Video Article

Identification of Small Molecule-binding Proteins in a Native Cellular Environment by Live-cell Photoaffinity Labeling

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Abstract

Identifying the molecular target(s) of small molecules is a challenging but necessary step towards understanding their mechanism of action. While several target identification methods have been developed and used to successfully elucidate the binding proteins of a variety of small molecules, these techniques have drawbacks that make them unsuitable for detecting certain types of small molecule-target interactions. In particular, non-covalent interactions that depend on native cellular conditions, such as those of membrane proteins whose structures may be perturbed upon cell lysis, are often not amenable to affinity-based target identification methods. Here, we demonstrate a method wherein a probe containing a photolabile group is used to covalently crosslink to the small molecule binding protein within the environment of the live cell, allowing the detection and isolation of the target protein without the need for maintenance of the interaction after cell lysis. This technique is a valuable tool for studying biologically interesting small molecules with unknown mechanisms, both in the context of basic biology as well as drug discovery.

Video Link

The video component of this article can be found at https://www.jove.com/video/54529/

Introduction

Bioactive small molecules fundamentally work by interacting with and altering the function of one or more "target" molecules, most commonly proteins, in the cell. In drug discovery, when an active compound is discovered through phenotypic screening, identification of the molecular target(s) of that compound is crucial, not just for understanding the mechanism of action and potential side-effects of the compound, but also for potentially discovering new biology underlying the disease model and paving the way for development of new mechanistic classes of therapeutics[1]. Although target identification is not required for a drug to be used therapeutically, in recent years there has been an increasing recognition that novel drug candidates are more likely to succeed in clinical trials, and therefore yield better returns on investment, if a validated target is known[2]. Thus, there has been a growing interest in methods for identifying small molecule target proteins.

A classical target identification experiment typically relies on affinity purification, where the small molecule of interest is immobilized onto a resin and incubated with whole cell lysates, after which unbound proteins are washed away and the remaining proteins are eluted and identified[3]. While this technique has been used to identify the targets of many small molecules[4], it is unsuitable as a universal target ID method for several reasons. First, the target protein must retain its native conformation upon cell lysis in order to retain its ability to bind to the small molecule. This can be particularly problematic for membrane proteins, which often undergo conformational changes after being removed from their native environment, or simply aggregate and precipitate out of solution. Second, the small molecule must be chemically modified in such a way that it can be immobilized onto the resin while maintaining its ability to bind the target protein. Deep binding pockets may therefore become inaccessible to a small molecule once it is fixed to the resin. Third, the binding affinity must be sufficiently high that the interaction is maintained during the washing steps, making identification of lower affinity interactions challenging. Fourth, environmental conditions such as pH, ion concentration, or the presence of other endogenous molecules can vary spatially within the cell and are sometimes prerequisites for drug-target interactions. Thus, finding the correct conditions to allow and maintain binding outside of the cell can require a significant amount of trial and error.

Photoaffinity labeling circumvents these issues by allowing the covalent binding of a small molecule and its target within the native context of a cell. Rather than immobilizing the small molecule to a large bulky resin, the molecule is instead chemically modified to install two small functional groups: a photoactivatable moiety which allows covalent crosslinking to the target protein when irradiated with a particular wavelength of light, and a reporter group that allows the target protein to be detected and subsequently isolated. Live cells are treated with the photoaffinity probe, the probe binds and covalently crosslinks to the target protein, and the probe-protein complex is then isolated intact. The specificity of probe binding to the target is demonstrated by performing a competition experiment in parallel, where an excess of the parent compound is used to compete away binding of the probe to the target protein.

The design and synthesis of photoaffinity probes varies greatly from one small molecule to another, and will not be covered in this protocol; however, several excellent discussions on the subject have been published[5-9]. The main consideration is that the probe retains the bioactivity of the parent compound, therefore presumably binding to the same target(s). Structure-activity relationship (SAR) studies must be performed to determine which parts of the molecule can be modified without loss of bioactivity. A variety of different chemical groups have been used as photoactivatable crosslinkers, including diazirine, benzophenone, and aryl azide, which each have advantages and disadvantages[10]. Likewise, there are multiple reporter tags that have been used to isolate probe-binding proteins. Reporter groups may be functional on their own, such as the commonly used biotin or fluorescent tags, or may be precursors that require further functionalization subsequent to the photocrosslinking step, which have the advantage of being smaller and thus less likely to compromise bioactivity[11].

