Chemistry & Biology
Review

Biochemical Target Isolation for Novices: Affinity-Based Strategies

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DOI 10.1016/j.chembiol.2010.05.015

Although a number of genomic and biochemical technologies are now used to elucidate the mechanisms of action of bioactive small molecules, affinity-based isolation of molecular targets is a classic, but still powerful, approach. This review highlights recent cases where biochemical isolation of target proteins of bioactive small molecules highlighted general strategies for a successful isolation and identification of molecular targets. This review is intended to be both an update on the most recent findings for those already active in the field of forward chemical genetics and a guide for scientists entering this burgeoning field.

Introduction

A number of approaches have been developed to determine modes of action of bioactive small molecules, including both natural products and synthetic molecules discovered by phenotypic screening of chemical libraries (Choi et al., 2003; Grozinger et al., 2001; Gumireddy et al., 2008; Kawazoe et al., 2004; Kwok et al., 2006; Mayer et al., 1999; Pelish et al., 2001; Shan et al., 2008; Zhang et al., 1999). Among the many approaches used to accomplish this goal, biochemical isolation of cellular protein targets using affinity resins is the classic and, perhaps, the most straightforward one. Preparation of the affinity resins requires structure-activity relationship studies of a small-molecule ligand of interest, which often suggest the sites appropriate for linker modification. It is important to note that very small ligands that have no sites appropriate for modification are not suited for affinity-based target isolation. Once a modification site is successfully identified, the small-molecule ligand is covalently attached to a solid support through a linker (Bach et al., 2005; Harding et al., 1989; Khersonsky et al., 2003; Oda et al., 2003; Snyder et al., 2005; Taunton et al., 1996; Zhang et al., 2007), or its biotinylated version is bound to avidin-agarose beads (Kaida et al., 2007; Lefkowitz et al., 1972; Low et al., 2005; Meng et al., 1999; Nguyen et al., 2003; Sato et al., 2007; Sche et al., 1999; Shimogawa et al., 2004; Sin et al., 1997). Typically, the proteins that bind directly to the small-molecule bait are purified from cell lysates by affinity chromatography, separated by SDS-PAGE, and excised from the gel. The isolated proteins are identified by partial tryptic digestion, mass-sequencing of the digested peptides, and database search of the sequences (Aebersold and Mann, 2003). The identified proteins then need to be validated as the actual molecular targets by independent molecular and cell biology experiments, to confirm that one or more of them is responsible for part or all of the effects of the small molecule.

Scientists involved in affinity-based target isolation have made significant efforts to increase the success rate. This review analyzes published examples of successful target isolation, with the goal of establishing "tips" for scientists beginning to use this approach. The rate-limiting step in biochemical target isolation is the final validation, which requires examination of individual candidate proteins from multiple viewpoints. A major problem with affinity-based target identification is the existence of nonspecific binding proteins, which are hard to remove completely, even after careful washing of the affinity resin. Nonspecific or less specific proteins have often confused scientists in academia and industry, especially novices in target isolation. The best way to accelerate final validation is to avoid isolating such false target proteins in the initial steps, or to exclude them at an early stage. Experimental evaluation of many false targets could consume valuable time, ultimately forcing graduate students to postpone their dissertation defenses, or a company to terminate further development of a pharmaceutical candidate. Thus, a key issue examined in this review is how to exclude nonspecific proteins and isolate bona fide target proteins.

Affinity and Abundance

In general, the higher the affinity of a small molecule for the target protein, the more successful the target isolation is assumed to be. High-affinity complexes tend to be maintained after extensive washing, which reduces the amount of nonspecific binding proteins in the sample. However, there are several issues that complicate this assumption.

