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J. Chang[a, †], Y. Kim[a, †] and H. J. Kwon[a, b, *]

Abstract: Identification of target proteins of natural products is pivotal to understanding the mechanisms of action to develop natural products for use as molecular probes and potential therapeutic drugs. Affinity chromatography of immobilized natural products has been conventionally used to identify target proteins, and has yielded good results. However, this method has limitations, in that labeling or tagging for immobilization and affinity purification often result in reduced or altered activity of the natural product. New strategies have recently been developed and applied to identify the target proteins of natural products and synthetic small molecules without chemical modification of the natural product. These direct and indirect methods for target identification of label-free natural products include drug affinity responsive target stability (DARTS), stability of proteins from rates of oxidation (SPROX), cellular thermal shift assay (CETSA), thermal proteome profiling (TPP), and bioinformatics-based analysis of connectivity. This review focuses on and reports case studies of the latest advances in target protein identification methods for label-free natural products. The integration of newly developed technologies will provide new insights and highlight the value of natural products for use as biological probes and new drug candidates.

1 Introduction

Natural products have exhibited potent and unique biological activities based on diverse structural complexity. Complex structures often imply multi-ligandable properties in these natural products. Therefore, identifying the multiple target proteins is mandatory for understanding the mechanism of action of natural products for drug development, as well as addressing potential adverse effects related to off-target actions. A fully described mechanism of action is crucial to the application of bioactive natural products to drug discovery. Affinity chromatography using appropriate tags has conventionally been used to identify binding proteins, and to explore mechanisms of action (Fig. 1A).[1-3] Affinity beads conjugated to suitable functional groups have been utilized for determining the binding proteins.[4,5] Various attempts have been made to induce immobilization of natural products through chemical modification; however, the lack of suitable types of or sites on functional groups available for modification and shadowing using labeling procedures[6] are major obstacles in determining the mechanisms of natural products.[7] Accordingly, alternative strategies for target protein identification without resorting to chemical modification are in high demand.[8] These studies have demonstrated the specific interaction of ligands, which can be directly detected through thermodynamic or proteolytic measurements when changes are induced. A number of recent studies[9-19] have identified the target proteins of natural products without using chemical modifications. The specific binding of a label-free natural product and its binding proteins can be detected by evaluating responses to thermal or proteolytic treatment.[12,20,21] Moreover, this new approach of target protein identification enables the identification of multiple targets for a natural product through proteome-wide analysis, which expedites the determination of mechanisms of action and facilitates the development of these compounds as clinical agents.[22] In this review, we will discuss several new strategies for cellular target identification and validation of natural products, without chemical modifications.

[a] *Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science & Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea.*

[b] *Department of Internal Medicine, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea.*

† These authors contributed equally to this work.

