Target identification of natural medicine with chemical proteomics approach: probe synthesis, target fishing and protein identification

Xiao Chen1,2, Yutong Wang1, Nan Ma3, Jing Tian1, Yurou Shao1, Bo Zhu1,2, Yin Kwan Wong3,4, Zhen Liang4, Chang Zou4 and Jigang Wang4,3,5

INTRODUCTION
Over the last 30 years, natural products have become an important source of new drugs for the treatment of various diseases. However, developing natural product-based new medicines through random moiety modification is a lengthy and costly process, due in part to the difficulties associated with comprehensively understanding the mechanism of action and the side effects. Identifying the protein targets of natural products is an effective strategy, but most medicines interact with multiple protein targets, which complicate this process. In recent years, an increasing number of researchers have begun to screen the target proteins of natural products with chemical proteomics approaches, which can provide a more comprehensive array of the protein targets of active small molecules in an unbiased manner. Typically, chemical proteomics experiments for target identification consist of two key steps: (1) chemical probe design and synthesis and (2) target fishing and identification. In recent decades, five different types of chemical proteomic probes and their respective target fishing methods have been developed to screen targets of molecules with different structures, and a variety of protein identification approaches have been invented. Presently, we will classify these chemical proteomics approaches, the application scopes and characteristics of the different types of chemical probes, the different protein identification methods, and the advantages and disadvantages of these strategies.

Signal Transduction and Targeted Therapy (2020) 5:72; https://doi.org/10.1038/s41392-020-0186-y

1School of Medicine & Holistic Integrative Medicine, and College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China; 2School of Biopharmacy, China Pharmaceutical University, Nanjing 210009, China; 3Artemisinin Research Center, and Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China; 4The First Affiliated Hospital of Southern University of Science and Technology, The Second Clinical Medical College of Jinan University, Shenzhen People’s Hospital, Shenzhen 518020, China and 5Department of Toxicology, School of Public Health, Guangxi Medical University, Nanning 530021, China
Correspondence: Zhen Liang (liang.zhen@szhospital.com) or Chang Zou (zou.chang@szhospital.com) or Jigang Wang (jgwang@icmm.ac.cn)
These authors contributed equally: Xiao Chen, Yutong Wang, Nan Ma

Received: 9 March 2020 Revised: 30 April 2020 Accepted: 30 April 2020
Published online: 21 May 2020

© The Author(s) 2020

Signal Transduction and Targeted Therapy
biology and the advent of the postgenomic era, an emerging and broadly applicable approach termed chemical proteomics was developed for target identification at the proteome level.31,32

As an important branch of proteomics, chemical proteomics integrates diverse approaches in synthetic chemistry, cellular biology and mass spectrometry.33 It is an approach to comprehensively fish and identify multiple protein targets of active small molecules, and it consists of two key steps: (1) probe design and synthesis and (2) target fishing and protein identification. In recent decades, five different types of probes and their respective target fishing methods with different scopes and characteristics for chemical proteomics approaches have been developed34 to screen targets for small molecules with different structures. After target fishing, there are also multiple protein identification methods that are suitable for different situations.35–37 Numerous pharmacological studies have applied chemical proteomics to identify drug targets and study their MOA.38–40 especially in the last few years.41 Hence, these studies provides us with a unique background to summarize the recent achievements in this field.

In the present review, we first briefly introduce the chemical proteomics approaches, including their classification and workflow, as well as their advantages and disadvantages. Second, as the initial step of target identification, we provide a glimpse of the synthetic processes of five different types of probes and describe in detail the probes’ application scope and characteristics, as well as their subsequent target enrichment schemes. In the third section of the review, different protein identification methods with distinct scopes, including gel separation and band identification, quantitative proteomics approach, and protein microarray, are described. In the last section, we provide some comments on the future direction of chemical proteomics for the target identification of natural products.

CHEMICAL PROTEOMICS IN TARGET IDENTIFICATION

Chemical proteomics is a postgenomic version of classical drug affinity chromatography that is coupled to subsequent high-resolution MS and bioinformatic analyses.42 As illustrated in Fig. 1a, chemical proteomics approaches can be divided into two categories according to their different workflows, namely, activity-based protein profiling (ABPP) and compound-centric chemical proteomics (CCCP).23

ABPP is a technology that combines activity-based probe and proteomics technologies to identify protein targets of small bioactive molecules to help elucidate their MOA and side effects.31,43 In a typical ABPP experiment (Fig. 1a), probes derived from the parent molecules are first designed and synthesized based on a structure–activity relationship (SAR) study of the parent molecules. The probes should be synthesized as follows: (i) the probes should retain the pharmacological activity of their parent molecules to ensure the accuracy of subsequent target identification; (ii) the probes should allow for easy enrichment of bound protein targets. Next, the probes are incubated with effector living cells, lysate or tissue homogenates, allowing them to completely bind their target proteins. After enrichment with chemical and biochemical techniques, the protein targets are identified through proteomics approaches. The final step is to validate the target information via SPR, MST, ITC, etc. and the corresponding pharmacological effects with the appropriate biological function assays.

CCCP originates from classic drug affinity chromatography, which has been in use for decades.45–46 Along with the development of proteomics techniques, CCCP merges the classic method with modern proteomics to identify protein targets of small bioactive molecules at the proteome level. Unlike ABPP (Fig. 1a), the first step of CCCP is to immobilize the drug molecule on a matrix,47 such as magnetic or agarose beads. The probe synthesis and immobilization processes will be described in detail in the next section. Similar to ABPP, immobilization should not influence the pharmacological activity of the drug of interest. Subsequently, lysates from cells or tissues are incubated with the affinity matrix, followed by extensive washing to remove nonspecific binders. After complete elution, the enriched proteins are identified with proteomics approaches, and the target information and corresponding pharmacological effects must also be confirmed.