In this protocol, we have used a photoaffinity probe containing a diazirine photocrosslinking group, and a terminal alkyne for the attachment of a reporter group through a Cu(I)-catalyzed Azide-Alkyne Sharpless-Hüisgen cycloaddition (or click) reaction[12-15]. The SAR studies, probe design and synthesis, and results of these studies have been published elsewhere[16-18].

Protocol

NOTE: This protocol was adapted from MacKinnon and Taunton[10] for use in live cells.

1. Preparation of Cultured Cells

1. Prepare sterile 6-cm cell culture dishes for the number of samples desired (see below).
   NOTE: One dish of cells is used per treatment condition, but if more protein is required 2 or 3 dishes can be prepared per condition and combined after UV irradiation.
   1. Prepare at least 3 dishes of cells; Negative control with DMSO only (D), Probe treatment only (P), and probe + competitor (C).
   2. Optionally, prepare a 4th dish as a no UV control, to be treated with probe but not subjected to UV light.
      NOTE: If multiple competitor molecules are to be tested, such as different analogs of the small molecule of interest, prepare additional competitor plates as necessary.

2. Add HEK293T cells to culture dishes in 4 ml culture media. Use 3.5 million cells per plate.
   NOTE: The number of cells should be determined for each cell type so that cells are almost 100% confluent at the beginning of the experiment, in order to achieve the maximum protein yield. For HUVEC, use 0.3 million cells per plate. A good approximation is 1/10 of the cells in a confluent 15 cm dish. HEK293T cells should be cultured in Dulbecco's Modified Eagles Medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin.
3. Return the cells to the culture incubator (37 °C, 5% CO_2) overnight.

2. Treatment of Cells and Photocrosslinking

1. The next day, pre-aliquot the following drugs into 1.5 ml microcentrifuge tubes:
   1. For the first treatment, add 4 µl of DMSO to 2 tubes and 4 µl of 10 mM competitor (*i.e.,* the unmodified parent compound) to 1 tube.
   2. For the second treatment, add 16 µl of DMSO to 1 tube and 16 µl of 50 µM photoaffinity probe to 2 tubes.
      NOTE: The optimal concentration may be different for different probes (see discussion), but between 200-500 nM is a good starting point (here we use 200 nM). The concentration of competitor should exceed that of the probe at least 20 fold (here we use 10 µM). The final concentration of DMSO should be equal in all plates and should not exceed 0.5% (20 µl volume).

2. Bring the cell plates into the cell culture hood and add the pre-aliquoted drugs from step 2.1.1 as follows: Aspirate 1 ml of culture media, resuspending the pre-aliquoted drug in the media, and gently adding it back to the plate drop wise. Add the DMSO to the DMSO (D) plate and the probe (P) plate, and add the competitor to the competition (C) plate. Return the plates to the culture incubator for 30 min.
3. With lights in the culture hood dimmed, add the pre-aliquoted probe from step 2.1.2 to probe (P) and competition (C) plates and add the DMSO to the DMSO (D) plate, as above. Return the plates to the culture incubator for 1 hr.
4. During the incubation, prepare the following items; A tray of ice that can fit all of the plates; ice-cold lysis buffer (PBS [pH 8.5] + 1x protease inhibitor cocktail [complete EDTA-free, diluted from 50x stock solution made in water]; 200 µl/plate); microcentrifuge tubes labeled D, P, or C for each of the different treatment conditions, on ice.
5. 15 min before the end of the 1 hr incubation, set up the UV lamp (365 nm) in the cold room and turn it on to warm up the bulb.
6. After 1 hr incubation with the probe, place dishes on ice. Wash the cells gently with 5 ml ice-cold PBS (pH 7.4) to remove excess probe. Re-cover the cells with 4 ml ice-cold PBS.
7. Place the dish of cells centered 3 cm under the UV lamp on top of an ice pack to minimize heating from the lamp. Irradiate for 3 min. Remove the dish to the tray of ice and repeat for all samples.
8. After irradiation, aspirate the PBS from the cells and add 200 µl of the ice-cold PBS (pH 8.5) with protease inhibitors to each plate. Detach the cells from the plate using a rubber scraper and transfer to the pre-labeled microcentrifuge tubes on ice.
9. Add SDS to a final concentration of 0.4% (10 µl of 10% SDS into 250 µl of sample).
   CAUTION: SDS powder is hazardous. Avoid inhaling SDS powder by wearing a mask over nose and mouth.
10. Lyse the cells by sonicating the suspension for 10 pulses (output 1, duty cycle 30%) and incubate on ice for 1 min before a second round of 10 pulses.
11. If needed, remove 2 µl of suspension and check under a light microscope to ensure complete cell lysis.
12. Boil the samples on a hot plate set to 95 °C for 5 min to complete cell lysis and denature all the proteins.
13. Measure the protein concentration in each sample using a spectrophotometric detergent-compatible protein assay, such as the Dc protein assay[19], according to the manufacturer's instructions, with a spectrophotometer set to measure absorbance at 750 nm.