*Corresponding author: Prof. Ho Jeong Kwon

Phone: 82-2-2123-5883; Fax: 82-2-362-7265; E-mail: kwonhj@yonsei.ac.kr

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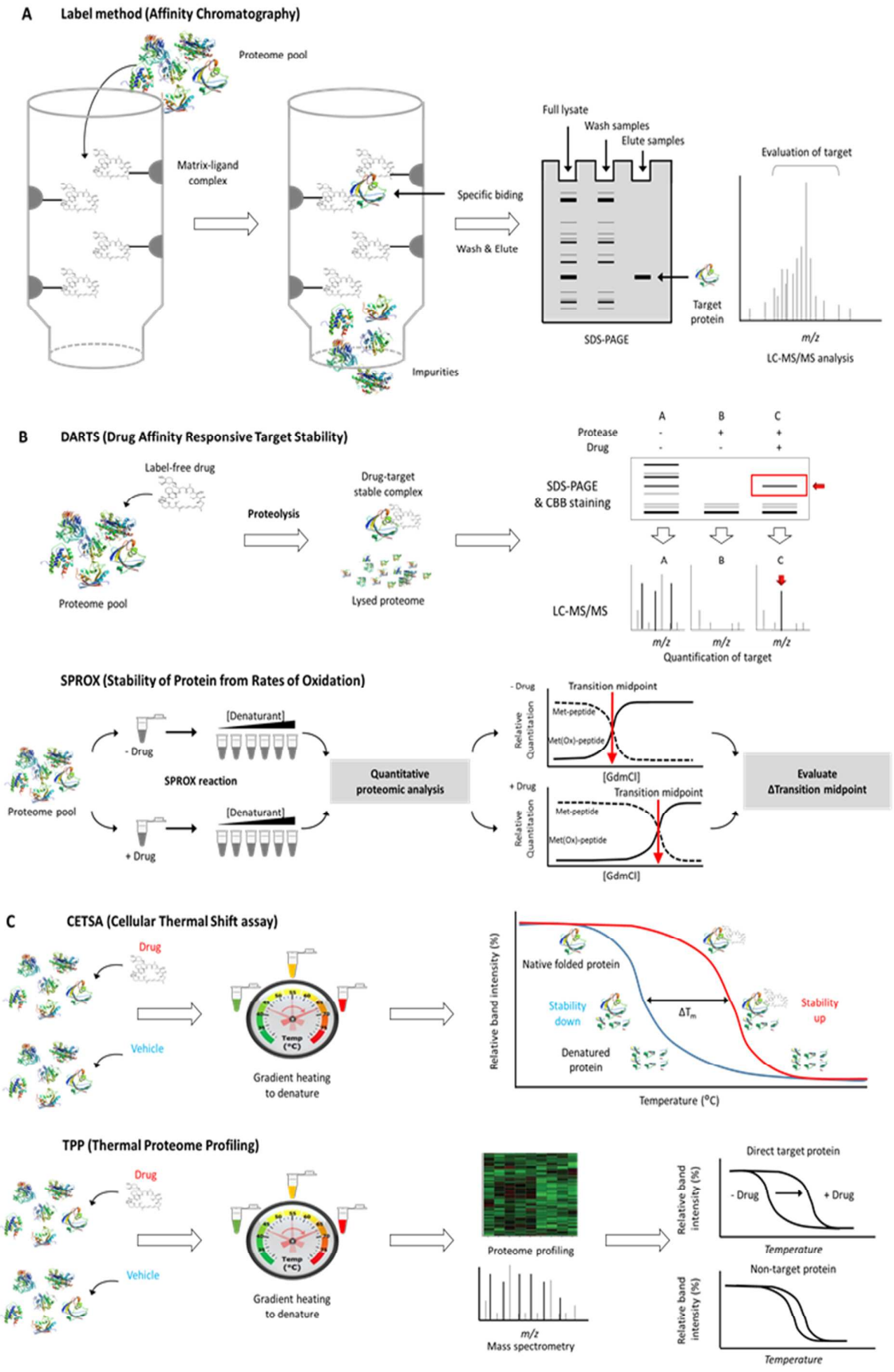
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Proteome profiling
m/z
Mass spectrometry
Direct target protein
Non-target protein
Temperature

Fig. 1 Labeled and label-free methods for target identification of natural products. (A) Affinity chromatography is a conventional method for separating target proteins from proteome mixtures, and is based on highly specific binding of a labeled chemical to target proteins. Owing to the high affinity between a matrix-ligand chemical complex and its target proteins, eluted mixtures containing the target proteins could be assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and LC-MS/MS analysis. (B) Label-free methods for detecting stabilization of target proteins. In proteome pool treated label-free compounds, stabilization and conformation of the compound-target complex were altered by protease-induced digestion (DARTS) or methionine oxidation (SPROX). Subsequently, interaction of target protein and drug can be visualized by SDS-PAGE with Coomassie brilliant blue (CBB) staining and detected by LC-MS/MS analysis. (C) Label-free methods for detecting ligand-induced thermal stabilization of target proteins. For detecting thermodynamic stability using CETSA, drug or vehicle-treated cell lysates/intact cells were heated to different temperatures and the target proteins were detected by western blotting. With CETSA, the advanced method of TPP analyzes thermodynamic stabilization using multiplexed quantitative MS to estimate ligand-target engagement in a cellular proteomic scale.

2. Advances in target identification and validation of label-free natural products

2.1. Direct methods for target identification of label-free natural products

2.1.1. Drug affinity responsive target stability (DARTS). DARTS is a recently developed, label-free method for direct target protein identification. It is based on the concept that ligand-bound proteins show altered stability in case of proteolysis compared to that of ligand-unbound proteins. The concept is that the structural stability of a target protein is altered by binding of its corresponding ligand, and the change in stability can be detected by the altered proteolytic pattern against different protease exposures by examination of the protected band through liquid chromatography-mass spectrometry (LC-MS/MS).[22] Protein targets of various natural products including FK506, rapamycin, didemnin B, resveratrol, and ecumicin were validated (Fig. 1B, Table 1) using the DARTS method.[12-14] DARTS does not require any chemical modification of the natural product for target identification. This allows natural products and natural product extracts to be used to determine their direct binding protein targets. Additionally, DARTS can be utilized for analyzing true interactions with low affinity, because washing is not included as an experimental step.[14,23] However, DARTS has limitations in identifying low-abundance proteins, as well as in validation of proteolysis of a cell lysate. Nevertheless, DARTS is a robust method for the determination of target proteins of natural products.[20,21]