As mentioned above, the chemical proteomics approach may possess many advantages, such as being unbiased and allowing high-throughput at the proteome level, but it also has limitations. With chemical proteomics, the mass of nonspecifically bound proteins and the active metabolites, in addition to the true target

![Fig. 1](image-url) a Comparison of activity-based probe profiling and compound-centric chemical proteomics. b General molecular structures of different types of chemical proteomics probes
proteins, may also be identified, leading to potential false-positive results. Furthermore, proteins that are insoluble in the buffers (e.g., PBS, Tris-HCl) used during the target enrichment process may pass unnoticed through the matrix. Comparing the two chemical proteomics approaches, in contrast to ABPP, the activation state of the identified proteins cannot be detected with CCCP, but CCCP is a more unbiased approach, allowing it to even identify targets with no enzymatic function, thereby facilitating the discovery of novel targets.

PROBE DESIGN AND SYNTHESIS

Designing and synthesizing the probe is the initial and pivotal step for target identification in chemical proteomics approaches. Generally, a probe consists of three parts, which are responsible for its respective functions: (i) a reactive group, which is derived from the parent drug molecule and ensures that it retains its pharmacological activity and ability to bind or modify protein targets; (ii) a reporter tag, such as biotin, an alkyne or a fluorescence group, for target enrichment or detection; (iii) a linker, sometimes cleavable, to connect the reactive group and the reporter tag, and it should be long enough to avoid steric hindrance.45,50 However, the structure of the probe may not always be constant. For example, in different chemical proteomics strategies for target identification, the probe might have one or even two of the components omitted. In this section, we will describe the diverse types of probes applied in chemical proteomics target profiling, as well as their design and synthesis, characteristics and scope of application.

Immobilized probe

In earlier studies, bioactive natural products were covalently immobilized on biocompatible inert resins, such as agarose and magnetic beads, to serve as bait for fish for target proteins in the active proteome (Fig. 1b). Due to the intrinsic properties of the beads, such as their macroscopic size and magnetism, the probe-fished proteins can be easily enriched, which is convenient for subsequent target identification. In the structure of bioactive natural products, various groups, such as sulfhydryl, amino and carboxyl groups, can be utilized for attachment to different active resins, which are commercially available.

For example, Schreiber et al.46 immobilized FK506 (tacrolimus), a natural immunosuppressant, to identify its protein targets in lysates obtained from calf thymus and human spleen cells. As shown in Fig. 2a, FK506 affinity matrices were prepared using an FK506 amino derivative. After complete incubation with cytosolic extracts of bovine thymus and human spleen, the matrix was competitively eluted with FK506, and a 14 K protein was enriched and identified. This led to the identification of a FK506-binding protein of 12 K (FKBP12), which functions as a protein folding chaperone for proteins containing proline residues. Another example is trapoxin, a microbially derived cyclotetrapeptide that inhibits histone deacetylation in vivo and causes mammalian cells to undergo cell cycle arrest.51 Because the epoxyketone side chain of trapoxin is indispensable for activity, Schreiber et al. chose to replace one of the phenylalanine residues of trapoxin’s cyclic core with a lysine that could then be covalently linked to a solid support. The matrix was incubated with nuclear proteins from bovine thymus, and the bound polypeptides were eluted by boiling the matrix in 1% SDS buffer. Six major polypeptides with apparent molecular sizes between 45 and 50 kDa were detected by SDS-PAGE and silver staining. In addition, the authors also employed trapoxin to competitively inhibit the binding between the polypeptides and the matrix to validate the specificity of the results (Fig. 2b). Other examples are illustrated in Fig. 2c.52-55

Although immobilized probes are easily synthesized and widely employed, one of their limitations, immobilization-induced steric hindrance, remains unsolved. The immobilization of the probes, which are always employed in CCCP, might influence the binding between the true targets and the reactive group, potentially leading to false-positive protein targets or the loss of potentially important protein targets. This could prove costly if an unsuitable protein target was selected for further evaluation.

Activity-based probe

To overcome immobilization-induced activity impairment, activity-based probes (ABPs) were developed for target identification in chemical proteomics. In the design of such probes, the first factor to consider is the activity of the drug molecule;51 in other words, the incorporation of the reporter group and the linker should not influence the bioactivity of the active molecule. Therefore, the SAR of the molecule should be studied or consulted before the start of the synthesis, and the probe’s pharmacological activity should be determined. Unlike immobilized probes, ABPs can interact with proteins in the active proteome before enrichment and even pass through the cell membrane to bind target proteins in living cells, potentially reflecting the true drug-target interactions under physiological conditions in cells.

However, non-immobilization raises an obvious question: How does one enrich or detect probes and target proteins is the probe is not immobilized? In the structure of ABP, a reporter group is present to overcome this problem (Fig. 1b). Among the diverse reporter groups, biotin is most frequently utilized due to its strong affinity with avidin, allowing its enrichment using either avidin or streptavidin beads. For instance, to elucidate the mechanism of resveratrol, a natural product exhibiting anticancer activity in mouse melanoma cells, our group synthesized a probe by connecting a biotin tag to resveratrol based on an SAR study and validated the probe activity with in vitro biochemical experiments (Fig. 3a). Subsequently, the probe was incubated with lysates of melanoma cells and then enriched with streptavidin beads. As a result, we identified histone deacetylase I (HDAC1) as the protein target of resveratrol in mouse melanoma cells and revealed an epigenetic regulation pathway of focal adhesion kinase.53 Other excellent examples are listed in Fig. 3b.56-64

