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Identification and validation of bioactive small molecule target through phenotypic screening

Yoon Sun Cho, Ho Jeong Kwon *

Chemical Genomics National Research Laboratory, Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science & Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea

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ABSTRACT

For effective bioactive small molecule discovery and development into new therapeutic drug, a systematic screening and target protein identification is required. Different from the conventional screening system, herein phenotypic screening in combination with multi-omics-based target identification and validation (MOTIV) is introduced. First, phenotypic screening provides visual effect of bioactive small molecules in the cell or organism level. It is important to know the effect on the cell or organism level since small molecules affect not only a single target but the entire cellular mechanism within a cell or organism. Secondly, MOTIV provides systemic approach to discover the target protein of bioactive small molecule. With the chemical genomics and proteomics approach of target identification methods, various target protein candidates are identified. Then network analysis and validations of these candidates result in identifying the biologically relevant target protein and cellular mechanism. Overall, the combination of phenotypic screening and MOTIV will provide an effective approach to discover new bioactive small molecules and their target protein and mechanism identification.

1. Introduction

Small molecules consisting of various structures from chemicals to peptides form the basis of drug discovery and development efforts. Small molecules that possess bioactivity are potential probes and/or drugs, especially if they possess flexibility and target specificity.[1] For example, small molecules with fluorescence can be used as probes for localization and detection markers. Other small molecules with a flexible structure can be diversified through conjugation with different active groups and additional probes, leading to improved activity and pharmacological properties. Small molecule libraries are diversely constituted, comprising natural products, synthetic products, and derivatives of previously identified bioactive small molecules.

The first step in the discovery of new bioactive small molecules is the selection of only bioactive compounds from among abundant sources of small molecules. However, a larger pool of small molecules with bioactivity is needed. Moreover, at present, most therapeutic drugs are limited to targeting receptors and antigens. Thus, diversifying the field of drug target proteins including undruggable targets would expand the range of bioactive molecule–protein target possibilities. Here, we propose screening and target protein identification methods for new bioactive small molecules with the potential for discovery and development as new drugs. This two-step procedure first employs phenotypic screening to discover new bioactive small molecules.[2] In the second step, the protein target of bioactive small molecules is identified through multi-omics-based target identification and validation (MOTIV).

2. Screening for bioactive small molecules

2.1. Target-based screening

The primary step in the discovery of new bioactive small molecules is screening, which may take two basic forms: target-based and phenotype-based. A number of screening methods have been employed, including high-throughput screening. To date, most screening has been carried out using target-based screening strategies. Target-based screening is effective in discovering bioactive small molecules that target one specific protein, such as a kinase,[3,4] histone deacetylase (HDAC),[5] or a G protein-coupled receptor.[6,7] Bioactive small molecules selected through target-based screening may strongly affect a specific target, but within the organism, they

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; CsA, cyclosporin A; HBC, 4-[3,5-bis-[2-(4-hydroxy-3-methoxy-phenyl)-ethyl]-4,5-dihydro-pyrazol-1-yl]-benzoic acid; HDAC, histone deacetylase; HUVEC, human umbilical vascular endothelial cell; KF, kahalalide F; MOTIV, multi-omics-based target identification and validation; ORF, open reading frame; SPR, surface plasmon resonance; ROS, reactive oxygen species; TsA, trichostatin A; UQCRB, ubiquinol-cytochrome c reductase binding protein.

* Corresponding author. Tel.: +82 2 2123 5883; fax: +82 2 362 7265.
E-mail address: kwonhj@yonsei.ac.kr (H.J. Kwon).

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may affect multiple proteins and signaling pathways rather than one single target or pathway. In living organisms, a protein functions by interacting with other proteins. Likewise, small molecules may have multiple cellular targets due to their chemical properties and the inherent connectivity of intracellular signaling mechanisms. Therefore, understanding the activity of a selected small molecule requires a more comprehensive screening method such as phenotypic screening.

2.2. Phenotypic screening

Phenotypic screening is a comprehensive screening method that allows the researcher to evaluate a small molecule's bioactivity at the cellular or organism level. Screening for angiogenesis and autophagy are examples of phenotypic screening (Fig. 1).