14. Normalize the protein concentration to 2.5 mg/ml (or if concentrations are lower than 2.5 mg/ml, normalize to the sample with the lowest protein concentration) by adding PBS pH 8.5 + 0.4% SDS as needed (for example, if samples are 5 mg/ml in 250 µl, add 250 µl of PBS pH 8.5 + 0.4% SDS to obtain a final concentration of 2.5 mg/ml).

3. Attachment of Fluorescent Tag by Click Chemistry for Visualization of Labeled Proteins

1. Remove 40 µl of the cell lysate and transfer to a new microcentrifuge tube. Keep the remaining lysates for reaction with biotin-azide (step 4).
2. Add the following reagents in order: 0.2 µl fluor-azide (1 mM stock solution in DMSO), 0.58 µl TCEP (100 mM stock in water with 4 equivalents NaOH added, prepared fresh), and 3.38 µl TBTA (1.7 mM stock in a 4:1 ratio of t-butanol to DMSO). Vortex to mix.
3. Add 1.14 µl $CuSO_4$-$5H_2O$ (50 mM in water) to start the reaction. Vortex briefly and incubate at room temperature for 30 min in the dark.
4. Add 50 µl of 2x SDS sample buffer to quench the reaction. At this point, run the samples directly on an SDS-PAGE gel[20] for best results, or store at -20 °C overnight if necessary.
5. If freezing samples, reheat for 5 min at 95 °C before loading onto the gel.
   NOTE: Since the molecular weight of the target protein is typically not known at this stage, it is advisable to run 2 gels with different acrylamide concentrations (*i.e.*, 8% and 15%), or a wide molecular weight range gradient gel (*i.e.,* 4-15%), to ensure complete molecular weight coverage.
6. When the dye front has reached the end of the gel, continue running the gel for an additional 5 min to ensure all of the excess unreacted fluor-azide has completely exited the gel.
7. Remove the gel to a container with ddH_2O and incubate for 10 min with gentle agitation, to wash away all excess fluor-azide.
8. Place the gel onto a glass plate and scan the gel using a Typhoon fluorescent gel scanner according to the manufacturer's instructions.