It is impossible to measure accurately the binding affinity of the small molecule and its target protein without knowing the identity of the target. In forward chemical genetics, the binding affinity must be estimated from the molecule's effective concentration in cell-based assays, such as EC_{50} (half maximal [50%] effective concentration) or IC_{50} (half maximal [50%] inhibitory concentration). For example, a drug with an EC_{50} or IC_{50} value in the pM range would be assumed to have higher affinity for its target than a drug with an EC_{50} or IC_{50} value in the μM range. Of course, the most potent molecule in cell-based assays may not always have the highest affinity. Some potent molecules could be "dirty," i.e., could bind to multiple targets, which act synergistically to produce strong pharmacological effects, and some may possess higher cell permeability or water-solubility than others. Further examination of compounds by multiple follow-up assays, including gene expression profiling, biochemical assays, and physical property evaluation, might help select the most appropriate for target identification.

Assuming that more potent molecules have higher affinities for their targets, the molecule with the lowest EC_{50} or IC_{50} value is

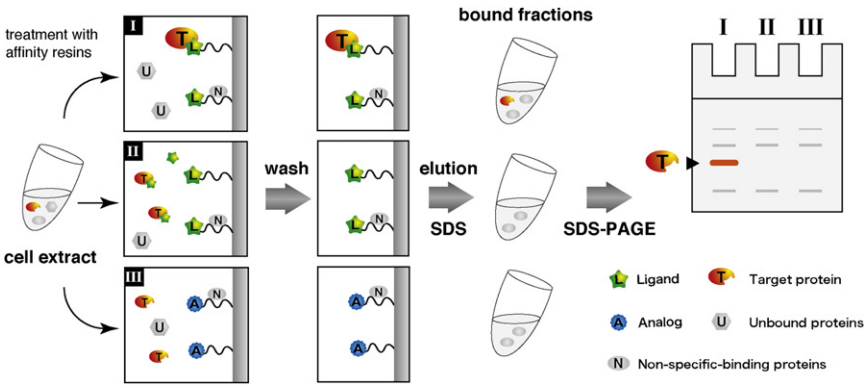


Figure 1. Identification of the Protein Target of a Bioactive Small Molecule
Affinity chromatography can be used to isolate the protein target T of a small-molecule ligand L. Negative control experiments can be designed to distinguish the protein target from nonspecific binding proteins N. Cell lysates are treated with affinity resin of the small-molecule ligand and the unbound proteins U are washed away.
(I) Elution by an SDS buffer. The purified sample contains specific and nonspecific proteins.
(II) Competition with excess amounts of free ligands. The specific target protein fails to bind to the affinity resin.
(III) Comparison with an inactive molecule A. The target protein is not supposed to bind to the resin. Comparison of band patterns on SDS-PAGE gels (I and II, or I and III) leads to identification of candidate target proteins.

usually considered best suited for target identification. Although a high affinity ligand is advantageous for isolating its complexes, its target protein is not necessarily easy to identify. A very low effective concentration, e.g., in the pM range, may reflect a low abundance of the target protein, making target identification difficult, due to its underrepresented band on an SDS gel.

What if cellular targets of the molecules with the EC_{50} or IC_{50} value in the low μM or high nM range need to be identified? Is it possible? From the perspective of their target proteins in cells, they could be either abundant or scarce. Isolation of a low abundance target with a low-affinity molecule would be extremely challenging. In contrast, isolation of a highly abundant target with a low affinity molecule remains possible. Although the affinity of the interaction is low, the high abundance of the target protein would, theoretically, increase the success rate of biochemical isolation and identification. Thus, the higher the affinity is and the more abundant the target is, the more likely it is to isolate the target (affinity-abundance theory): the balance between protein abundance and effective concentration is an important factor in the success of biochemical target isolation.