In addition to the biotin tag, fluorescent tags are also widely used as the reporter group for target identification. Fluorescent-modified probes allow the efficient and rapid detection of target proteins, but it cannot be enriched like biotin tags.65 However, in some cases, due to the large size of biotin, biotin can interfere with the original activity of the bioactive drug molecule. In addition, endogenous biotinylated proteins in the active proteome can interfere with identification by generating false-positive protein targets.56,66

Click chemistry probe

With advancements in bioorthogonal chemical reactions, especially the development of the click reaction, the limitations of biotinylated probes have been largely alleviated. In fact, the incorporation of an orthogonally reactive group in the structure of natural products has been one of the most widely used strategies for target identification in the last decade. With the orthogonal reaction group, such probes can undergo bioorthogonal click reactions with their complements (e.g., azide to alkyne, strained alkyne to tetrazine, tetrat to cyclopropane, etc. and vice versa), thereby covalently connecting probes to affinity tags (biotin-azide, biotin-cyclopropane, etc.) or fluorescent tags (rhodamine-azide, FITC-azide, etc.) for subsequent enrichment and target identification (Fig. 1b). Due to their relatively small sizes, these orthogonally reactive groups have little or no influence on the intrinsic pharmacological activity of the natural products, and the probes can easily reach the cytoplasm to bind target proteins in situ before the click reaction and enrichment.

Many research groups, such as Tate’s group at Imperial College, Sieber’s group at the Technical University of Munich and Lin’s group at the National University of Singapore, have made great
achievements in the target identification of natural products with click chemistry probes, including acivicin,\(^7\) curcumin,\(^6\) andrographolide,\(^7\) artemisinin,\(^7\) zerumbone\(^7\) and cholesterol.\(^7\) Taking artemisinin as an example (Fig. 4b), Wang et al. utilized a click chemistry probe of artemisinin to identify its protein targets in \textit{Plasmodium falciparum} and made two important findings: (i) heme, rather than free ferrous iron, is predominantly responsible for artemisinin activation; and (ii) artemisinin may kill the parasite through a promiscuous targeting mechanism. Because modifying artemisinin’s structure without influencing its activity is quite difficult, the authors synthesized a click chemistry probe derived from artesunate, an analog of artemisinin that also exhibits antimalarial activity. After activity validation, the probe was incubated with malaria parasites to fully bind the target proteins. Then, the target–probe complex was modified with a biotin tag through a click reaction and enriched with streptavidin beads. Finally, a total of 124 parasite proteins were identified, of which 33 proteins had previously been reported. Moreover, taking OAT (ornithine metabolism, arginine and proline metabolism) as a representative target, the activation mechanism of artemisinin was studied. It was observed that the probe itself did not bind to OAT, and its binding required the addition of hemin and was further enhanced in the presence of vitamin C, Na\(_2\)S\(_2\)O\(_4\) or glutathione, which reduce hemin to heme. By contrast, the addition of ferrous iron had no detectable effect on probe-OAT binding, revealing that heme, rather than free ferrous iron, has a predominant role in artemisinin activation.

**Fig. 2** Workflow of target identification with immobilized probes of natural medicines. \textbf{a} Identification of FK506 protein targets with an immobilized FK506 probe. \textbf{b} Identification of trapoxin protein targets with K-trap. \textbf{c} Structures of other reported immobilized probes of natural medicines.
Another example is ferroptocide, a small molecule chemically derived from the diterpene natural product pleuromutilin that rapidly and robustly induces ferroptotic death of cancer cells. After biological evaluation, Llabani et al. synthesized a click chemistry probe of ferroptocide to identify the protein targets in HCT 116 cells (Fig. 4c). The cells were pretreated with 20 μM ferroptocide or DMSO for 60 min and then incubated with 20 μM ferroptocide probe for 30 min; a click reaction with biotin-azide and enrichment with streptavidin magnetic beads followed. On-bead trypsin digestion coupled to LC/LC–MS/MS analysis provided a list of over 300 targets. With subsequent CRISPR knockout studies, the authors found that ferroptocide is an inhibitor of thioredoxin, a key component of the antioxidant system in the cell, and positively modulates the immune system in a murine model of breast cancer.

Photoaffinity probe
All the click chemistry probes mentioned above possess active groups that can covalently modify the amino acid residues in target proteins, leading to steady binding between the probes and the targets during the click reaction and enrichment. However, some natural products, such as resveratrol, interact with their protein targets through noncovalent secondary bonds, including hydrogen bonds, ionic bonds and hydrophobic interactions. For these bioactive molecules, simple click chemistry probes are unsuitable for target identification because the binding between the active molecule and the target protein can be disrupted during the click and enrich processes due to their noncovalent interactions. For such cases, the photoaffinity labeling technique (PAL) was developed. Photoaffinity probes generally consist of a click chemistry probe skeleton for target binding and enrichment and a photoaffinity group for fixing the binding between the probe and the targets (Fig. 1b). After incubation with the active proteome, the photoaffinity probe generates highly active free radical intermediates to covalently bind the target protein under certain wavelengths of light, and this is followed by click chemistry and target enrichment. Frequently used photoaffinity groups include benzophenone, aryl azide, and diazirine, and of these, benzophenone is the most widely applied due to its stability and ease of synthesis. In recent years, diazirine-based photoaffinity probe synthesis has attracted much interest due to its small size and...
high efficiency. Unfortunately, photoaffinity groups can intrinsically bind some nonspecific proteins (e.g., diazirine to voltage-dependent anion channels),\textsuperscript{82,83} which may affect the accuracy of the results.