4. Attachment of Biotin Tag by Click Chemistry for Affinity Purification of Labeled Proteins

1. Of the remaining lysates from step 3.1, use the maximum amount after protein normalization such that all samples are the same volume (for example, if after normalization to 2.5 mg/ml the resulting sample volumes are 500, 550, and 600 µl, use 500 µl of each).
2. Pre-clear the lysates by adding to 50 µl high-capacity streptavidin agarose beads, pre-washed 2x with PBS (pH 7.4). Incubate 1 hr at 4 °C with rotation.
3. Pellet the beads by centrifugation at 1,000 x g for 3 min. Remove the supernatant to a new microcentrifuge tube on ice and discard the beads.
4. Remove 1-2% of the DMSO sample to a new tube labeled "input". Add an equivalent volume of 2x SDS sample buffer and store at -20 °C.
5. Per 500 µl of lysate, add the following reagents: 1.38 µl biotin-azide (10 mM stock in DMSO), 5.5 µl TCEP, and 32.5 µl TBTA. Vortex to mix.
6. Add 11 µl $CuSO_4$-$5H_2O$ per 500 µl of lysate and vortex briefly. Incubate at room temperature for 30 min.
7. Add 4 sample volumes of acetone chilled to −20 °C. Vortex the samples and incubate overnight at −80 °C to completely precipitate the proteins and remove unreacted biotin-azide.
8. Centrifuge the samples at 17,000 x g for 15 min at 4 °C to pellet the precipitated proteins.
9. Aspirate the supernatant completely, and resolubilize the proteins by sonication in 150 µl PBS (pH 7.4) + 1% SDS.
10. Add 600 µl of PBS (pH 7.4) to dilute the concentration of SDS to 0.2%.
11. Add the samples to 30 µl pre-washed high-capacity streptavidin agarose beads and incubate 1 hr at 4 °C with rotation.
12. Pellet the beads by centrifugation at 1,000 x g for 3 min. Aspirate the supernatant containing unbound proteins and discard.
13. Add 1 ml of wash buffer (400 mM NaCl, 50 mM Tris, 0.2% SDS, pH 7.4) to the beads and incubate 5 min at room temperature with rotation.
14. Repeat steps 4.12 and 4.13 3 times.
15. Aspirate the wash buffer completely from the beads, and add 30 µl of 2x SDS sample buffer.
16. Incubate for 5 min on a 95 °C heat block to release the proteins from the beads.
17. Centrifuge the beads for 1 min at 13,000 x g at room temperature.
    NOTE: At this point, the samples can be stored at -20 °C until ready to run SDS-PAGE. If freezing samples, reheat for 5 min at 95 °C before use.
18. Carefully pipette the sample buffer containing proteins off of the beads, and load onto SDS-PAGE[20] (see important considerations below). The beads can be saved for reboiling later if necessary.
    NOTE: For protein detection by silver stain, better results are obtained using a 1 mm thick gel. If a band is to be cut out of the silver-stained gel for mass spectrometry (MS) analysis, prepare all reagents fresh from sterile solutions and leave an empty well in between samples on the gel. For every band cut from the probe lane, a parallel slice should be taken from the DMSO lane so that background proteins can be subtracted. For validation of protein ID by western blot, it is critical to also load the input sample from step 4.4 when running the SDS-PAGE gel.
19. Follow a standard protocol for either silver staining[21] or Western blot[22] to detect the target protein(s).

Representative Results

The results shown here were obtained with a photo-affinity probe of the antifungal drug itraconazole, the use of which has been previously published[16]. These results demonstrate the use of the live-cell photoaffinity labeling technique to successfully identify a major itraconazole-binding protein as the 35 kDa membrane protein Voltage-Dependent Anion Channel 1 (VDAC1).

The above protocol was performed in HEK293T cells using the itraconazole probe for photolabeling and unmodified itraconazole as the competitor molecule. After the samples were prepared as described, they were subjected to SDS-PAGE in order to separate the proteins by molecular weight. Molecular weight marker units on the left are in kilodaltons (kDa). The first lane is the DMSO control sample (D), the second lane is the probe sample (P), and the third lane is the competition sample (C). Bands present in the DMSO only lane are considered background. In analyzing these data, note that specific binding proteins should be absent in the DMSO control sample, present in the probe sample and decreased in the competition sample. In this case, the main protein band detected only in the probe sample was a band of approximately 35 kDa (**Figures 1** and **3**), which was identified by mass spectrometry as VDAC1. The identity of this protein was confirmed in **Figure 5** by Western blot using a specific VDAC1 antibody. Taken together, these results allow us to conclude that VDAC1 is a major binding protein of itraconazole in these cells.

Figure 1 demonstrates the visualization of proteins after labeling with a fluorescent tag (from step 3 in protocol); **Figure 3** demonstrates the visualization of proteins by silver stain after labeling with biotin tag and performing affinity purification (from step 4 in protocol); and **Figure 5** demonstrates the validation of the protein ID using a specific antibody for VDAC1. **Figures 2** and **4** exemplify what happens if certain steps in the protocol are not performed correctly (noted in figure legends).