In an example from our own work, we were able to purify human GLO1, an abundant metabolic enzyme that catalyzes the conversion of methylglyoxal to D-lactate, as a second target of indomethacin, a clinically used antiinflammatory drug (Sato et al., 2007). Although the K_D value of the interaction was in the low μM range, purification and identification of GLO1 was possible, due to its high abundance. The molecular targets that underlie the anti-inflammatory effects of indomethacin are known to be COX proteins. Nevertheless, further cell-based experiments suggested that inhibition of GLO1's enzymatic activity is responsible for the clinically observed synergy between indomethacin and anticancer drugs (Duffy et al., 1998; Hixson et al., 1994; Hull et al., 2003; Maca, 1991; Raveendran et al., 1992; Ruegg et al., 2003).

There have been cases to which the affinity-abundance theory does not apply. The affinity-abundance theory applies to small molecules that inhibit the function of the target proteins, such as enzyme inhibitors. In some cases of successful target identification, however, the complexes between small molecules and their target proteins activate or actively exert biological functions. In such cases, interaction of a small molecule with a subpopulation of the target protein could trigger biologically significant effects, allowing a low concentration of a high-affinity ligand to exert its biological effects by binding to an abundant molecular target. Tight interactions and abundant targets are the most favorable conditions for target isolation. One prominent example is the isolation of FKBP using FK506 (Harding et al., 1989). This clinically used immunosuppressive drug exerts its biological activity in the pM range, and its interaction with its target protein, FKBP, is very tight. FK506 has a low effective concentration, but FKBP is highly abundant in cells. Later studies showed that the complex between FK506 and FKBP inhibits a less abundant protein, calcineurin, to exert its immunosuppressive activity (Liu et al., 1991).

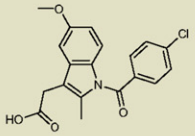
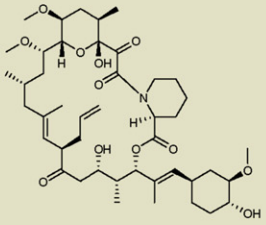
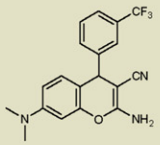
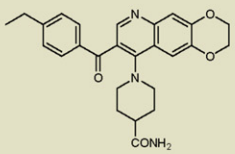
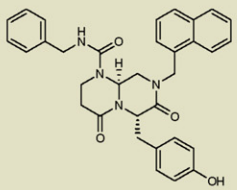
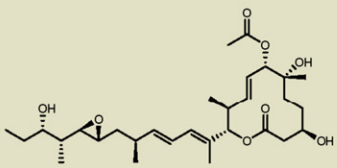
A quick way to validate an abundant target of a molecule with a low IC_{50} is to carry out an siRNA knockdown experiment. If the complex between the small molecule and the target elicits or activates biological functions, the knockdown of the target should impair the molecule's action. We recently found this to be the case with chromeceptin, a blocker of adipogenesis, whose molecular target is MFP-2, an abundant peroxisomal protein (Choi et al., 2006). Knockdown of MFP-2 in cells impaired chromeceptin's action, indicating that MFP-2 is required for chromeceptin to function. Our recent studies indicate that the complex between MFP-2 and chromeceptin binds to and impairs an enzyme required for fat synthesis (unpublished results).

Competition

A well designed, negative control experiment is important in target isolation, to distinguish target proteins from nonspecific proteins. An example of such an experiment is a competition experiment, in which excess amounts of a free compound are added to cell lysates (Figure 1). When the lysates are treated with an affinity resin, the free compounds compete with compounds on the resin, lowering the recovery of proteins that bind selectively to the immobilized compound. Proteins with lower recovery in the negative control experiment are more likely to become target candidates for validation.