Angiogenesis screening consists of assessing invasion and tube formation of human umbilical vascular endothelial cells (HUVECs) grown on a matrix.[8,9] When stimulated with vascular endothelial growth factor or low oxygen physiological condition such as hypoxia, HUVECs invade through the matrix to construct new vessels. HUVECs also form tube-like structures to create and stabilize the newly built blood vessels. If a small molecule inhibits both of these phenotypes and also inhibits HUVEC growth, then it is a potential angiogenesis inhibitor.

Autophagy screening consists of detecting an increase in autophagosomes within a cell.[10] Autophagosomes are easily detected using acidic vacuole staining markers, such as lysotracker or monodansylcadaverine. High-content screening makes it possible to quantify increases in autophagosomes in live cells. The overall morphology, growth, and death of cells are also easily examined quantitatively. Autophagy has been characterized as apoptosis-independent cell death,[11] but its mechanism is still unclear. However, the potential of autophagy as a target in the treatment of diseases such as cancer and atherosclerosis has motivated the discovery of many novel autophagy controllers. Autophagonizer, a novel synthetic compound, was recently discovered by phenotypic screening with high-content screening.[12] This unique small molecule induces autophagy in mammalian cells, but is not inhibited by known autophagy inhibitors. Its mechanism seems to be different from that of rapamycin, which inhibits the mTOR signal to induce autophagy, but its target is still under investigation.

Phenotypic screening also identifies new targets for bioactive small molecules, thereby expanding the otherwise restricted field of compounds available for new drug development. Unknown target proteins encountered through phenotypic screening can be identified using various target identification methods involving genomics, proteomics or MOTIV, a combination of 'omic' techniques.