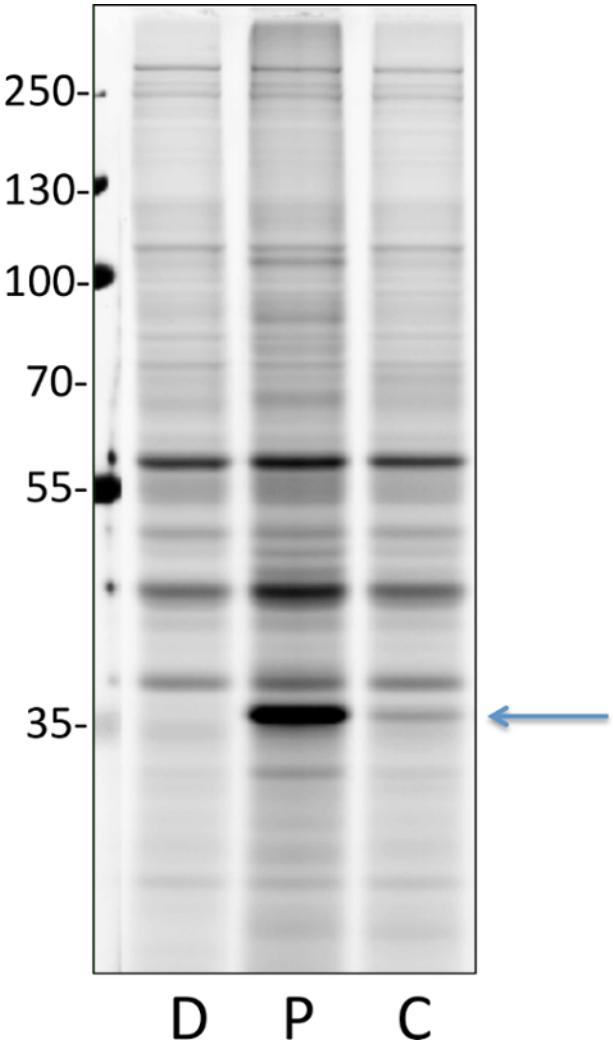

Figure 1. A representative fluorescence-scanned SDS-PAGE gel (from step 3 in protocol). D = DMSO only; P = probe only (200 nM); C = competition (10 μM). Molecular weight marker units on the left are in kilodaltons (kDa). The arrow points to the major photolabeled protein band at approximately 35 kDa that is specifically present in the probe lane and is competed away by excess parent compound, indicating that it is a specific binding protein. Please click here to view a larger version of this figure.

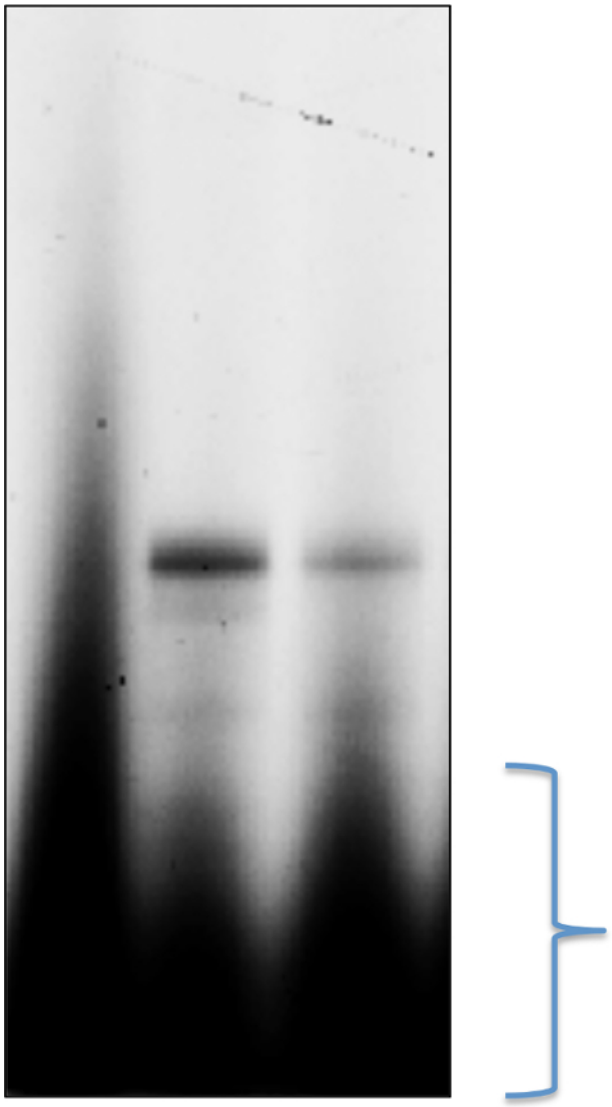

Figure 2. A fluorescence-scanned gel. The excess fluor-azide has not been completely removed from the gel (steps 3.6 and 3.7), causing large black smears to appear at the bottom of the gel. Please click here to view a larger version of this figure.