An excellent example of a well designed, negative control experiment was provided by the isolation of class Ia phosphatidylinositol 3-kinase (PI3K) as a molecular target of quinostatin, an inhibitor of cellular S6 phosphorylation (Yang et al., 2007). Yang and coworkers used an affinity resin in which the polyethylene glycol-modified quinostatin was immobilized on agarose beads (Table 1). When the bound proteins were analyzed by

Table 1. Chemical Structures and Molecular Targets of Bioactive Small Molecules Discussed in This Review

Name of molecules	Structure of molecules	Target proteins	Phenotypes
Indomethacin		Cyclooxgenase 1, 2 (COX1, COX2)	anti-inflammatory
		Glyoxalase 1 (GLO1)	synergistic effects for anti-tumor drugs
FK506		FK506 binding protein (FKBP)	immunosuppressiion
Chromeceptin		Multifunctional protein 2 (MFP-2)	inhibiton of adipogenesis and insulin/IGF signaling
Quinostatin		Class Ia phosphatidylinositol 3-kinase (PI3K)	inhibiton of cellular S6 phosphorylation
ICG-001		Cyclic AMP response element-binding protein	down-regulation of β-catenin/T cell factor signaling
Pladienolide B		Splicing factor 3b	anti-tumor activity

SDS-PAGE, a number of bands were observed. Competition experiments revealed that a single 85-kD band disappeared in the presence of 100-μM free quinostatin. After in-gel trypsin digestion and mass-sequencing, the 85-kD band was identified to be class Ia PI3K regulatory subunits, p85α and p85β. One problem with this approach is that bioactive small molecules or

pharmaceutical candidates are often lipophilic in nature, usually have low water solubility, and are not soluble enough in lysates for competition to occur. Quinostatin was water-soluble at least up to 100 μM. However, competition was not always observed with a number of small molecules with low water-solubility that we have tested in the laboratory.

Control Molecule

Another classic approach to biochemical target isolation is to use a control molecule that lacks biological activity (Figure 1). The proteins isolated by an active molecule are compared with those isolated with an inactive molecule; proteins isolated by the active molecule, but not by the inactive one, are identified as target candidates. In principle, this approach is powerful for discriminating targets from nonspecific or irrelevant proteins. However, false negative controls are a potential pitfall. Different inactive molecules have different reasons for being inactive, including low solubility, low cell permeability, and low affinity for the target protein. Inactive molecules with low solubility or cell permeability might be able to bind to the target protein in cell lysates in vitro. Moreover, high-affinity molecules could exhibit negative activity, if simple binding is unable to modulate the function of the target protein. The use of such inactive molecules would still purify the target proteins, leading to incorrect conclusions.

The most appropriate negative control molecule would be an inactive, chemically analogous molecule with physical properties similar to those of the active molecule. For example, an inactive chiral isomer of the active molecule would serve as an excellent negative control. Chiral pairs of small molecules usually have identical physical properties, except for their optical rotation. Both chiral isomers undergo the same binding to nonspecific proteins, through noncovalent or electrostatic interactions. However, the isomers are distinct with respect to stereospecific molecular recognition by a target protein.

In our example, an inactive epimer of the proapoptotic natural product, aurilide, was used as a negative control to isolate the mitochondrial protein responsible for the biological activity of the marine natural product (Table 2) (unpublished data). In another example, an inactive enantiomer was used as a negative control for detecting the target of a plant natural product, a jasmonate glucoside, which controls the nyctinastic leaf movement of the leguminous plant, *Albizzia saman* (Nakamura et al., 2008). Nakamura and coworkers used the photo-affinity probe of the natural product to successfully detect and isolate the molecular target in the cell membrane.

A potential problem with the chiral isomer approach is that at least one stereo-center that is critical for the activity is needed in the chemical structure. Although many natural products are chiral, pharmaceutical candidates tend to be achiral, due to the cost-effectiveness of achiral isomers in pharmaceutical production. The isolation of ornithine-δ-aminotransferase by diazonamide A provides an example in which a close analog, but not a chiral isomer, was used as a negative control (Wang et al., 2007). Diazonamide A is an antimitotic natural product that blocks spindle assembly in mammalian cells. To identify its molecular target, Wang et al. (2007) synthesized a *seco* analog of diazonamide A (Table 2). The chemical structure of the inactive analog was identical to that of diazonamide A, except the analog lacked the σ-bond connecting carbons 16 and 18. The *seco* analog served as an excellent negative control in the target identification of diazonamide A.