Cravatt’s group has utilized photoaffinity probes directly in living mammalian cells to globally map the binding proteins of cholesterol, an essential structural component of cellular membranes that serves as a precursor of several classes of signaling molecules.\textsuperscript{84} Based on cholesterol’s structure, they first designed and synthesized a set of sterol probes (Fig. 5a), each of which contained a photoactivatable diazirine group at the 6 position of the steroid core, which is a modification that has previously been shown to minimally perturb the biophysical properties of cholesterol. Then, living human cells were incubated with the probes and irradiated with 365 nm UV light to covalently cross-link the probe with the targets. After biotin modification through a click reaction and enrichment with streptavidin beads, the target proteins were identified with a quantitative proteomics approach. Over 250 cholesterol-binding proteins, including receptors, channels, and enzymes involved in many established and
previously unreported interactions, were identified. Other examples of photoaffinity probes are illustrated in Fig. 5b.85–93

Nonlabeling approach

In all the types of probes described above, the addition of an exogenous group could interfere with the pharmacological activity of the natural product. Moreover, for some natural products, their structures have no active sites suitable for modification, thereby limiting the application of these probe-based methods. For this reason, some nonlabeling chemical proteomics approaches, such as drug affinity responsive target stability (DARTS),94,95 stability of proteins from rates of oxidation (SPROX),96,97 cellular thermal shift assay (CETSA),98,99 and thermal proteome profiling (TPP),100 were developed for target identification. DARTS identifies targets by detecting enzymolysis changes based on the fact that the binding of the drug molecule stabilizes target proteins to trypsin-induced hydrolysis. For instance, Piazza et al.101 utilized a DARTS-based approach to identify the protein targets of three metabolites: adenosine 5′-triphosphate (ATP), L-phenylalanine (L-Phe) and phosphoenolpyruvate (PEP). In the study (Fig. 6a), proteomes were extracted under conditions that preserve the structures of native proteins, and the extracts were exposed to the small molecule of interest. Samples were subjected to limited proteolysis with the broad-specificity protease proteinase K to generate structure-specific protein fragments. The fragments were then digested with the sequence-specific protease trypsin to generate peptide mixtures amenable to bottom-up proteomic analysis. The peptides were analyzed with MS, and the targets were identified by comparing the peptides in the presence and absence of the small molecule. A total of 231 targets were observed for ATP, and 129 and 41 protein targets were identified for PEP and L-Phe, respectively.

Unlike DARTS, SPROX detects the oxidation level of methionine in proteins to identify targets due to changes in antioxidant ability.
following the binding of the molecule. CETSA covers a wider range of applications than DARTS and SPROX, such as target identification in living cells, cell lysates and tissues. It is based on the thermodynamic stability alterations induced by the drug molecule’s binding. To overcome the challenges of low sensitivity and throughput in CETSA, TPP was developed. It is derived from CETSA but also allows the identification of off-targets and biomarkers. For these nonlabeling methods, the natural products need not be modified or attached to exogenous groups, allowing complete retention of their intrinsic bioactivities. Although nonlabeling chemical proteomics approaches have been widely applied in the target identification of natural products and medicines, they suffer from a few drawbacks, such as the tedious condition groping process and insufficient target sensitivity against nonlabeling molecules.

TARGET IDENTIFICATION
After probe synthesis and subsequent target enrichment, the target proteins are identified with proteomic analysis by mass spectrometry, which is a valuable tool. In the early days of research, mass spectrometry was applied to identify specific bands in SDS electrophoretic gels or spots in two-dimensional electrophoretic gels. Because the targets are confirmed by comparing the gray values of proteins in different groups (probe vs control), this method may lead to low-abundance target loss and nonspecific results. To overcome these challenges, quantitative proteomics approaches for measuring abundance changes of many proteins in multiple samples have been developed. In addition, with the development of chip technology, protein microarrays have also been employed for target identification in chemical proteomics approaches. In this section, we will describe several different protein identification methods in detail, including their workflows, features and practical applications.

Gel separation and band identification
Briefly, the target proteins in the active proteome are enriched with molecule-derived probes and subsequently separated through SDS-PAGE or two-dimensional electrophoresis. Coomassie brilliant blue staining or silver staining can be used to visualize the separated proteins. Then, the gels containing the enriched proteins from different groups (always probe vs control) were aligned to identify the distinct bands or spots, followed by gel excision and in-gel digestion. Finally, the target proteins were identified by identifying the postdigested peptides with mass spectrometry (Fig. 6b). In the example mentioned above, our
group identified resveratrol’s targets in mouse melanoma cells with this method (Fig. 3a). After probe synthesis and target enrichment, the binding proteins were eluted with SDS loading buffer. Then, the targets were separated by SDS-PAGE, followed by Coomassie brilliant blue staining, and we discovered two distinct bands compared with the control lane (DMSO). The two bands were excised and identified as acetyl-CoA acetyltransferase 1 (ACAT1) and HDAC1. With in vivo and in vitro experiments, we confirmed that resveratrol inhibits focal adhesion kinase (FAK) expression by interacting with HDAC1. Although the method has been widely used in protein identification, it has two disadvantages: (i) some low-abundance but vital target proteins are still invisible after Coomassie brilliant blue staining or even silver staining, resulting in target loss; and (ii) some distinct bands or spots, especially bands, contain more than one protein, so the nonspecific binding in these bands or spots may also be identified.

Quantitative proteomics
To overcome the deficiencies in-gel separation and band identification, quantitative methods have been incorporated into chemical proteomics. By comparing the relative abundance of proteins between different samples or against appropriate negative controls, proteins with a higher abundance ratio (>1.5 or 2, different values have been used) than a threshold are identified as specific targets, thus avoiding nonspecific binders. Due to the precision of the method, low-abundance targets can also be identified. Quantitative proteomics approaches mainly include stable isotope labeling by amino acids in cell culture (SILAC), chemical labeling approaches and label-free approaches. SILAC has become the most frequently used quantitative proteomics approach in target identification since its development in 2002. It uses isotopically labeled amino acids to stably label proteins during cell culture and determines the relative quantity of peptides by comparing the molecular weight shifts after MS.