2.1.2. Stability of proteins from rates of oxidation (SPROX). Similar to DARTS, SPROX is another method of target identification based on detection of ligand-induced stabilization of target proteins (Fig. 1B, Table 1).[19,24] Instead of detecting proteolytic patterns, SPROX measures the levels of methionine oxidation of target proteins, which describes ligand-induced thermodynamic changes. When a complex protein pool is incubated with a compound of interest or a solvent control, an oxidizing agent (hydrogen peroxide) is treated in the presence of a chemical denaturant [guanidinium hydrochloride (GdmCl)] to oxidize methionine. After a quenching oxidation reaction and generation of tryptic peptides, a tandem LC-MS/MS technique, such as multidimensional protein identification technology (MudPIT), is applied to quantify the rate of selective methionine oxidation. Plots of the levels of non-oxidized and oxidized methionine-containing peptides against the concentration of GdmCl show that the ligand-bound proteins have a larger transition midpoint shift than the control samples. The immunosuppressant cyclosporine A was subjected to SPROX with yeast lysate in a proof-of-concept study, and SPROX identified the previously known target proteins, cyclophilin A and UDP-glucose-4-epimerase, as well as eight new protein targets.[18,19] In addition, the known target of resveratrol, a cytosolic aldehyde dehydrogenase, was verified using SPROX, and six newly identified targets were discovered.[9] SPROX has limitations in that it requires methionine residues modulated by the ligand for determining thermodynamic changes, and only sufficiently concentrated proteins can be evaluated for detection of a transition midpoint shift. Recently, an advanced methodology termed stable isotope labeling with amino acids in cell culture (SILAC)-based SPROX was introduced to expand target protein coverage at a proteomic scale.[22,25]

2.1.3. Cellular thermal shift assay (CETSA). CETSA was recently introduced to evaluate binding of a ligand to its cognate target in cells and tissue samples. This method is based on the ligand-induced thermodynamic stabilization of target proteins (Fig. 1C, Table 1).[15,26] The compound or vehicle-treated cell lysates and intact cells were heated to different temperatures and the target protein in the soluble fractions was separated from precipitated destabilized protein and detected by western blotting. The ligand-target interaction can be plotted against temperature to display obvious shifts in the melting curves. In addition, an isothermal dose-response fingerprint ($ITDRF_{CETSA}$) was generated for estimating relative differences in ligand concentration, in which cell lysates were treated with different ligand concentrations at fixed temperatures and heating times to assess drug concentration effects and establish a similar extent of target engagement.[11,15] The known biological targets of various anti-cancer agents, including methotrexate, raltitrexed, and TNP-470, were verified using CETSA in cell lysates, intact cells, or tissue samples.[15] In a recent report, quinone reductase 2 (NQO2) was also validated through CETSA as a reactive oxygen species-generating non-specific target of the analgesic and antipyretic compound acetaminophen, which is one of the most used drugs worldwide.[16] The physical interaction between ligand and target protein and the target specificities in intact cells can be monitored using this method; however, the method is not applicable to highly inhomogeneous proteins or for proteins in which unfolding of the ligand-binding domain does not induce aggregation and denaturation, such as is exhibited by DNA binding and chaperone proteins.[16,26-28]

2.1.4. Thermal proteome profiling (TPP). TPP is an advanced method of CETSA, which is able to identify proteins that exhibit the ligand-induced thermal stability at higher temperatures, combined with multiplexed quantitative MS to estimate ligand-target

engagement at a cellular proteomic scale (Fig. 1C, Table 1). To facilitate proteome-wide profiling of protein thermal stability, the neutron-encoded isobaric mass tagging reagent, TMT10, is used for high-resolution MS, resulting in full melting curves for a large proportion of expressed soluble proteins. The target proteins involved in modes of action as well as the off-target effects of compounds in cells can be examined through the melting curves determined by TPP. As a proof-of-concept study, TPP was applied to confirm the interactions between kinase inhibitors, including staurosporine, a broad-specificity inhibitor originally isolated from a bacterium, and their known spectrum of targets.[17] Treatment of cell extracts with staurosporine induced reproducible thermal shifts of numerous kinases, along with other regulatory subunit proteins of kinase complexes, allowing reliable target identification. In addition, TPP has been utilized to identify off-targets of several kinase inhibitors including the melanoma drug vemurafenib, which is known to target BRAF and causes phototoxicity as a side effect.[17] A heme biosynthesis enzyme, ferrochelatase (FECH), was revealed as an off-target of several kinase inhibitors, and was found to be photosensitive.[10,17]