Another potential negative control is a molecule that is inactivated by attachment of a linker at a position critical for biological activity. This approach was used to isolate glycogen synthase kinase-3β (GSK-3β) as a molecular target of TWS119, an achiral synthetic molecule that induces neurogenesis in murine embryonic stem cells (Ding et al., 2003). TWS119 was linked through its anilino group, which does not affect biological activity, to an affinity resin. Another affinity resin, in which an analogous and active molecule, TWS113, was linked through a nonpermissive site for biological activity, was used as a negative control (Table 2). Direct comparison of the bound proteins identified GSK-3β as the most likely protein target of TWS119.

Elution

Selective elution of bound proteins from the affinity resin is another option for target isolation (Figure 2). Elution of bound proteins with protein denaturing agents, such as SDS, often gives highly complex results on a SDS-PAGE gel, due to nonspecific proteins, which could conceal faint bands of bona fide molecular targets at low abundance. The nonspecific proteins could also increase the number of potential target candidates to validate. On the other hand, elution of bound proteins using a free, bioactive small molecule would permit selective elution under mild conditions.

One successful example of selective elution is the isolation of cyclic AMP response element-binding protein as a molecular target of ICG-001, a small molecule that downregulates signaling by β-catenin/T cell factor (Emami et al., 2004). To identify the molecular target of ICG-001, an affinity resin was prepared by binding the biotinylated derivative of ICG-001 to avidin agarose resin (Table 1). The proteins bound to the ICG-001 affinity resin were specifically eluted with free ICG-001. When the bound proteins were eluted with a denaturing elution buffer, a number of nonspecific proteins were detected. Selective elution, using free ICG-001, limited the number of proteins, permitting identification of cyclic AMP response element-binding protein, a low abundance transcription factor.

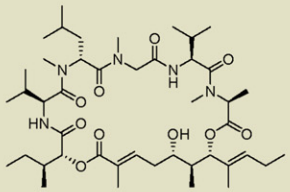
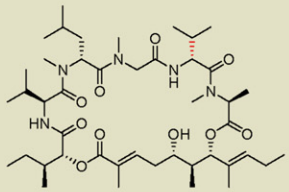
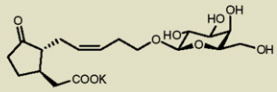
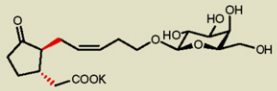
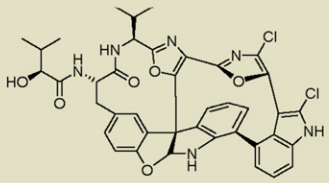
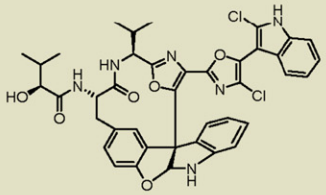
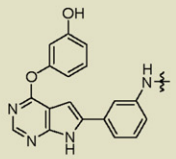
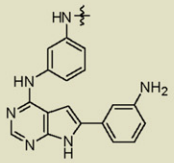
Successful use of the selective elution approach depends on the solubility of the small molecule. Selective elution is feasible only when the bioactive small molecule is highly water soluble. Moreover, target proteins cannot be eluted if they react covalently with the small molecule.

Linker

Recent studies showed the importance of the linker between the small molecule bait and the affinity resin. A variety of polymethylene linkers and polyethylene glycol (PEG) linkers with different lengths is commercially available and many have been popular choices. Recent studies suggested that hydrophilic PEG linkers are preferable to polymethylene linkers, because the PEG conjugates exhibit more desirable physical properties and usually reduce binding of nonspecific proteins (Sato et al., 2007) .