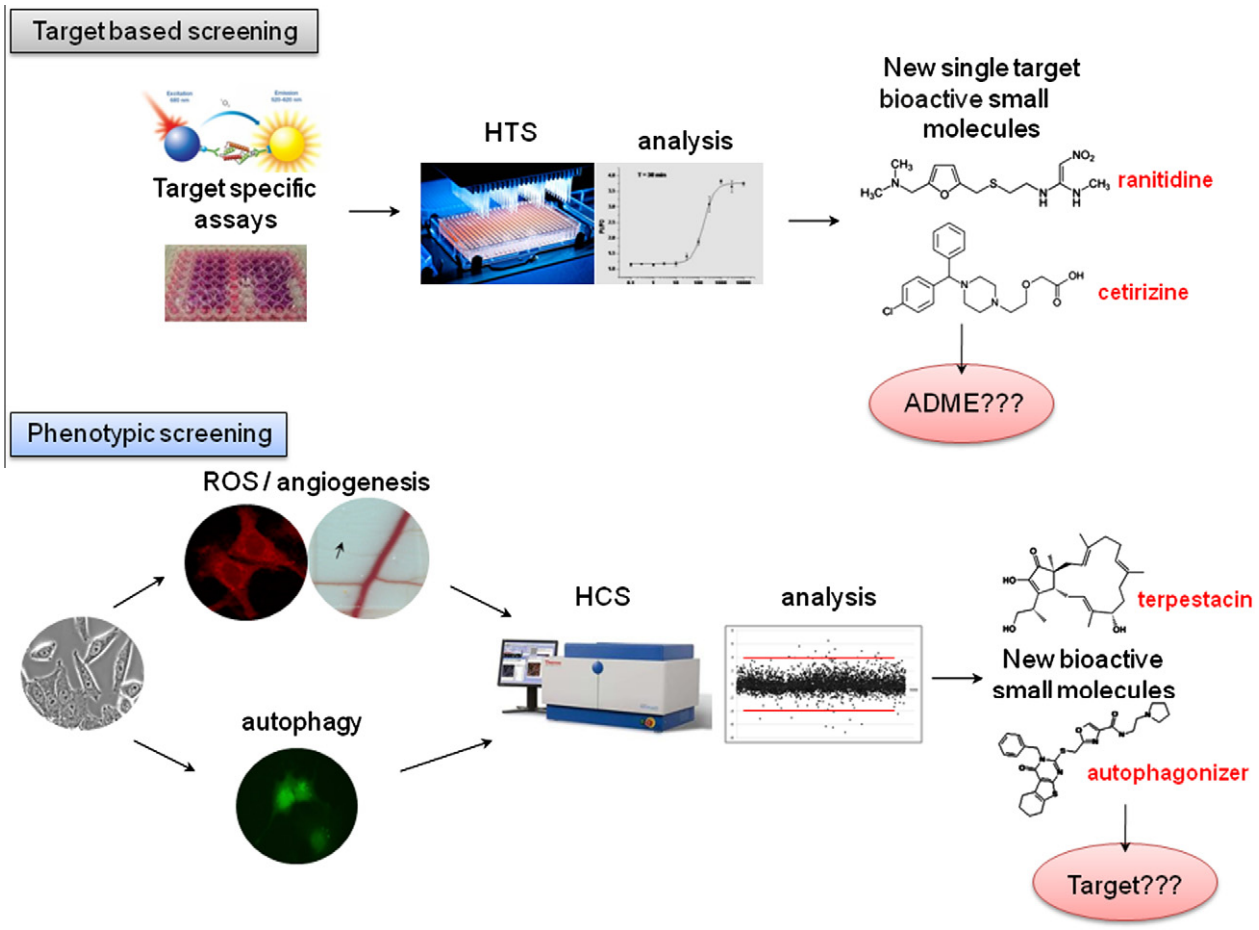

Figure 1. Target-based screening and phenotypic screening schemes. Target-based screening identifies only one specific signaling pathway affected by small molecules. However, absorption, distribution, metabolism, and excretion (ADME) factors should be examined as part of therapeutic drug development efforts. Examples include ranitidine, a histamine H2-receptor antagonist used to treat peptic ulcer disease and gastroesophageal reflux disease, and ceterizine, a racemic selective H1 receptor inverse agonist used for the treatment of allergies, hay fever, angioedema, and urticaria. Phenotypic screening starts with cell-based screening. ADME factors are resolved, but the target protein and signaling pathways are not revealed. Examples include terpestacin, reactive oxygen species (ROS) and angiogenesis inhibitor that targets ubiquinol-cytochrome c reductase binding protein (UQCRB), a component of mitochondria complex III, and autophagonizer, an autophagy inducer that acts through an unknown mechanism. (HTS: high throughput screening, HCS: high contents screening.)

3. Target identification and validation

Identification of the protein target of a bioactive small molecule is the most important step in new drug development because small molecules typically exert their bioactive effects through interactions with proteins. The interaction between the small molecule and its target protein is the key to understanding the small molecule's cellular mechanism. Methods for identifying targets of bioactive small molecules can be divided into two major categories: genetics/genomics-based and proteomics-based. Combining these two methods creates a new and effective target identification and -validation system termed MOTIV.

3.1. Genomics/genetics-based target identification

The classical method for identifying targets is genetics/genomics-based target identification, which involves analysis of mutations and genomic alterations—introduced or naturally occurring—in living cells or organisms. Genetic modifications introduced by specific gene deletion or overexpression of a specific protein can be used to analyze the sensitivity or resistance of a bioactive small molecule. In this approach, the set of individual gene-mutated organism strains forms the mutated genomic library. Examples of this system include the deletion mutants of yeast *Saccharomyces cerevisiae* consisting of the molecular barcoded yeast open reading frame (ORF) library (MOBY-ORF),[13] and the ORF library of overexpression mutants of yeast *Schizosaccharomyces pombe* (ORFeome).[14,15] Both mutant ORF libraries are suitable for screening genes resistant or sensitive to a bioactive small molecule. Resistant genes especially are easier to detect if they do not affect the growth of the organism. As proof of concept, FK506 binding protein 12 and target of rapamycin deletion mutants were shown to be resistant to rapamycin. In the overexpressed library of *S. pombe* ORFeome, each gene is tagged with yellow fluorescent protein, making it possible to examine the effect of leptomycin B, an inhibitor of the nuclear export protein Crm1, on the localization of the corresponding protein.

3.2. Target identification by forward chemical proteomics

Chemical proteomics consists of forward and reverse chemical proteomic methods, both of which use affinity-based interaction. Forward chemical proteomics is the discovery of a bioactive small molecule followed by identification of its target protein.[16] Target protein identification involves the use of various probes and linkers on the small molecule. Probe-linked small molecules mixed with cell lysates or proteins expressed in vitro can be easily used in affinity pull-down methods. Proteins in such mixtures that bind directly to the bioactive small molecule can be identified by mass spectrometry, immunoblotting, and other molecular detection methods. For example, FK506 and cyclosporin A (CsA) are immunosuppressants with different receptors; FK506 forms a complex with FK506 binding protein, and CsA forms a complex with cyclophilin. However, these two complexes were found to have the common target, calcineurin.[17] Calcineurin, a Ca^{2+}- and calmodulin-dependent phosphatase, was identified from a group of proteins that bind to FK506 and CsA complexes. Calcineurin co-precipitated with calmodulin in pull-down assays and co-migrated with calmodulin in electric mobility shift assays. Its phosphatase activity is inhibited by FK506 and CsA complexes, a fact that helps explain the function of immunosuppressants and the workings of their Ca^{2+}-dependent pathways.