2.1.5. Pros and cons of direct methods for target identification of label-free natural products. Based on proteomic target identification methods, DARTS and SPROX are similar, in that these methods detect the ligand-induced changes in the folding and thermodynamic stability of targets.[14] DARTS can be used to elucidate the interaction of ligand-target proteins using western blotting or Coomassie blue staining of SDS gel. When the sample had a low abundance of target protein, the DARTS method could not easily visualize the target protein. Additionally, when the target protein is related to membrane proteins, resistance to proteolytic digestion could misrepresent the stability, because the ligand might nonspecifically bind with the target protein or minimally affect the stability of membrane proteins.[29] SPROX also has several additional limitations. For instance, this method requires relatively large concentrations of compound (in the μM to mM range), although flexibility is improved on down-stream quantitative proteomics.[30] Additionally, only methionine containing peptides are detectable with SPROX analysis, and peptides without methionine residues might not be affected by the differential oxidation rates used for determining thermodynamic changes.[14] CETSA and $ITDRF_{CETSA}$ have a major advantage, in that they are based on western blotting detection with excellent availability of high-affinity antibodies to the exposed epitopes in denatured proteins. Additionally, multiplexing with several antibodies against other relevant proteins can increase output and provide information on selectivity and off-target effects of the ligands. However, this advantage might be a drawback, due to the requirement of monoclonal antibodies with highly specific activity on target proteins, as well as clear differences in molecular sizes for accurate quantification. Furthermore, with interference from phenol red and biotin in the cell culture medium or from the compounds of interest themselves, some artifacts regarding color or fluorescence could also be problematic.[11] Above all, CETSA enables verification of the engagement and potency of known targets whereas it has weakness in respect to detecting unknown targets.[10] To overcome the limitations of CETSA, TPP was combined with CETSA and ITDR for multiplexed quantitative mass spectrometry, which allows for the monitoring of changes in protein thermal stability in the proteome. This aspect is useful for understanding the therapeutic mechanism of natural products. However, further developments are required for the target identification of membrane proteins and tissue samples.[17] Because membrane proteins are unstable in detergent-free cell extraction conditions, improvement of experimental conditions with mild detergents for better assessment

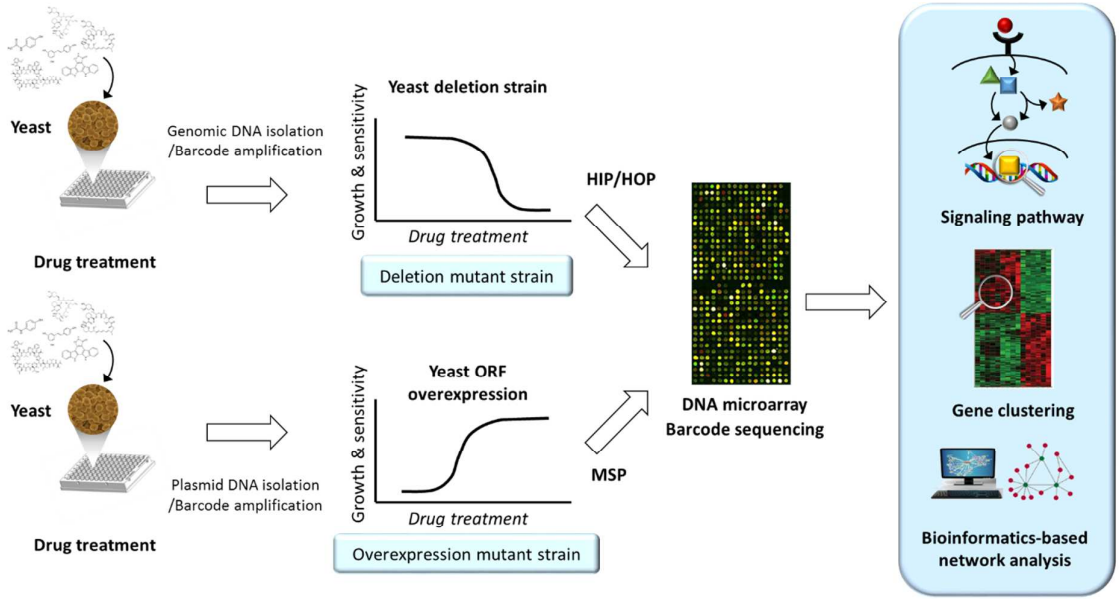

Fig. 2 Indirect methods for target identification of label-free natural products using genetics and genomics based profiling. Yeast pools, including a homozygous or heterozygous deletion strain and an ORFeome overexpressed strain, were cultured and treated with the compounds. Polymerase chain reaction (PCR) was applied for barcode amplification from purified DNA and its quantification was analyzed by the microarray and barcode sequencing method. From sensitivity and resistance data of compounds, target protein and related proteins were identified by analyzing signaling pathway, gene clustering, and bioinformatics-based networks.

of membrane proteins will be the subject of future work.[10,11]

2.2. Indirect methods of target identification of label-free natural products

2.2.1. Genetics and genomics based approach. Yeast is a valuable organism used as eukaryotic model for mammalian diseases and mechanism studies. At least 31% of the proteins encoded by yeast genes are homologous to human genes and approximately 50% of the human genes implicated in heritable diseases have yeast homologs.[31,32] Furthermore, yeast is easy to manipulate because of genetic tractability and stable growth, and has been widely used for target identification of natural products using genetic and genomics profiling information (Fig. 2, Table 1).[33,34] Sequencing of the complete *Saccharomyces cerevisiae* genome has contributed to the construction of complete deletion mutants via homologous recombination.[35-37] Mutant yeast strains (including haploids, homozygous diploids, or heterozygous diploids) allow for genome-wide profiling approaches of natural product-gene interactions to be performed with haploinsufficient profiling (HIP) and homozygous profiling (HOP).[26,38-40] At sub-lethal doses of natural products, hypersensitive deletion mutants have been applied to identify molecular targets and specific chemical-genetic interactions. Furthermore, the HIP method was utilized to establish the genome-wide deletion mutant collection with *Schizosaccharomyces pombe* strains.[41-43]

