one state as the reference with a light isotope label and adding a heavy isotope label to the other. The two samples are then mixed and analyzed by MS. The ratio between the two isotopic distributions (one for the light reagent and one for the heavy reagent) can be determined from the mass spectra and used to calculate the relative protein quantities. This quantitative approach using stable isotope labeling with MS analysis has proven particularly advantageous for the discrimination of proteins specifically associated with the target population from non-specifically co-purified contaminants by comparative quantitation between positive column binders and negative column binders [44] (Fig. 3). This integrated strategy using affinity techniques and quantitative proteomics should be of wide utility for identification of the targets of biologically active small molecules. This methodology provides a new tool for chemical proteomics in post-genomic medicinal science.

6. Conclusion

In conclusion, a compound-based affinity approach to chemical proteomics can be used for unbiased large-scale profiling of protein target selectivity; this is impossible with currently available drug screening panels. The new technique can be applied for the rapid and large-scale identification of primary targets of drug candidates and, more generally, protein–ligand pair interactions, allowing us to obtain binder fingerprints on a proteome-wide scale. Such information is potentially very useful for optimization of lead compounds. The data may also serve to define previously unknown protein functions, based on the phenotypes induced by compounds.

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