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Los Angeles

Small Molecule Target Identification using
Drug Affinity Responsive Target Stability (DARTS)

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular and Medical Pharmacology

by

Brett Eugene Lomenick

2013

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ABSTRACT OF THE DISSERTATION

Small Molecule Target Identification using Drug Affinity Responsive Target Stability (DARTS)

by

Brett Eugene Lomenick

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2013

Professor Jing Huang, Chair

Small molecule target identification is a monumental task. Knowledge of the binding interactions between small molecule compounds and cellular macromolecules such as proteins is crucial for understanding their biological properties and mechanisms of action, yet discovering these interactions remains a considerable challenge. To overcome the limitations of current target ID methods, we developed a novel technique that can identify direct binding interactions using the native, unmodified compounds. This method, called drug affinity responsive target stability (DARTS), takes advantage of the stabilization of protein structure upon drug binding that provides the target protein with resistance to protease degradation. When a small molecule is incubated with protein lysates, it binds and protects target proteins from proteolysis while degradation of non-target proteins is unaffected. Using proteomics, target proteins are then

identified based on their enrichment in the compound-treated sample versus a control sample post-proteolysis. After demonstration of DARTS using a variety of known drug-protein pairs, DARTS was used to discover that the protein translation initiation machinery is a novel target of resveratrol. Then, to improve the sensitivity of DARTS, it was combined with a powerful gel-free fractionation technique and mass spectrometry analysis, which allowed for the identification of several potential new targets for the anti-cancer marine natural product didemnin B (DB). The TCP1 chaperonin complex (TRiC) was validated to be a target of DB using both biochemical and cell-based assays. Importantly, this is the first non ATP-mimetic compound reported to bind and inhibit TRiC, and could help design more specific and potent inhibitors of this complex. Finally, we demonstrated that DARTS can also be used for mapping the binding domains of small molecules within their target proteins. DARTS provided the first experimental evidence that DB binds both domains 1 and 2 of the eukaryotic elongation factor-1 alpha and verified the binding of pateamine A and hippuristanol to the N-terminal and C-terminal domains of the eukaryotic translation initiation factor 4a, respectively. Collectively, the results presented in this dissertation establish DARTS as a powerful technique for identifying and studying protein-small molecule interactions that will have a profound impact on the chemical biology field.

The dissertation of Brett Eugene Lomenick is approved.

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To my family for their infinite inspiration and support throughout life

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CHAPTER 1

INTRODUCTION

Modern medicine and pharmacology are dependent on a mechanistic understanding of drug action at the cellular and molecular level. Central to this is the knowledge of which macromolecules are acted upon in the cell by each small molecule compound used therapeutically or for experimental purposes. Moreover, naturally occurring metabolites also interact with a plethora of cellular macromolecules, which is vital for their physiological functions. The majority of the relevant interactions that bioactive small molecules have with cellular components are with proteins (1). Despite the fundamental importance of protein-small molecule interactions and the existence of many methodologies to discover them, identification of the binding targets of small molecules remains a daunting task (2).

SMALL MOLECULE TARGET IDENTIFICATION

Methodology for the identification of the binding targets of small molecule compounds falls into two general categories: affinity-based and affinity-free methods (3). Affinity-based methods include all those that directly identify proteins based solely on their binding to the small molecule. On the other hand, affinity-free methods do not directly identify bound proteins but identify candidate targets and pathways based on indirect cellular or biochemical signatures produced by the small molecule (4).

Affinity-based Target ID Approaches

The oldest and most commonly used target identification method is affinity chromatography (Figure 1.1). In this approach the small molecule is attached to a solid support either directly (e.g. Affi-Gel agarose beads) or indirectly (e.g. biotinylated small molecule and streptavidin-agarose beads) and incubated with cell or tissue lysates to pull down the specific binding proteins (4). Most often the bound proteins are then analyzed by SDS-PAGE and gel staining, followed by identification using mass spectrometry. As most matrices and small molecules may pull down many non-specific protein binders or proteins not involved in the compound's mechanism of action, a structurally related but inactive analog of the small molecule is often used simultaneously as a negative control. Additionally, competitive binding or elution with an excess of the active compound versus an inactive analog can also be performed to determine specific and non-specific binders.