To date, many studies have applied chemical proteomics approaches along with SILAC to identify protein targets of various natural products as well as natural medicines. For instance, Kalesh
et al. applied a “spike-in” SILAC method to identify the cellular targets of zerumbone (Zer), a phytochemical with diverse biological activities ranging from anti-inflammatory to anticancer properties. As shown in Fig. 7b, the authors initially synthesized a click chemistry probe of zerumbone (Yn-Zer), and the probe’s activity was confirmed to be similar to that of zerumbone. In parallel, lysates of HeLa cells labeled with $^{15}$N$_4^{13}$C$_6$-arginine and $^{15}$N$_2^{13}$C$_6$-lysine (heavy cells) were treated with 20 μM Yn-Zer, whereas lysates from cells cultured in normal medium (light cells) were treated with 20 μM Yn-Zer, 20 μM Yn-Zer combined with 75 μM Zer, 20 μM Yn-Zer combined with 100 μM Zer, and 20 μM Yn-Zer combined with 150 μM Zer. The addition of Zer competitively inhibited the binding between the target proteins and Yn-Zer. With SILAC, the relative amounts of the fished proteins (compared to the heavy group) were determined, and proteins with lower relative amounts with increasing Zer concentration were identified as specific targets. Finally, a total of 62 proteins that are involved in vital biological processes showed statistically significant enrichments, with many of these proteins having key roles in regulating apoptosis and cell survival.

Compared with SILAC, isobaric tags for relative and absolute quantification (iTRAQ), a typical chemical labeling approach, can be used to perform stable isotope labeling of peptides digested from proteins and utilizes labeling reagents to quantify reporter ions fragmented by MS/MS, affording many advantages. For example, Wang et al. utilized a clickable activity-based probe derived from andrographolide, a natural product with known anti-inflammatory and anticancer effects, to enrich protein targets in live cancer cells (Fig. 8b). In the assay, the cells were first incubated with the clickable probe or with DMSO as a negative control. Following sequential probe binding, biotin modification through a click reaction, avidin pull-down and thorough washing, the target proteins were digested with trypsin. The resulting peptides were labeled with their respective iTRAQ regents (control group with 113 and 114, whereas probe group with 116 and 117) and pooled for further identification and quantification via LC–MS/MS. The results were also validated through cell migration and invasion assays, revealing that andrographolide has a potential novel application as a tumor metastasis inhibitor.

For the label-free quantitative proteomics approach, protein abundance is calculated by detecting the MS signal densities of peptides digested from the specific protein or the number of MS/MS signals corresponding to peptides and proteins. Due to the missing labeling process, this method is much simpler and more cost-efficient. However, it has disadvantages in accuracy and throughput, especially in some promiscuous cases, such as samples with heavy backgrounds, compared to quantitative labeling proteomics approaches. Moreover, in the label-
free approach, only one MS run can be done per sample, so samples of different groups have to be examined in separate MS runs, which might lead to operating error, leading to reduced accuracy in the results.

Protein microarray
In addition to MS-based approaches to identify the protein targets of drug molecules of interest, protein microarrays are another approach that has been utilized frequently. The main function of protein microarray technology is investigating the functional properties of immobilized proteins, the interaction of proteins, and their enzyme activity, and it has also been utilized to study the interactions between proteins and natural products or medicines for decades.132,133 Combined with chemical proteomics, this method can also be applied for target identification, providing a platform for analyzing the interactions between small molecules and thousands of proteins.134 In a typical protein microarray approach (Fig. 6c), diverse proteins are first immobilized on a miniature high-density array, followed by the labeling of the molecule to be tested with an affinity tag, such as biotin, a fluorophore, a photoaffinity group or a radioactive isotope, to allow the molecule-linked proteins to be easily traced. Notably, this method is high-throughput, allowing the identification of target and off-target proteins in the whole proteome on the microarray in a single run.135,136 However, it also possesses certain disadvantages. For example, active molecules need to be modified with tags that might influence their intrinsic activities. Along with the development of mass spectrometry techniques, protein microarrays are always combined with mass spectrometry to overcome modification-induced activity alterations.137

SUMMARY AND OUTLOOK
In the development of new natural medicines, target identification facilitates the determination of the MOA and side effects, accelerating this process from discovery to market. Along with the development of chemical biology and proteomics, the chemical proteomics approach has become a popular method in target identification of small active molecules, especially natural products, providing an important theoretical basis for novel natural medicine research and development. In most cases, natural products need to be modified with reporter tags to facilitate enrichment or detection, which might influence their intrinsic pharmacological activities, thereby leading to a biased target result. Moreover, some nonlabeling chemical proteomics approaches for target identification are not well qualified in promiscuous cases, and their low accuracy and throughput limit their broad application. Therefore, developing a highly accurate nonlabeling chemical proteomics approach with high-throughput is imperative. Although some studies have simultaneously applied two or more different strategies for target identification to avoid nonspecific binding and narrow target collection and obtained a more accurate target list, the MOA predicted from the targets must be validated by biochemical methods. Collectively, the use of chemical proteomics will continue to be a key tool to drive the discovery of new therapeutic compounds of natural origin.