Using the over-expressed library of the yeast ORFeome in high-copy plasmids[44-46], multi-copy suppression profiling (MSP)[47,48] was developed to explore the mechanisms of natural products and molecular targets to complement HIP and HOP methods.[49] This approach was based on an assumption that over-expression of a natural product target results in increased resistance to the natural product. To easily recognize each open reading frame (ORF) and compare the chemical-genomic profiles, a fluorescent protein was tagged at its locations in the yeast proteome.[44] Specific responses can be analyzed after treatment with various natural products or small molecules, and are detectable with localisome profiling. Additionally, by insertion of a molecular barcode containing unique oligonucleotides in a yeast strain, oligonucleotide microarray systems or barcode sequence platforms can quantify and profile the fluctuating quantities of each DNA barcode, which leads to identification of the genes regulating sensitivity and resistance to natural products.[39] Target identification using yeast is limited, as some pathways and target proteins are not entirely conserved between human and yeast genomes. With additional validation procedures using mammalian and human systems, genetic and genomic profiling will provide genetic and molecular insight into the determination of natural product targets and genetic networks.[50-52]

Recently, small interfering RNA (siRNA) and short hairpin RNA (shRNA) have been utilized as genetics- and genomics-based approaches for target gene knock-down, leading to manipulation of target protein levels for functional validation of natural product interactions.[46,50] Target protein levels decrease in knock-down conditions, because transcription of the target gene is inhibited. Interfering RNA increases the efficiency and sensitivity of natural products by excluding any off-target effect in the cells, based on the phenotypic changes *in vitro*. Validation methods using interfering RNA showed that target over-expression could diminish the effects of natural products on target proteins with an activated phenotype, by increasing the levels of target proteins.[53-57] Although these shRNA and siRNA methods have increasingly been used in target identification of natural products, they risk off-target effects and false positive results induced from the heterogeneous guide sequences.[58]

Recently, a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 genome editing approach from adaptive immune defenses of bacteria and archaea was discovered and applied to overcome off-target effects and to improve effective gene editing.[59-62] The Cas9 nuclease is targeted to specific genomic loci by a 20-nucleotide guide sequence, but can tolerate certain mismatches in the DNA target, and thereby promote undesired off-target mutagenesis.[63,64] This promising strategy enables a wide variety of genome editing applications, which require high specificity. With huge possibilities, these engineering systems of CRISPR-Cas9-mediated gene-deletion methods have been introduced to elucidate the mechanism of action of natural compounds and approved agents for target identification.[65-67] To identify physiological targets of small molecules, DrugTargetSeqR was developed by combining high throughput sequencing, computational mutation discovery, and CRISPR-Cas9-based genome editing to evaluate genetic and epigenetic mechanisms causing drug resistance.[68] Based on these applications, Kasap *et al.* validated some drugs undergoing clinical trials as anti-cancer agents. Ispinesib is an inhibitor of kinesin-5[69-71], which directly and physiologically targets kinesin-5 in human cancer cell lines. The cytotoxic drug YM155 showed some indications related to mutagenic agents and resistance with cell proliferation.[72] Further, Smurnyy *et al.* established a novel method for drug-target validation using next-generation sequencing of drug-resistant clones and CRISPR-Cas9 editing in mammalian cells.[73] This approach demonstrated that disrupting a functional *HPRT1* allele and point mutation of *ERCC3* by gene editing-induced drug resistance.

2.2.2. Bioinformatics-based analysis using Connectivity Map (CMap) application. Bioinformatics is an integrated and innovative field combining computer science and information processing. By enhancing the accuracy of the results to reduce cost and time, this approach allows for exploration of human diseases at a molecular level, and assists in explaining disease phenomena and their genetic aspects using computer techniques.[74-76] Bioinformatics mapping and protein-interaction database analysis could be a novel method for determining target protein interactions of natural products by elucidating novel pathways and cellular complexes. The Connectivity Map (CMap) approach was established by the Broad Institute (http://www.broadinstitute.org). Gene-expression and genome-wide transcriptional data of 1,309 bioactive small molecule-treated human cells and genetic reagents are described in CMap. This search engine queries gene-expression signatures to connect small molecules, genes, and disease responses. With pattern-matching software to obtain signature data, CMap could be utilized to elucidate connections among small molecules sharing a