Length is an important factor in determining the usefulness of a specific linker. Our laboratory recently examined the effects of

Table 2. Comparison of Structures between Bioactive Small Molecules and Their Negative Control Molecules

Structure of Molecule	Structure of Control Molecule	Target Protein
 Aurilide	 epimer	A mitochondrial protein
 Jasmonate glucoside	 enantiomer	A cell membrane protein
 Diazonamide A	 *seco* analog	Ornithine-δ-aminotransferase
 TWS119	 TWS113	Glycogen synthase kinase-3β

Structural differences in chiral isomers are highlighted in red.

linker length on recovery rates of target proteins from an affinity resin prepared by binding a biotinylated small molecule bait to avidin agarose resin (Sato et al., 2007). PEG-based linkers with different lengths (11–32 Å) were inserted between a small molecule bait and biotin, and the resulting conjugates were compared in terms of target recovery. In this particular case, longer PEG linkers exhibited higher recovery of the molecular target from cell lysates. Furthermore, elongation of the linker, by insertion of a long, rigid polyproline helix between a small-molecule bait and a biotin tag, boosted the capacity of affinity purification (Figure 3) (Sato et al., 2007). The rigid polyproline helix may project a small-molecule bait away from the biotin-avidin complex to permit its interaction with protein targets. The polyproline-rod approach has now been used in target identification programs of five pharmaceutical companies.

Localization

Introduction of a fluorophore to a small molecule can reveal the subcellular localization of the bioactive molecule, and suggest the location of a molecular target. This in term can narrow down the search field and reduce compositional complexity of the sample. For example, if the molecule is localized in the nucleus, nuclear extracts would be the appropriate lysates for target isolation. Although designing a fluorescent derivative that is as bioactive as its parent molecule can be somewhat time-consuming, the fluorescent probe is a powerful tool for

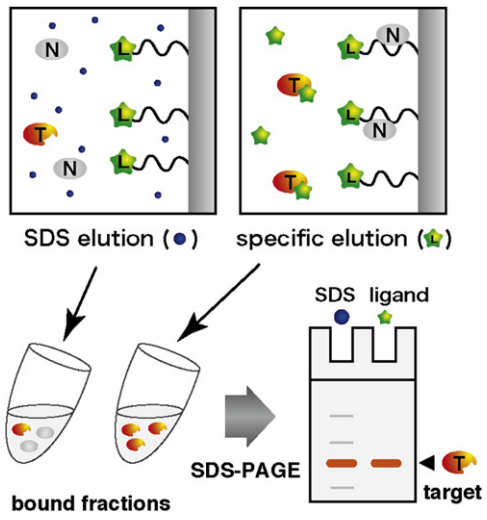


Figure 2. Specific Elution by a Free Ligand
Elution of the bound proteins with protein denaturing agents, such as SDS, often gives highly complex results on an SDS-PAGE gel, due to nonspecific proteins. Elution of bound proteins with a free ligand reduces the detection of nonspecific proteins.

selecting organelle-enriched lysates, which greatly reduces background levels in protein purification. It should be noted that another way to reduce background levels is prepurification of the cell lysates by conventional biochemical methods such as ion-exchange columns.

One excellent example of the use of a fluorescent probe is the isolation of splicing factor SF3b as a molecular target of the antitumor natural product pladienolide B (Table 1) (Kotake et al., 2007). Although pladienolide showed prominent antitumor activity, the target protein remained unclear. Kotake and coworkers prepared three probes (H^3-labeled, fluorescence-tagged, and photoaffinity-biotin-tagged) by modifying the acetoxy group of pladienolide (Kotake et al., 2007). First, the H^3-labeled and fluorescence-tagged probes were used to determine that the subcellular localization of pladienolide was in nuclear speckles, suggesting a splicing- or transcription-related protein as a binding protein of pladienolide. The photoaffinity-biotin-tagged probe was then incubated and reacted with partially purified nuclear extracts, leading to identification of spliceosome-associated proteins 145 (SAP145, SF3b subunit 2) and 130 (SAP130, SF3b subunit 3) as targets of pladienolide.