Another example of the application of forward chemical proteomics is the HDAC inhibitor trapoxin.[18] Trapoxin, a cyclic tetrapeptide derived from a fungal product, was shown to cause the accumulation of highly acetylated core histones in mammalian cell lines. Similar to trichostatin A (TsA), trapoxin induces morphological normalization in v-sis-transformed NIH3T3 cells. However, trapoxin irreversibly inhibits histone deacetylase; its epoxide group is the only chemically reactive moiety, and it forms a covalent bond between trapoxin and the enzyme molecule. Its chemical structure is different from that of TsA and *n*-butyrate, another HDAC inhibitor. Although trapoxin has a different mode of action from TsA, TsA-resistant cell lines are also resistant to it. Like TsA, trapoxin induces cell-cycle arrest in both G_1 and G_2 phases. These biological results suggest that accumulation of hyperacetylated histones due to HDAC inhibition is the mechanism underlying the action of both TsA and trapoxin.

Nanobeads are representative of a new class of tools used for target identification. Novel magnetic nanocarriers for affinity purification were originally developed by Handa et al.[19] These nanocarriers are approximately 180-nm in diameter and consist of 40-nm magnetite particles. The substantial magnetization of these relatively large 40-nm magnetite particles enables magnetic nanocarriers to be dispersed in a wide range of organic solvent without disrupting their core/shell structure. Moreover, such nanocarriers are capable of immobilizing a variety of small molecules. As proof of concept, methotrexate, an anti-cancer agent that acts through inhibition of dehydrofolate reductase, was used as a ligand. Magnetic nanobead immobilization of methotrexate followed by pull-down from crude extracts of HeLa cells resulted in the isolation of a 27-kDa protein, subsequently identified as dehydrofolate reductase by western blotting. Thus, new methods of immobilization with nanobeads are emerging as target identification methods.

3.3. Target identification by reverse chemical proteomics

Reverse chemical proteomics is the opposite of forward chemical proteomics. In this approach, the organism's cDNA is amplified first, then presented to the small molecule in a manner that links the molecule's target protein directly to its corresponding gene. Using reverse chemical proteomics, it is possible to identify new target protein candidates that act through different cellular mechanisms.[1] This makes it possible to screen for new target protein candidates from a single phenotype. A major method of reverse chemical proteomics is phage display. In phage display, a pool of T7 phage expressing a human cDNA library is prepared such that each phage expresses a single cDNA. Probe-linked small molecules are immobilized and then exposed to the human cDNA-expressing T7 phage pool to allow binding of the immobilized molecule to cDNA-encoded peptides displayed on the phage surface. After repeated rounds of binding and washing, phages that express peptides that bind the immobilized small molecule with high affinity can be isolated, and the target can easily be identified by DNA sequencing and validated through analysis and characterization of the corresponding human cDNA.

The power of the reverse chemical proteomics method is exemplified by 4-[3,5-bis-[2-(4-hydroxy-3-methoxy-phenyl)-ethyl]-4,5-dihydro-pyrazol-1-yl]-benzoic acid (HBC), a newly synthesized curcumin derivative unexpectedly found to lack the CD13-inhibitory activity of its parent compound.[20] By applying the phage display technique to immobilized biotin-HBC, the target protein was identified as calmodulin.

A second example is terpestacin, a natural product isolated from *Embellisia chlamydospora*.[21] Phenotype screening revealed that terpestacin possessed anti-angiogenic activity, which manifested as inhibition of invasion, tube formation, and chorioallontoic membrane angiogenesis. But the mechanism underlying terpestacin's anti-angiogenic effect was mysterious. Using the phage display method, the protein target of terpestacin was identified as ubiquinol-cytochrome c reductase binding protein (UQCRB). UQCRB is a mitochondrial complex III protein that is involved in mitochondrial electron transport and contributes to the production of reactive oxygen species (ROS). Surface plasmon resonance (SPR) analyses

confirmed that terpestacin and UQCRB were direct binding partners. Furthermore, molecular validation showed that the anti-angiogenic action of terpestacin was due to inhibition of hypoxia-inducible factor stability and ROS production in hypoxia (Fig. 2).