ACKNOWLEDGEMENTS
We gratefully acknowledge financial support from the National Natural Science Foundation of China (81903588, 81803456 and 81841001); the Major National Science and Technology Program of China for Innovative Drug (2017ZX09101002-001-001-05); the Fundamental Research Funds for the Central Public Welfare Research Institutes (ZKXT16003); the Natural Science Foundation of Jiangsu Province (BK20190799, China); the Science and Technology Foundation of Shenzhen (JCYJ20180303154128430); the International Cooperation Foundation of Shenzhen (GJHZ2018092817602104); the Shenzhen Economic and Information Committee “Innovation Chain and Industry Chain” integration special support plan project (20180225112449943); and the Shenzhen Public Service Platform on Tumor Precision Medicine and Molecular Diagnosis.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

REFERENCES
1. Newman, D. J. & Cragg, G. M. Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. 70, 461–477 (2007).
2. Guo, Z. The modification of natural products for medical use. Acta Pharmac. Sin. B 7, 119–136 (2017).
3. Carlson, E. N. Natural products as chemical probes. ACS Chem. Biol. 5, 639–653 (2010).
4. Rodrigues, T., Reker, D., Schneider, P. & Schneider, G. Counting on natural products for drug design. Nat. Chem. 8, 531 (2016).
5. Gouyette, A. Synthesis of deuterium-labelled elliptinium and its use in metabolic studies. Biomed. Environ. Mass Spectrom. 15, 243–247 (1988).
6. Caille, P. et al. Phase II trial of elliptinium in advanced renal cell carcinoma. Cancer Treat. Rep. 69, 901–902 (1985).
7. Jones, R. N., Fritzche, T. R., Sader, H. S. & Ross, J. E. Activity of retapamulin (SB-275033), a novel pleuromutilin, against selected resistant gram-positive cocci. Antimicrob. Agents Chemother. 50, 2583–2586 (2006).
8. Berman, A. Y., Motechin, R. A., Wiesenfeld, M. Y. & Holz, M. K. The therapeutic potential of resveratrol: a review of clinical trials. NPJ Precis. Oncol. 1, 35 (2017).
9. Bonnefont-Rousselot, D. Resveratrol and cardiovascular diseases. Nutrients 8, 250 (2016).
10. Xia, N., Daiber, A., Förstermann, U. & Li, H. Antioxidant effects of resveratrol in the cardiovascular system. Br. J. Pharmacol. 174, 1633–1646 (2017).
11. Kunnumakkara, A. B. et al. Curcumin, the golden nutraceutical: multtargeting for multiple chronic diseases. Br. J. Pharmac. 174, 1325–1348 (2017).
12. Chen, Y. et al. Platinum complexes of curcumin delivered by dual-responsive polymer nanoparticles improve chemotherapeutic efficacy based on the enhanced anti-metastasis activity and reduce side effects. Acta Pharmac. Sin. B 9 (2019), https://doi.org/10.1016/j.apsb.2019.10.011.
13. Zhang, T. et al. Inhalation treatment of primary lung cancer using liposomal curcumin dry powder inhalers. Acta Pharmac. Sin. B 8, 440–448 (2018).
14. Ding, Y. et al. Discovery and development of natural product oridonin-inspired anticancer agents. Eur. J. Med. Chem. 122, 102–117 (2016).
15. Zhu, L., Li, M., Liu, X., Du, L. & Jin, Y. Inhalable oridonin-loaded poly (lactic-coglycolic) acid large porous microparticles for in situ treatment of primary non-small cell lung cancer. Acta Pharmac. Sin. B 8, 80–90 (2017).
16. Chai, X.-Y. et al. Six insecticidal isoryanodane diterpenoids from the bark and twigs of Itoa orientalis. Tetrahedron 64, 5743–5747 (2008).
17. Liu, L., Li, A.-L., Zhao, M.-B. & Tu, P.-F. Tetratones and flavonoids from Pyrola ciliatella. Chem. Biodivers. 4, 2932–2937 (2007).
18. Jin, W. et al. Simultaneous analysis of multiple bioactive constituents in Rheum tanguticum Maxim. ex Balf. by high-performance liquid chromatography coupled to tandem mass spectrometry. Rapid Commun. Mass Spectrom. 21, 2351–2360 (2007).
19. Chen, X. et al. Target identification with quantitative activity based protein profiling (ABPP). Proteomics 17, 1600212 (2017).
20. Comess, K. M. et al. Emerging approaches for the identification of protein targets of small molecules—a practitioners’ perspective. J. Med. Chem. 61, 8504–8535 (2018).
21. Schenone, M., Dankñi, V., Wagner, B. K. & Clemons, P. A. Target identification and mechanism of action in chemical biology and drug discovery. Nat. Chem. Biol. 9, 232 (2013).
22. Zhong, C. et al. Identification of bioactive anti-angiogenic components targeting tumor endothelial cells in human injection using multidimensional pharmacokinetics. Acta Pharmac. Sin. B 9 (2019), https://doi.org/10.1016/j.apsb.2019.12.011.
23. Rix, U. & Superti-Furga, G. Target profiling of small molecules by chemical proteomics. Nat. Chem. Biol. 5, 616–624 (2009).
24. Majumder, A., Biswal, M. R. & Prakash, M. K. One drug multiple targets: An approach to predict drug efficacies on bacterial strains differing in membrane composition. ACS Omega 4, 4977–4983 (2019).
25. Klessig, D. F., Tian, M. & Choi, H. W. Multiple targets of salicylic acid and its derivatives in plants and animals. Front. Immunol. 7, 206 (2016).
26. Zon, L. I. & Peterson, R. T. In vivo drug discovery in the zebrafish. Nat. Rev. Drug Discov. 4, 35 (2005).
27. Lamb, J. et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science 313, 1929–1935 (2006).
28. Hillemeier, M. E. et al. The chemical genomic portrait of yeast: uncovering a phenotype for all genes. Science 320, 362–365 (2008).
Caligiuri, M. et al. MasP2; triple-hybrid trap for quantitative proteome fingerprinting of small molecule-protein interactions in mammalian cells. J. Cell. Biol. 13, 711–722 (2006).