Outlook

In this review, we briefly summarized lessons from successful affinity-based target identification of bioactive small molecules. Although there are many excellent reviews for target identification, this review focused on discussing the strategies to avoid isolation of false targets in six aspects: affinity and abundance, competition, control molecule, elution, linker, and localization, about which scientists entering the field often ask questions. Identifying molecular targets of bioactive small molecules remains one of the technical challenges in forward chemical genetics and phenotype-based drug discovery. Although the affinity-based biochemical approach needs to be combined with a number of nonbiochemical approaches for lowering the risk of target identification, the direct association between the bioactive small molecule and its putative target protein must be confirmed in each individual case, and the classic biochemical approach remains powerful. Recent advances in mass spectrometry, genomics, and analytical techniques have greatly improved biochemical isolation and validation of molecular targets. What we lack are methods to isolate target proteins that are inactive, insoluble, or least abundant in cell lysates, and to isolate, identify, and validate nonprotein molecular targets of small molecules. Continued research and method

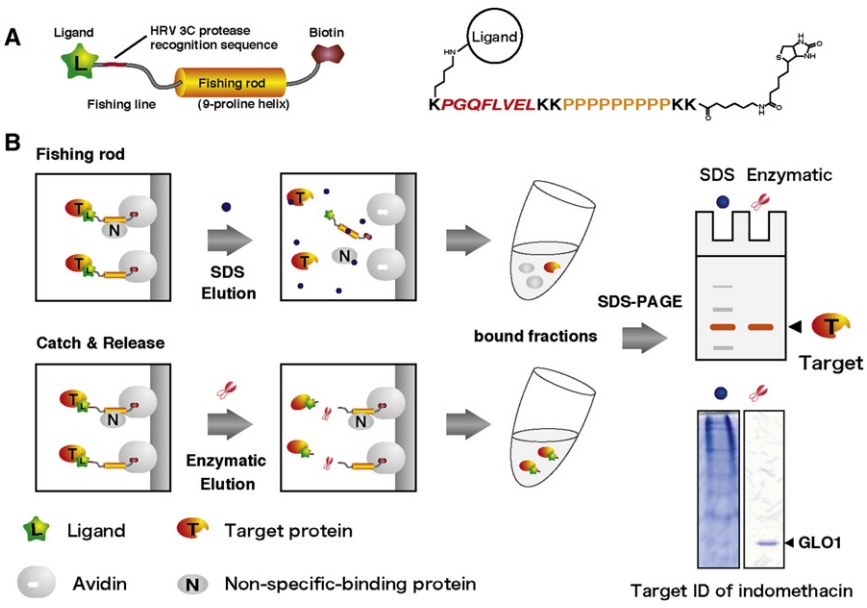


Figure 3. Polyproline Approach to Isolating Protein Targets of Bioactive Small Ligands
(A) The model structure (left) and the amino acid sequence (right) of a polyproline linker.
(B) Enzymatic elution with HVC3C protease at 4°C greatly reduces the detection of nonspecific proteins. Using this technology, human GLO1 was identified as a second target of indomethacin, a clinically used antiinflammatory drug (lower right panels).

development will lead to successful identification of additional molecular targets.

ACKNOWLEDGMENTS

We are grateful to the MEXT (JSPS) KAKENHI (18390002 and 18032042 to M.U., and 21870016 to S.S.), the Uehara Memorial Foundation (M.U.), and the Naito Foundation (S.S.). A.M. is an Inoue Fellow supported by the Inoue Foundation of Science. The Uesugi research group participates in the Global COE program "Integrated Material Sciences" (#B-09).

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Choi, Y., Kawazoe, Y., Murakami, K., Misawa, H., and Uesugi, M. (2003). Identification of bioactive molecules by adipogenesis profiling of organic compounds. J. Biol. Chem. *278*, 7320–7324.

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