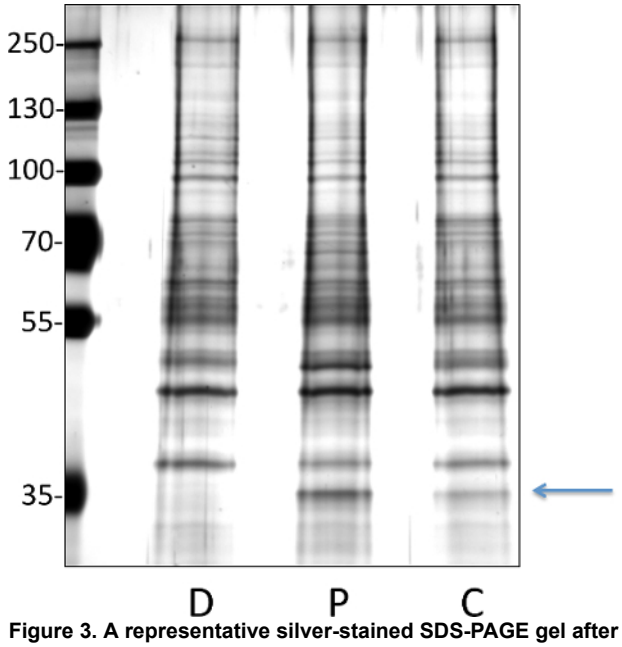

Figure 3. A representative silver-stained SDS-PAGE gel after biotin pull-down (from step 4 in protocol). The arrow points to the same band that was visualized in **Figure 1**, which was subsequently excised from the gel and subjected to MS analysis for protein identification. Marker units on the left are in kilodaltons (kDa). Note the empty space in-between each lane, in order to avoid cross-contamination of samples during gel loading and band excision. Please click here to view a larger version of this figure.

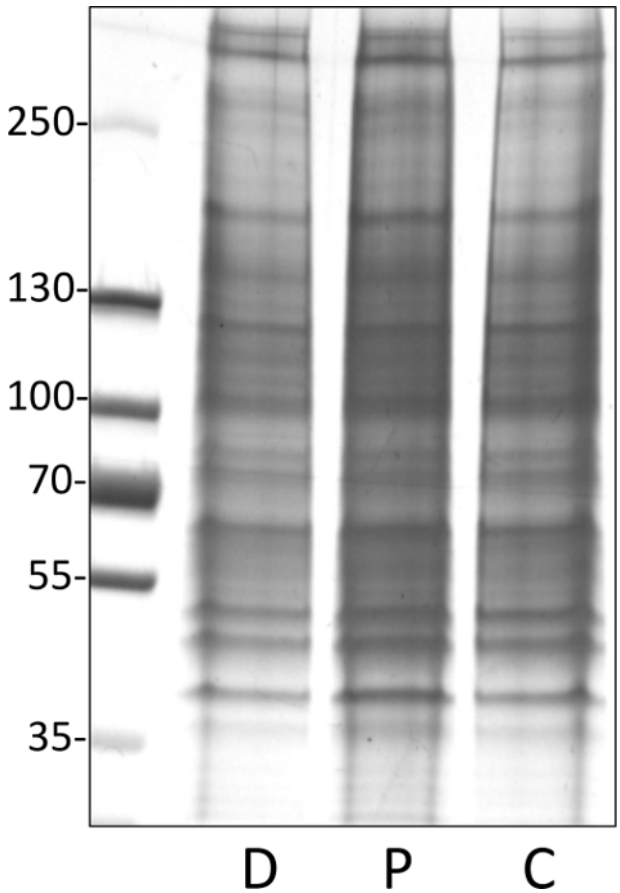

Figure 4. Silver staining. A silver-stained gel with very high background staining, due to insufficient pre-clearing of the lysates (step 4.2) and/or washing of the beads (step 4.13). Also note the lack of space between the lanes. Marker units on the left are in kilodaltons (kDa). Please click here to view a larger version of this figure.