Cohen, A. A. et al. Dynamic proteomes of individual cancer cells in response to a drug. Science 322, 1511–1516 (2008).

Wang, S. et al. Advanced activity-based protein profiling application strategies for drug development. Front. Pharmacol. 9, 1–9 (2018).

Lum, K. M. et al. Mapping protein targets of bioactive small molecules using lipid-based chemical proteomics. ACS Chem. Biol. 12, 2671–2681 (2017).

Bantscheff, M., Scholten, A. & Heck, A. J. R. Revealing promiscuous drug-target interactions by chemical proteomics. Drug Discov. Today 14, 1021–1029 (2009).

Yang, Y., Fonovic, M. & Verhelst, S. H. L. Cleavable linkers in chemical proteomics applications. Methods Mol. Biol. 1191, 185–203 (2017).

Izhak, D. N. et al. SILAC-based quantitative mass spectrometry-based proteomics quantifies endoplasmic reticulum stress in whole HeLa cells. Dis. Model. Mech. 12, dmm-040741 (2019).

Jing, Y., Wan, J., Angelidaki, I., Zhang, S. & Luo, G. iTRAQ quantitative proteomic analysis reveals the pathways for methanation of propionate facilitated by magnetite. Water Res. 108, 212–221 (2017).

Moulder, R., Bhosale, S. D., Goodlett, D. R. & Lahesmaa, R. Analysis of the plasma proteome using iTRAQ and TMT-based Isobaric Labeling. Mass Spectrom. Rev. 37, 583–606 (2018).

West, G. M. et al. Mass spectrometry-based thermal shift assay for protein-ligand binding analysis. Anal. Chem. 82, 5573–5581 (2010).

Akter, S. et al. Chemical proteomics reveals new targets of cysteine sulfenic acid reductase. Nat. Chem. 14, 995–1004 (2018).

van Rooden, E. J. et al. Mapping in vivo target interaction profiles of clinical drugs by high-throughput mass spectrometry. J. Proteome Res. 18, 751–760 (2019).

Itzhak, D. N. et al. SILAC-based quantitative mass spectrometry-based proteomics quantifies endoplasmic reticulum stress in whole HeLa cells. Dis. Model. Mech. 12, dmm-040741 (2019).

Chen, X. et al. Comparative profiling of analog targets: A case study on resveratrol for mouse melanoma metastasis suppression. Theranostics 8, 3504–3516 (2018).

Bar-Peled, L. et al. Chemical proteomics identifies druggable vulnerabilities in a genetically defined cancer. Cell 171, 696–709 (2017).

Hu, L., Fawcett, J. P. & Gu, J. Protein target discovery of drug and its reactive intermediate metabolite by using proteomic strategy. Acta Pharmacol. Sin. 2, 126–136 (2012).

Speers, A. E. & Cravatt, B. F. Activity-based protein profiling (ABPP) and click chemistry (CC)—ABPP by MudPIT mass spectrometry. Curr. Protoc. Chem. Biol. 1, 29–41 (2009).

Cuatrecasas, P., Wilchek, M. & Anfinsen, C. B. Selective enzyme purification by affinity chromatography. Proc. Natl Acad. Sci. USA 61, 636 (1968).

Harding, M. W., Galat, A., Uehling, D. E. & Schreiber, S. L. A receptor for the immuno-suppressant FK506 is a cis-tyr-tyryl-prolyl isomerase. Nature 341, 758 (1989).

Crews, C. M., Collins, J. L., Lane, W. S., Snapper, M. L. & Schreiber, S. L. GTP-dependent binding of the antiproliferative agent didemnin to elongation factor 1 alpha. J. Biol. Chem. 269, 15411–15414 (1994).

Knockaert, M. et al. Intracellular targets of cyclin-dependent kinase inhibitors: identification by affinity chromatography using immobilised inhibitors. Chem. Biol. 7, 411–422 (2000).

Sieber, S. A. & Cravatt, B. F. Analytical platforms for activity-based protein profiling—exploiting the versatility of chemistry for functional proteomics. Chem. Commun. 22, 2311–2319 (2006).

Yang, P. & Liu, K. Activity-based protein profiling: recent advances in probe development and applications. ChemBioChem 16, 712–724 (2015).

Taunton, J., Hassig, C. A. & Schreiber, S. L. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 272, 408–411 (1996).

Li, J. et al. Artemisinins target GABA(A) receptor signaling and impair a cell identity. Cell 168, 86–100 (2017).

Capolupo, A. et al. Determination of gymnemic acid I as a protein biosynthesis inhibitor using chemical proteomics. J. Nat. Prod. 80, 909–915 (2017).

Cassiano, C. et al. In cell scalaridial interactor profiling using a bio-orthogonal clickable probe. Chem. Commun. 50, 6043–6045 (2014).

Margarucci, L. et al. Chemical proteomics-driven discovery of oleocanthal as an Hsp90 inhibitor. Chem. Commun. 49, 5848–5846 (2013).

Zhao, Q. et al. Natural products triptolide, celastrol, and withaferin A inhibit the shigeneractivity of peroxiredoxin I. Chem. Sci. 6, 4124–4130 (2015).

Dong, T. et al. Ainsladmer A selectively inhibits Ikkalpha/beta by covalently binding a conserved cysteine. Nat. Commun. 6, 6522 (2015).

Sin, N. et al. The anti-angiogenic agent fumagillin covalently binds and inhibits the methane monoxygenase, MetA-F2. Proc. Natl Acad. Sci. USA 94, 6099–6103 (1997).
Target identification of natural medicine with chemical proteomics... Chen et al.