A final example is provided by marine natural products, which are also under investigation as a new source of natural products with bioactivity.[22] Kahalalide F (KF) is a natural product isolated from *Elysia rufescens* that possesses anticancer activity. It is currently in phase II clinical trials as an anticancer drug against solid tumors, but its direct target protein was initially uncharacterized. Using the phage display method, RPS25 (ribosomal protein S25) was identified as a candidate KF target protein. Five different clones of RPS25 were isolated from different human cDNA libraries and all were validated with phage binding assays, which showed that the number of phage bound to KF increased in a time- and concentration-dependent manner. KF-RPS25 binding was validated by SPR. In addition, an analysis of fluorescent KF localization showed that KF localized to cellular ribosomes, consistent with the role of RPS25 as part of the small subunit of the eukaryotic ribosome. Thus, the direct interaction between KF and RPS25 has been demonstrated, but further studies are required to interpret the relevance of RPS25 binding to the function of KF.

Finally, data from diverse genomic/proteomic methods of analyzed compounds constitute their profiles.[23,24,26] Profiles of various compounds are compared with each other, and overlapping targets and new functions of targets can be identified. Comparison of compound effects allow which group of patients respond well and which group of patients should be restricted from the compound treatment.

3.4. MOTIV

Effective target identification requires considerable knowledge about the bioactive small molecule and robust identification methods. Various methods are currently applied for target identification as noted above. Genomics and proteomics methods are applied to