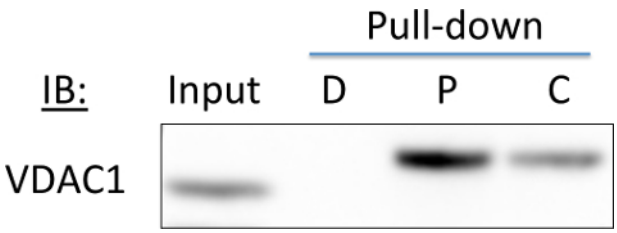

Figure 5. Western blot of the photolabeled 35-kDa protein, identified by MS as VDAC1 after biotin pull-down (from step 4 in protocol). The signal is present in the probe lane and decreased in the competition lane, as in **Figures 1** and **3**, confirming the identity of the protein as VDAC1. It is important that the input fraction (from step 4.4) is run alongside the pull-down samples, to ensure that the antibody works and the protein of interest can be detected in the lysate. A slight increase in molecular weight may be observed in the pull-down samples due to the added size of the covalently attached probe. Please click here to view a larger version of this figure.

Discussion

Different approaches to identifying the targets of small molecules can be broadly grouped into two categories: top-down, where the cellular phenotype of the drug is used to narrow down its potential targets based on their known functions, or bottom-up, where the target is identified directly by chemical or genetic means[3]. Top-down or phenotypic studies can identify certain cellular processes affected by the drug (*e.g.*, transcription/translation/DNA synthesis, cell cycle block, signaling pathway activation/inhibition, *etc.*) that might underlie the ultimate phenotype of the small molecule, which helps narrow down the list of potential targets to proteins involved in those processes. However, a major disadvantage of the top-down approach is that it relies on what is already known about these processes, and is therefore biased against targets whose functions have not been characterized or have not been previously described to be involved in a given process.

The bottom-up approach circumvents this problem by identifying the target directly in an unbiased manner. This can be accomplished by genetic means; for example, by screening an shRNA library for knockdown cell lines that confer resistance or hypersensitivity to the drug, or by isolating drug-resistant cell lines and using genomic sequencing to determine which genes contain mutations[1]. These types of experiments can identify key proteins or pathways related to the activity of the drug, but do not necessarily prove direct binding to the identified protein. Chemical approaches, on the other hand, typically involve the use of a chemical probe to bind to the target protein directly and allow its isolation and identification. Designing a probe that retains its ability to bind to the target protein requires the use of SAR studies to identify a position on the molecule that can be modified without significant loss of activity. It also requires that the probe be able to bind its target either covalently or with high enough affinity that it can be isolated from other cellular proteins (*i.e.,* affinity purified), and that these proteins retain their ability to bind the small molecule outside of their native cellular environment. This requirement can be problematic for certain targets; for example, integral membrane proteins often require a specific lipid environment to maintain their active conformation, and may become aggregated or improperly oriented for ligand binding once cells are lysed. The method described in this manuscript avoids these potential pitfalls of affinity purification by allowing the covalent modification of the target protein within the native cellular environment, so that subsequent manipulations cannot interrupt the drug-target interaction[10].

Successful implementation of the described protocol depends on several factors. It is important that the target protein of the small molecule be sufficiently abundant in the cell type chosen for photolabeling. The cell type most potently affected by the small molecule can be a good starting place, but it may be necessary to test multiple different cell types to find one with high target protein yields. Alternatively, enrichment of different protein populations by subcellular fractionation may increase the target yield while decreasing background labeling. Detection of fluorescently tagged proteins is in general much more sensitive than protein detection by silver stain; therefore, bands detected by fluorescence may not be detectible by silver staining after the biotin pull-down. Scaling up the experiment by using multiple dishes of cells per treatment condition can help to overcome this detection problem. Increasing the concentration of probe can also help to improve pull-down yields. Specific bands may also be obscured by high background, in which case more stringent washing, multiple pre-clearing steps, or the use of beads of a different composition (such as magnetic beads instead of agarose) may be helpful. However, if the target protein is too low in abundance it may not be possible to detect by silver stain. Additionally, if the target protein does not migrate as a sharp band on SDS-PAGE (such as heavily glycosylated proteins) it will also be more difficult to visualize. Further modifications to the protocol could be made to overcome these problems, such as performing whole-sample proteomics or SILAC (Stable Isotope Labeling by Amino acids in Cell culture) to identify labeled proteins after pull-down, eliminating the need to cut a band out of a silver-stained gel.