90. Lehmann, J., Richers, J., Pöthig, A. & Sieber, S. A. Synthesis of ramariolide natural products and discovery of their targets in mycobacteria. *Chem. Commun.*, **53**, 8671–8674 (2017).

91. Leicht, J., Richards, J., Pöthig, A. & Sieber, S. A. Synthesis of ramariolide natural products and discovery of their targets in mycobacteria. *Chem. Commun.*, **53**, 107–110 (2017).

92. Li, Q., Cui, L., Wang, C. & Lei, X. Isozyme and protein identification of bice biologically interacting proteins. *ACS Cent. Sci.*, **3**, 501–509 (2017).

93. Shi, H., Cheng, X., Sze, S. K. & Yao, S. P. Proteome profiling reveals potential cellular targets of staurosporine using a clickable cell-permeable probe. *Cell Chem. Commun.*, **47**, 11306–11308 (2011).

94. Lemos, S. M. et al. Mixed isoto-type photoaffinity reagents for identification of small-molecule targets by mass spectrometry. *Angew. Chem. Int. Ed.*, **45**, 4239–4333 (2006).

95. Lomenick, B. et al. Target identification using drug affinity responsive target stability (DARTS). *Proc. Natl. Acad. Sci. USA*, **106**, 21984–21989 (2009).

96. Lomenick, B., Jung, G., Wohlschlegel, J. A. & Huang, J. Target identification using drug affinity responsive target stability (DARTS). *Curr. Protoc. Chem. Biol.*, **3**, 163–180 (2011).

97. Strickland, E. C. et al. Thermodynamic analysis of protein-ligand binding interactions in complex biological mixtures using the stability of proteins from rates of oxidation. *Nat. Protoc.*, **8**, 148 (2013).

98. DeArmond, P. D., Xu, Y., Strickland, E. C., Daniels, K. G. & Fitzgerald, M. C. Thermodynamic analysis of protein–ligand interactions in complex biological mixtures using a shotgun proteomics approach. *J. Proteome Res.*, **10**, 4948–4958 (2011).

99. Molina, D. M. et al. Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science*, **341**, 84–87 (2013).

100. Jafari, R. et al. The cellular thermal shift assay for evaluating drug target interactions in cells. *Nat. Protoc.*, **9**, 2100 (2014).

101. Savitski, M. M. et al. Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science*, **346**, 1255784 (2014).

102. Piazza, I. et al. A map of protein-metabolite interactions reveals principles of chemical communication. *Cell*, **172**, 358–372.e23 (2018).

103. Kost, G. et al. A novel anti-cancer agent, 1-(3,5-dimethoxyphenyl)-4-[(6-iodo-2-naphthalenecarboxamido)ethyl]pyrazine (RX-5902), interferes with β-catenin function through Y593 phospho-p68 RNA helicase. *J. Cell Biochem.*, **116**, 1595–1601 (2015).

104. Ryan, D. J., Spraggins, J. M. & Caprioli, R. M. Protein identification strategies in MALDI imaging mass spectrometry: a brief review. *Curr. Opin. Chem. Biol.*, **48**, 64–72 (2019).

105. Brunet, M. A. & Roucou, X. Mass spectrometry-based proteomics analyses using the OpenProt database to unveil novel proteins translated from non-canonical open reading frames. *J. Proteome Res.*, **16**, e59589 (2019).

106. Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai, B. S. & Mishe, S. M. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophor. Int. J.*, **20**, 601–605 (1999).

107. Raikos, V., Hansen, R., Campbell, L. & Euston, S. R. Separation and identification of hen egg protein isoforms using SDS–PAGE and 2D gel electrophoresis with MALDI-TOF mass spectrometry. *Food Chem.*, **99**, 702–710 (2006).

108. Ong, S.-E., Foster, L. J. & Mann, M. Mass spectrometric-based approaches in quantitative proteomics. *Methods*, **29**, 124–130 (2003).

109. Mann, M. Functional and quantitative proteomics using SILAC. *Nat. Rev. Mol. Cell Biol.*, **7**, 952–958 (2006).

110. Zhu, W., Smith, J. W. & Huang, C.-M. Mass spectrometry-based label-free quantitative proteomics. *Biomed. Res. Int.*, **2010**, 840518 (2009).

111. Tempel, M. F. et al. Protein microarray technology. *Drug Discov. Today*, **7**, 815–822 (2002).

112. Wilson, D. S. & Nock, S. Recent developments in protein microarray technology. *Angew. Chem. Int. Ed.*, **42**, 494–500 (2003).

113. Wang, D.-Y. et al. Target identification of kinase inhibitor alisertib (MLN8237) by using DNA-programmed affinity labeling. *Chem. Eur. J.*, **23**, 10906–10914 (2017).

114. Rodríguez-Furlan, C., Zhang, C., Raikhel, N. & Hicks, G. R. Drug affinity responsive target stability (DARTS) to resolve protein–small molecule interaction in arabidopsis. *Curr. Protoc. Plant Biol.*, **2**, 370–378 (2017).

115. Ehe, B. K. et al. Identification of a DYSR1A-mediated phosphorylation site within the nuclear localization sequence of the hedgehog transcription factor GLI1. *Biochem. Biophys. Res. Commun.*, **491**, 767–772 (2017).

116. Kleiner, R. E., Hang, L. E., Molloy, K. R., Chait, B. T. & Kapoor, T. M. A chemical proteomics approach to reveal direct protein–protein interactions in living cells. *Cell Chem. Biol.*, **25**, 110–120 (2018).

117. Yang, F., Gao, J., Che, J., Jia, G. & Wang, C. A dimethyl-labeling-based strategy for site-specifically quantitative chemical proteomics. *Anal. Chem.*, **90**, 9576–9582 (2018).