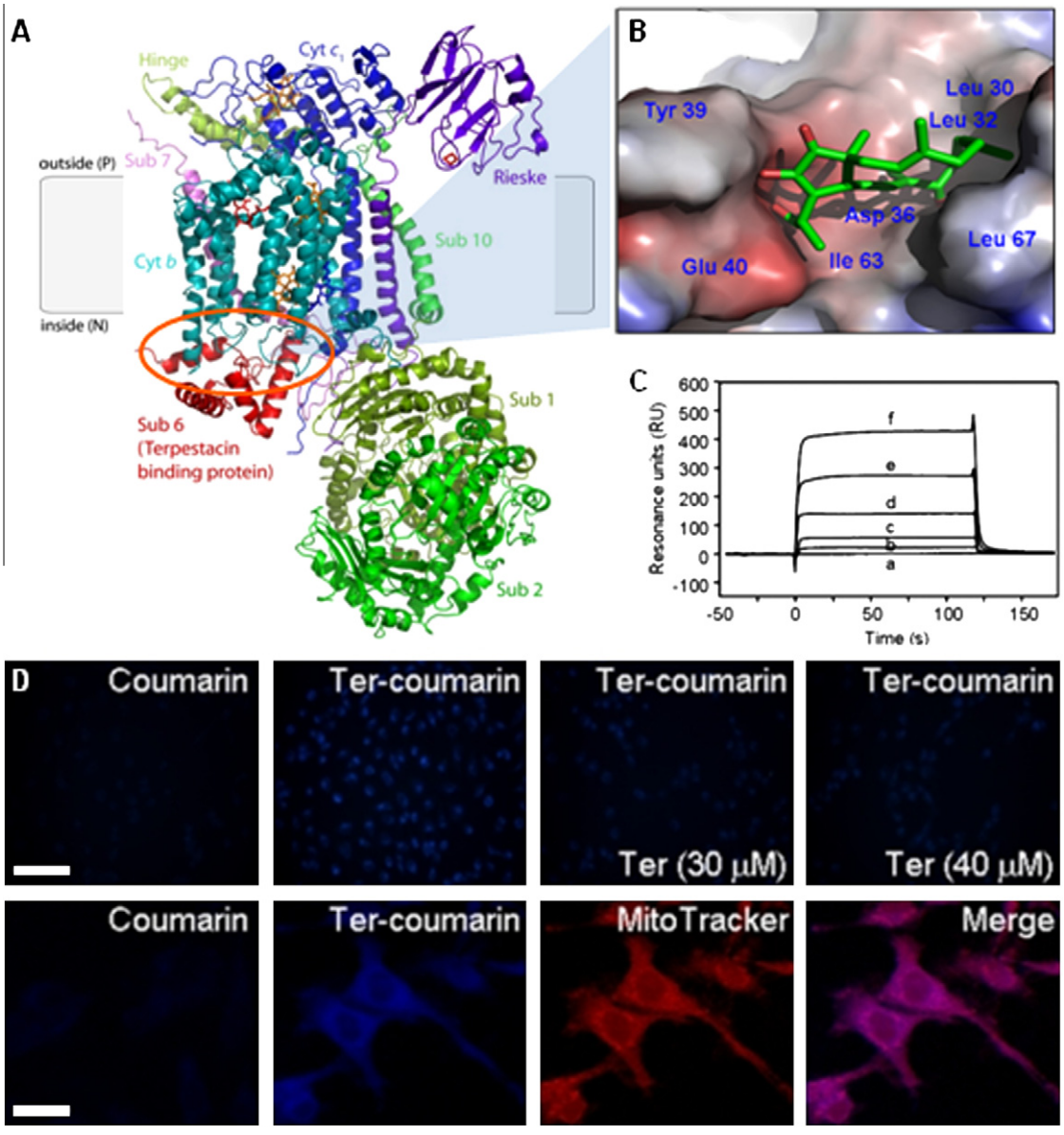

Figure 2. Example of reverse chemical proteomics. The target protein of terpestacin was identified as UQCRB through phage display biopanning. (A) Electrostatic surface representation of the hydrophobic pocket of UQCRB bound to terpestacin. (B) Docking model analysis of terpestacin (red) and UQCRB (blue). (C) SPR result validating the direct binding of terpestacin and UQCRB. (D) Terpestacin-coumarin localized with Mitotracker, indicating that terpestacin interacts with UQCRB in the mitochondria.

yeast for new drug discovery and target validation,[25] chemical genomics which includes both genomics and proteomics is used for anticancer drug discovery,[26] and genomics and proteomics methods are under development for new drug target discovery.[27,28] MOTIV constitutes a systematic target identification and validation method that relies on the characteristics of the bioactive small molecule. MOTIV is a combination of chemical proteomics and genomics methods, followed by an analysis of network organization of target protein candidates (Fig. 3). Compared to single target identification technique, MOTIV provides larger number of target candidates. Furthermore cross-validation of the numerous candidates resulting from various 'omics' methods makes the resulting target proteins more reliable and suitable for validation. First, bioactive small molecules are divided into either genomics method or chemical proteomics method of target identification. Secondly, the target identification methods are carried out, resulting in a list of target candidates. Thirdly, the target candidates are validated. Lastly, the candidates are analyzed in signaling network constructions, resolving which cellular mechanism the small molecule is involved in.

First, as noted above, some small molecules are capable of linking probes for affinity based methods, whereas others are not. The MOTIV system directs small molecules to the appropriate method for target protein identification based on structure and activity considerations. Small molecules capable of linking additional probes such as biotin for immobilization are suitable for both genetic/genomic and chemical proteomic methods. Direct small molecule and target protein interactions will be easily tested with various pull-down assays and phage display technology. However, the activity of the probe-linked small molecules must be tested first. Many small molecules may be suitable for probe linking, but if probe linking is not possible, the genetics/genomics-based target identification method is recommended. Genomics based methods consist of genetically modulating the organism first, and then treating the small molecule for activity analysis, so probes are not necessary. Mutated yeast collections as mentioned previously are the representative examples. Likewise, bioactive small molecules are sentenced to the appropriate target identification methods.