Another important consideration is the concentration of the probe to be used in the experiment. Ideally, the lowest amount of probe that produces a detectable signal should be used. Higher concentrations will give higher signal but also higher background labeling, and once all of the binding sites on the highest affinity target become saturated there may be additional lower affinity targets that begin to be detected. The optimal concentration should therefore be determined empirically by starting with a relatively high concentration (1-2 μM) and titrating back until only one or two proteins are labeled and the competition is obvious.

The potency of the probe may be taken into consideration in choosing the starting concentration; however, depending on the target abundance and mode of inhibition, there may not be a good correlation between the probe activity and target detection (for example, a probe with low nanomolar inhibition will not necessarily pull down enough protein at low nanomolar concentrations to be detectable). Thus, it is preferable to determine the most appropriate concentration empirically using detection of labeled proteins as a readout. The concentration of competitor used should exceed that of the probe by at least 20 fold, but care should also be taken not to exceed the solubility limit of the competitor.

In the case that multiple proteins are pulled down by the probe, identification of the functionally relevant protein becomes more complicated. However, there are several ways to help narrow down the potential target proteins. As mentioned above, titrating back the probe concentration can help improve the specificity of labeling. As the concentration of probe drops, higher affinity binding proteins should retain their signal intensity while lower affinity proteins should disappear. Deconvolution of multiple potential targets can also be aided by the use of chemical analogs of the parent compound with varying degrees of activity. In theory, for a functionally relevant target, there should be a correlation between binding

to the target and activity of the analog. For example, competition by inactive analogs of the parent compound can be used to rule out binding proteins that are not involved in the phenotypic effects of the compound. Likewise, active analogs, or other small molecules that induce the same phenotype as the parent compound, can be used to help rule in relevant binding proteins. Along the same lines, use of an inactive or structurally unrelated photoaffinity probe can help rule out irrelevant proteins.

Validation of the target protein first requires verification of the protein identified by MS. This can be easily accomplished by western blot following the biotin pull-down, assuming a high quality antibody for the target protein is available. However, when working with an unfamiliar protein it is easy to waste significant time and money on bad antibodies. It is therefore critical that the antibody be validated, and that it detects a specific band in the lysate of the cells being used for labeling (*i.e.,* the input sample). In addition, because the protein yield after pull-down can be low, the antibody has to be highly sensitive, and depending on the antibody high concentrations may be necessary (up to 1:100 dilution in some cases). To avoid these issues, a tagged version of the protein can be expressed in the cells before photolabeling, and the tag can then be used to detect the protein after pull-down.

Once the protein ID is confirmed, functional validation of the target can be accomplished by genetic and/or pharmacologic manipulations, or functional assays of the target of interest, to show that the activity of the target is affected by small molecule binding. The binding site can be mapped by identifying the amino acid modified by the probe, or determining the 3-dimensional structure of the bound small molecule by X-ray crystallography or NMR spectroscopy. Ideally, if a mutant can be found that loses its ability to bind the small molecule, it should be able to abolish the activity of the small molecule when expressed in place of the wild-type protein. It may also be desirable to show specificity of the binding to the target over other structurally related or "off-target" proteins.

This general protocol can be used in any application requiring direct measurement of small molecule-binding to a protein target, as long as a probe can be made that retains the bioactivity of the parent compound. Potential applications include, but are not limited to, identifying molecular targets of new compounds, surveying potential off-targets of existing drugs, confirming the direct binding of a small molecule to a particular protein of interest, determining competition of binding by other small molecules at the same binding site on a protein, and discovering new receptors of naturally derived or endogenous ligands.

Disclosures

The authors have nothing to disclose.

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