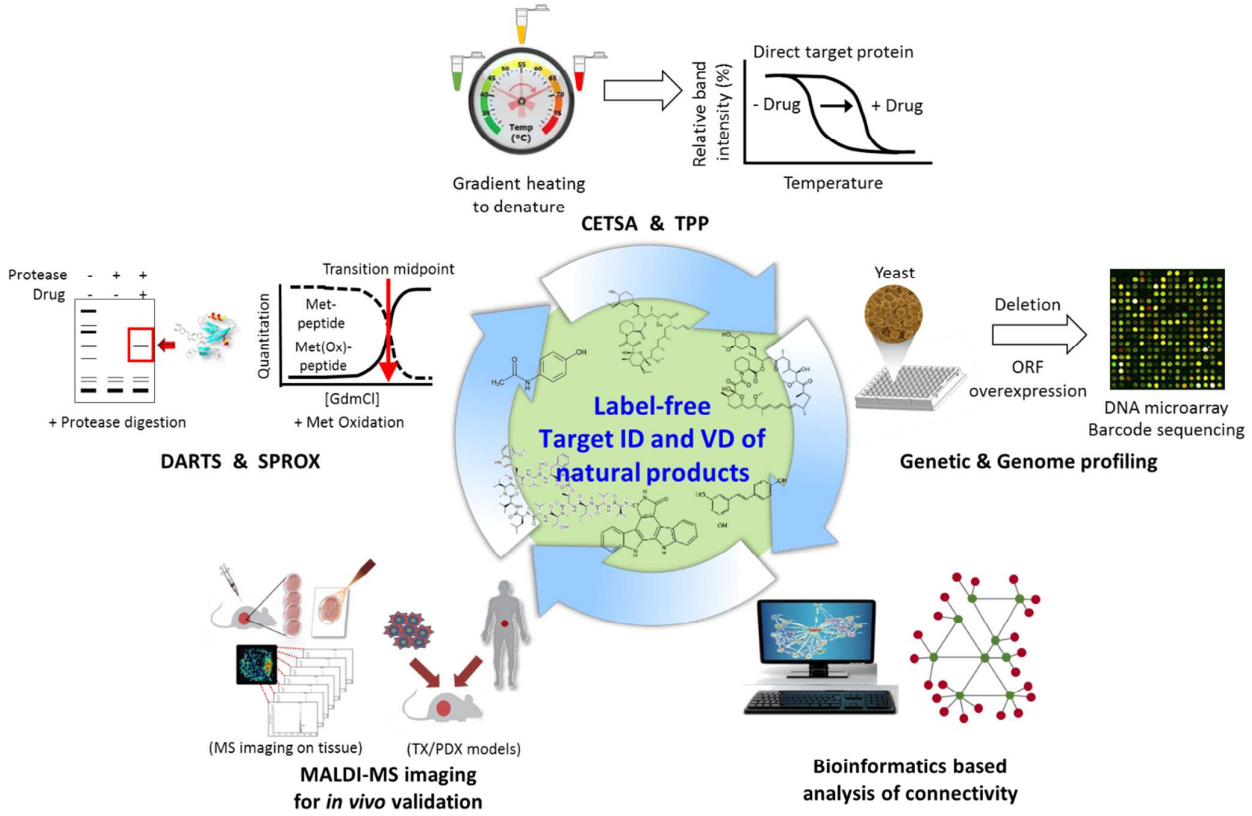

Fig. 3 Perspectives using target identification and validation methods of natural products with label-free approaches. Label-free methods could be a promising strategy for identifying target proteins and mechanism of action of natural products. Furthermore, biologically relevant multiple targets of natural products without chemical modification can be identified by virtue of newly developed methods that accelerate the application of natural products for development of chemical probes and new drugs.

mechanism of action, chemical and physiological processes, and diseases and drugs. Owing to common transitory features, this simple pattern-matching algorithm could be applied to discover functional connections among natural products, genes, and diseases (Table 1).[77-81] With widespread availability of CMap, these logistics are utilized for identifying mechanisms of action, drug repurposing, and lead discovery with natural compounds. In an earlier work, Tiedermann *et al.* suggested that pristimerin inhibited proteasome chymotrypsin-like activity and NF-κB using the CMap tool.[82] Mukherjee *et al.* used a similar method to identify that COX inhibitors and HDAC inhibitors (i.e. resveratrol) regulate T-cell activation-dependent posttranscriptional effects and RNA binding proteins.[83] Hieronymus *et al.* demonstrated that the androgen receptor signaling inhibitors celastrol and gedunin regulated upstream HSP90 and its associated mechanisms *in vitro*.[84]

From an efficient pattern-matching algorithm, a "connectivity score" ranging from +1 to -1 is assigned to describe the interaction between a particular gene signature and all reference sets. The score shows the closeness or connection between the expression profiles. The positive or negative score reflects that a new compound with potential to become a drug agent could be influenced by the expression pattern of the phenotype of interest between a query signature and a reference profile for drug repositioning, finding new potential drug compounds, and systemic study in cellular mechanisms.[81] With the basic methodology described by the CMap data, novel methods were additionally established. For example, probabilistic multi-source connectivity mapping methods was introduced and the drug-treated transcriptional response data derived from CMap was provided as a promising strategy for repositioning of approved and generic drugs for alternate uses in cancer treatment.[85,86] Accordingly, combining these strategies with bioinformatics-based analysis for target identification and mechanism study of natural products without using chemical modifications is a very promising method. CMap provides a valuable low cost, systematic, and high throughput strategy to describe various possibilities between mechanisms of drug actions and intrinsic biological states,[81] although wet experiments should always be performed for validation.[87]