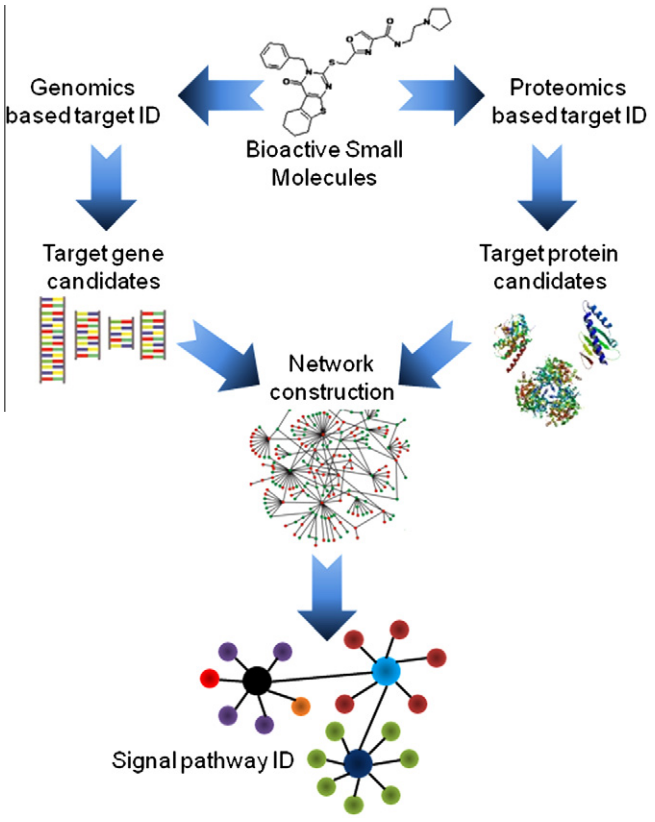

Figure 3. Multi-omics-based target identification and validation (MOTIV) scheme. Genetics/genomics and/or proteomics-based target identification methods are applied to newly discovered small molecules. Then, target protein candidates are chosen for validation. Candidates from both methods are analyzed with respect to intracellular network connections to interpret which signaling pathway is involved in the bioactivity of the small molecule.

After carrying out the target identification examinations, numerous target protein candidates will arise. Bioactive small molecules that underwent both genetic/genomic and chemical proteomic target identification methods will result in candidates that do or do not overlap. These candidates identified through one method serve to cross-validate those of the other. Both target identification methods yield many target protein candidates, making a probable database for the small molecule.

Thirdly, by analyzing the relationships among the candidates, a signaling network can be formed that establishes the relevance of candidates with the endpoint phenotype obtained from phenotypic screening. Validations based on this signaling network can reveal biologically relevant target proteins and related signaling pathways.[29–31] The signaling pathway could result in one specific pathway, or suggest new pathways that have not been validated before, since new targets can be identified from phenotypic screening and target identification methods. The resulting network provides insight into the cellular mechanisms of the target proteins and the endpoint phenotype. New receptors and proteins that have not been previously considered can be exploited as new bioactivity controllers, expanding the pool of potential new drug candidates. Overall, MOTIV provides a systematic target identification and validation method. Importantly, the results obtained using this system open up new avenues for bioactive small molecule targeting in therapeutic drug development since a small molecule will eventually affect the entire cellular mechanism.

3.5. Target protein validation

Target identification is always followed by validations to eliminate false-positive target candidates. The signaling pathways discovered by MOTIV provide the starting point of target protein validation. Validation assays consist of a variety of methods, including biochemical, biological, and biophysical interaction-detection methods; endpoint phenotype detection; and the use of yeast, zebrafish, or other model organisms. These diverse validation assays establish the biological relevance of the small molecule and its target proteins. For example, validation through phage-binding and competition assays confirmed the direct binding of calmodulin-expressing phage and HBC. SPR confirmed the interaction of HBC with full-length calmodulin, and demonstrated that Ca^{2+} is required for binding. Finally, docking simulations suggested that HBC fits into the hydrophobic pocket of calmodulin. Validation of the terpestacin target also included phage binding and competition assays as well as SPR analyses. Treatment of cells with coumarin-linked terpestacin was also used to assess terpestacin colocalization with MitoTracker, a mitochondria marker. By targeting UQCRB, terpestacin inhibits hypoxia-induced ROS generation and activation of hypoxia-inducible factor, thereby inhibiting tumor angiogenesis. Likewise, validations are required for reliable target protein identification.