4. Conclusions and future perspectives

Historically, natural products from plants, bacteria, and fungi have inspired the development of valuable therapeutic agents. Natural products are not given the credence in modern drug discovery that history dictates they should receive. This is partly attributed to the

difficulties in understanding modes of action regarding their therapeutic effects. Exploitation of protein-natural product interactions is critical for understanding the mechanisms of action and therapeutic effects of natural products. Identification of the target proteins of natural products starts by tagging the natural products of interest to elucidate their binding partners among proteomes. Since tagging the natural product is time-consuming and laborious, strategies for identifying target proteins without labeling or chemical modification have been in high demand in modern drug development. Even after the identification of the direct target proteins of a natural product using the novel approaches described above, subsequent functional analysis should be implemented to validate the biological relevance of the identified target, as well as to explore new roles of the target protein in biological systems of interest.[2,51] The known functions of several target proteins have been validated and correlated with the natural product-induced phenotypes. Since a variety of information is available on functionality, *in silico* (computer-based) protein-natural product interactions[88], and natural product side effects[89], proper experiments for validation and differentiation of non-specific binding are essential for unbiased conclusions regarding target identification. Furthermore, accurate identification of target proteins must be considered with protein-protein binding in protein interaction database networks.[90] In addition, the identified target proteins should be validated *in vitro* and *in vivo* for future perspectives and drug development. Surface plasmon resonance (SPR)[56] and isothermal calorimetry (ITC)[55] have been widely used as *in vitro* methods for the assessment of the biophysical interaction of natural products with target proteins. For the analysis of interactive status using SPR, natural products are immobilized at the surface of the SPR chip.[91,92] If natural products of interest are difficult to chemically modify or immobilize, ITC could be an alternative method of monitoring the direct interaction of natural products and target proteins without using chemical modification. With ITC, label-free natural products can be used to determine the dissociation constant (K_D value) for natural products and target proteins.[62,63]

Furthermore, Analysis of tissues by direct measurement of a natural product and its metabolite using matrix-assisted laser desorption ionization (MALDI) is an emerging technology that has been developed to validate the interaction of a natural product with its target protein *in vivo*. MALDI-MSI is an analytic mass spectrometry technique, which provides a global register of the identities of ion peaks identified at each sample location.[93-97] MALDI-MSI can be applied to detect a full mass scan of all ion masses, or selected ion monitoring of a single specific ion mass similar to other MS analysis methods. Furthermore, MALDI-MSI can be employed to exhibit the spatial distributions of all the detected compounds as one integrated image by automating the procedure at the X, Y position of the two-dimensional space of sample tissues.[98-100] Moreover, this validation method can provide an accurate identification of natural products or drugs administered to patients, as well as endogenous proteins, peptides, and lipids, without any labeling or chemical modifications.[99,101] MALDI-MSI was used for *in vivo* validation of the interaction between a compound and target protein using sunitinib, a receptor tyrosine kinase inhibitor (RTKI), using known target receptor tyrosine kinases (RTKs).[102-104] This study provided direct evidence that MALDI-MSI could be a powerful means for validating interactions between a compound and its target protein *in vivo*. Furthermore, this could be a promising method for providing information on label-free compounds with regard to absorption, distribution, metabolism, and excretion (ADME), and on metabolites and target proteins after *in vivo* administration to patients.[94,95,105,106].

Recent advances in target identification and validation methods for natural products with label-free approaches are introduced herein. Notably, these new approaches utilize a common property of ligand-induced stabilization of the target protein and integration of the latest omics technologies with bioinformatics. It is also noteworthy that both the on- and off-target effects of natural products can be detected on an unbiased proteomic scale by monitoring ligand-induced stabilization of target proteins. Furthermore, prediction of the side effects of newly identified natural products can be accomplished by detecting non-specific targets. These label-free methods of target identification have a marked advantage in that no chemical modification is required for determining direct binding proteins; the methods are applicable to any natural compound independent of mode of action. Using these strategies, acquiring a valuable set of data on direct binding of natural products with their innate structures would become the primary strategy for determining molecular mechanisms. Clinically relevant non-specific targets of natural products can be identified without using chemical modification. Newly advanced solutions based on classical physicochemical properties, including ligand-induced thermodynamics together with multi-omics-based informatics approaches may improve the drug discovery process. Genetics- and genomics-based profiling and bioinformatics-based CMap are being explored as additional options for target identification of natural products with no chemical modification. Depending on chemical or biological properties of natural products, a suitable target identification method should be designed and studied for better understanding of mode of actions of natural products. This will accelerate the application of natural products in the development of chemical probes and new drugs (Fig. 3).

5. Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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Table 1. Examples of affinity based label-free techniques for target identification and validation of natural products and compounds

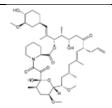
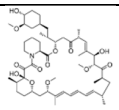
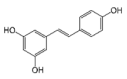
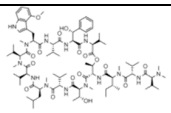
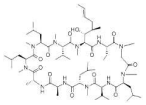
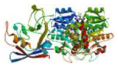
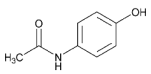
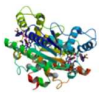
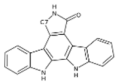
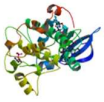
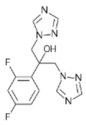
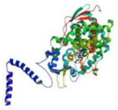
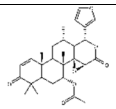
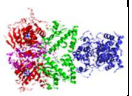
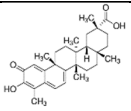
Label-free techniques		Compounds		Target protein		References
Direct methods	DARTS	FK506		FKBP12		12-14
		Rapamycin		FKBP12 / mTOR		12-14
		Resveratrol		Tif1 (yeast eIF4A)		12-14
		Ecumicin		ClpC1		21
	SPROX	Cyclosporine A		Cyclophilin A/ UDP-glucose-4-epimerase		19,24
	CETSA	Acetaminophen		NQO2 (off-target)		16
	TPP	Staurosporine		PKC		17
	Genomic profiling	Fluconazole		Erg11		38,107,108
Indirect methods	CMap	Gedunin		HSP90		77,78
		Celastrol				

Table 2. Pros and cons among the direct methods for target identification of label-free natural products

Direct methods	Pros	Cons	References
DARTS	•No chemical modification on natural products •Cell lysates can be applicable for target ID •Can be utilized for analyzing true interactions having low affinity	•Difficult to identify low abundance protein targets in cell lysates •Should be validated with steps for preparation and proteolysis of cell lysates •Missing stability and proteolytic resistance related to nonspecific binding	12-14,29
SPROX	•Irreversible reaction of proteins oxidation •Can be detect drug-induced thermodynamic changes in a dose-dependent manner •Can provide flexibility on down-stream quantitative proteomics	•Require large concentrations of drugs •Can detect only methionine containing peptides	14,18,30
CETSA	•Can utilize intact cells without any steps or treatments •Based on western blotting and high selectivity of antibodies to target proteins	•Difficult to detect some proteins containing unfolded biding sites •Off-target effect and non-specificity of antibodies •Problems about color and fluorescent of experimental agents	10,11,15,26
TPP	•Can be analyzed to estimate ligand-target engagement at a cellular proteomic scale by quantitative MS	•Should be established database of proteome pool •High cost and labor •Difficult to membrane proteins because its stability and solubility	10,17

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Figure legends

Fig. 1. Labeled and label-free methods for target identification of natural products. (A) Affinity chromatography is a conventional method for separating target proteins from proteome mixtures, and is based on highly specific binding of a labeled chemical to target proteins. Owing to the high affinity between a matrix-ligand chemical complex and its target proteins, eluted mixtures containing the target proteins could be assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and LC-MS/MS analysis. (B) Label-free methods for detecting stabilization of target proteins. In proteome pool treated label-free compounds, stabilization and conformation of the compound-target complex were altered by protease-induced digestion (DARTS) or methionine oxidation (SPROX). Subsequently, interaction of target protein and drug can be visualized by SDS-PAGE with Coomassie brilliant blue (CBB) staining and detected by LC-MS/MS analysis. (C) Label-free methods for detecting ligand-induced thermal stabilization of target proteins. For detecting thermodynamic stability using CETSA, drug or vehicle-treated cell lysates/intact cells were heated to different temperatures and the target proteins were detected by western blotting. With CETSA, the advanced method of TPP analyzes thermodynamic stabilization using multiplexed quantitative MS to estimate ligand-target engagement in a cellular proteomic scale.

1 **Fig. 2. Indirect methods for target identification of label-free natural products using genetics and genomics based profiling.** Yeast pools, including a homozygous or heterozygous deletion strain and an ORFeome overexpressed strain, were cultured and treated with the compounds. Polymerase chain reaction (PCR) was applied for barcode amplification from purified DNA and its quantification was analyzed by the microarray and barcode sequencing method. From sensitivity and resistance data of compounds, target protein and related proteins were identified by analyzing signaling pathway, gene clustering, and bioinformatics-based networks.

2 **Fig. 3. Perspectives using target identification and validation methods of natural products with label-free approaches.** Label-free methods could be a promising strategy for identifying target proteins and mechanism of action of natural products. Furthermore, biologically relevant multiple targets of natural products without chemical modification can be identified by virtue of newly developed methods that accelerate the application of natural products for development of chemical probes and new drugs.