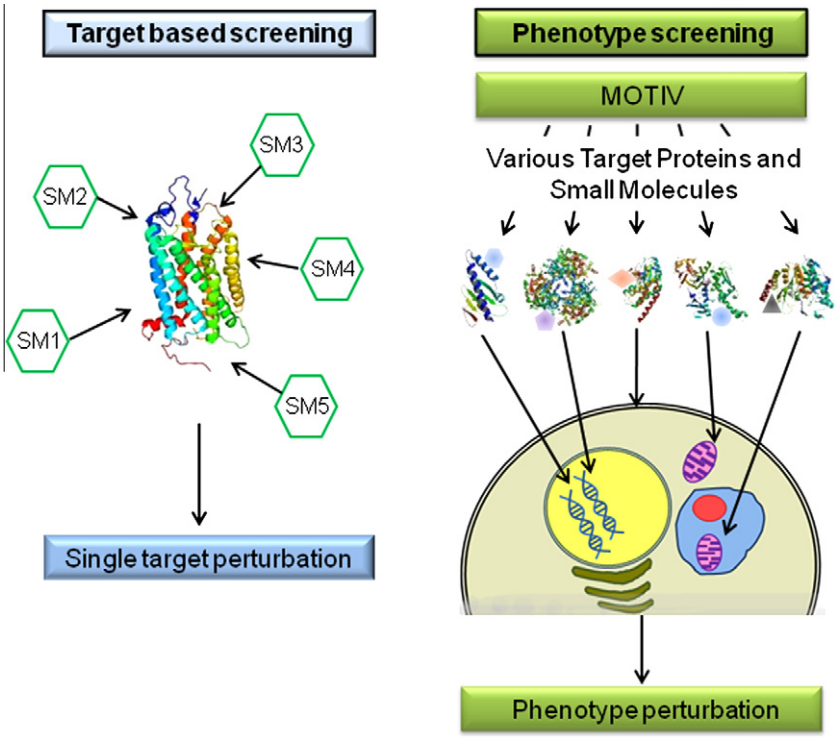

Figure 4. Perspectives of phenotypic screening and MOTIV. Target-based screening is restricted to a single target protein pathway, and is limited in its ability to predict what will really happen within the organism. However, phenotypic screening and MOTIV yield several potential target proteins and signaling pathways for a given bioactive small molecule. These target proteins produce a variety of signals that collectively manifest as a single phenotype. The result is an expanded field of targets from various signaling pathways that can be exploited for the development of therapeutic bioactive small molecules. (SM: small molecule.)

4. Perspectives and conclusion

Bioactive small molecules are potential probes and drugs. The primary step in the discovery of new bioactive small molecules is screening. Phenotypic screening is a method for discovering new small molecules with bioactivity at a comprehensive, organism level. Since screening for drug discovery is exclusively target-based, differences in the phenotype of an organism provide a new pool of bioactive small molecules. Thus, phenotypic screening is typically the first method to discover a new pool of bioactive small molecules.

The second step in bioactive small molecule discovery is target protein identification. Identification of protein targets of bioactive small molecules is necessary to convincingly demonstrate that the candidate compound is a viable probe or drug candidate. Target identification is the most time-consuming and difficult step in the discovery of new bioactive small molecules. Here, we introduced MOTIV, a systematic and effective approach for identifying protein targets of bioactive small molecules. In this system, chemical proteomics- and/or genetics/genomics-based target-identification methods are applied as appropriate, given the properties of the small molecule under investigation. Small molecules that resist modification or addition of probes lend themselves to genetics/genomics-based methods, whereas both genetics/genomics- and chemical proteomics-based methods can be applied to small molecules that are readily conjugated with immobilizing probes. These two methods go hand in hand to compensate for each other's limitations. Overall, the MOTIV system is suitable for discovering small molecule target proteins and validating their functions (Fig. 4).

The identification of target protein candidates provides the first clue to the signaling mechanism modulated by the small molecule. Furthermore, a network analysis of target protein candidates provides additional insight into the small molecule's bioactivity, connecting related signal pathways and the endpoint phenotype. Because these target proteins are not from a restricted pool of proteins, as they are in target-based screening, the outcome is open-ended: novel targets and unexpected signaling mechanisms might ultimately explain the link between the bioactive small molecule and the endpoint phenotype. This concept is important in that, in a real live organism, small molecules rarely affect only a single protein, but instead impact many other signals within the cell.

The last step in the discovery process is validation of target proteins to eliminate false-positive candidates. As noted above, validation assays may take a variety of forms, but must establish the biological relevance of the small molecule and its target proteins if the bioactive small molecule under study is to be developed into an effective therapeutic drug.

Bioactive small molecules are potential drug candidates. But developing them into therapeutic drugs requires effective screening, target identification, and validation procedures. Bioactive small molecules can be effectively discovered by phenotypic screening and MOTIV. The combination of phenotypic screening and MOTIV provides a clearer understanding of the bioactivity of small molecules at the organism level and widens the field of target protein candidates. In so doing, phenotypic screening and MOTIV expand the discovery of drug candidates and eventually new drug developments.

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