The large number of non-specific binders encountered in many affinity chromatography experiments confounds identification of the true targets of the small molecules. To reduce this effect, extensive washing with stringent buffers is often required. However, weaker interactions often will not survive such treatment and will be lost. A recent advance in target identification combines affinity chromatography with SILAC mass spectrometry (5). Using light versus heavy labeled cell lysates, affinity chromatography is performed with the immobilized active compound versus an immobilized inactive analog (6). Alternatively, competitive binding or elution could be performed with free active versus inactive compounds using a single immobilized compound. The proteins pulled down from each sample are then combined and analyzed directly by LC-MS/MS using the isotope ratios for relative quantification. Proteins specifically bound by the active compound will show enrichment in that sample over the control,

whereas all the non-specific binders will be present in equal amounts. Since visualization of enriched target proteins on an SDS-PAGE gel is unnecessary, SILAC allows for easier identification of specific versus non-specific binders. This also reduces the need for extensive washing and may permit identification of weaker interacting proteins.

Although affinity chromatography has a long history of successfully identifying the targets of hundreds of small molecules, it is often tedious and technically complicated. First, it requires that the small molecule has at least one derivatizable functionality which, when modified, does not significantly alter the binding affinity and specificity to its target protein(s). Additionally, linkage of the small molecule to the matrix must not prevent binding to the target protein(s) via steric hindrance. While ascertaining whether modification of functional groups affects binding can often be foretold from structure-activity relationship studies using bioactivity as an indirect predictor of binding, the latter requirement cannot be predicted at all. The necessity of modifying each small molecule for attachment to a solid support has prevented the identification of untold numbers of small molecule targets by affinity chromatography.

A number of similar affinity-based target ID methods exist that utilize either covalent or non-covalent binding to the target protein for identification. The one commonality of these methods is that they all require derivatization of the small molecule, which is inherently limited as described above. The least damaging type of modification that can be made to a small molecule, however, is the addition of a radiolabel. This simply involves replacing a hydrogen or other unnecessary functional group (as determined by SAR) with a tritium or other radioactive isotope such as I-125. Such limited modifications are unlikely to cause significant disruption to target protein binding. Upon binding to its target protein, the presence of the radiolabel can then be used as a tracer to follow throughout a protein purification process to isolate and identify the

target (7). One potential limitation of this approach is that the binding affinity and specificity need to be fairly high. For a compound with multiple targets isolation of each becomes even more tedious and technically challenging. Furthermore, the affinity to the target protein must be strong enough such that a significant portion of the radiolabeled small molecule remains bound to its target protein throughout the purification process, which often lasts for several days.

One powerful approach to overcome the latter limitation is to engineer the small molecule to covalently attach to its target protein upon binding. While some compounds themselves already covalently attach to their targets, a variety of small reactive functional groups can be incorporated into the compound for this purpose (8). Most notably, photoaffinity and activity-based probes will become covalently linked to the target protein upon UV light exposure or by the activity of the enzyme active site, respectively (9-11). An advantage of these probes is that target protein labeling can be performed both *in vitro* and in live cells, with subsequent purification then performed after cell lysis (12). In addition to using these compounds with protein purification schemes, they can also have a second functional group incorporated for attachment of an affinity handle (e.g. biotin) for affinity chromatography (13). Although this approach improves the ability to identify multiple and weaker binding targets, they still require a considerable investment of effort for each individual small molecule, and there is no guarantee that such modifications will not alter target binding.

Affinity-free Target ID Approaches

Unlike the affinity-based methods that rely on direct binding to identify target proteins, affinity-free approaches utilize indirect cellular and biochemical signatures produced by treatment with the small molecule of interest. The primary advantage of such methods over most affinity-based methods is that the native, unmodified small molecule is used. However, most

affinity-free methods are limited to molecules with a specific mechanism of action or to those capable of generating specific cellular effects. Moreover, these methods simply identify candidate targets to which direct binding must be subsequently validated using biochemical approaches.

The classical genetics approach to target identification relies on isolation of drug-resistant mutant clones, with the goal being to identify single residue changes within the binding target protein that disrupt drug binding (14-16). Although many genetic and epigenetic changes beyond those that directly affect target protein binding may lead to drug resistance, this approach has demonstrated considerable success in yeast and prokaryotes (17-19). More recently, promising results have been obtained using human colon cancer cells as well, although the considerable number of passenger mutations hampers determination of the key resistance mutation in each clone unless candidate target proteins are already known (20-21). While powerful, selection for drug-resistant mutants can only be used for cytostatic and/or cytotoxic compounds. However, demonstration that a mutation within the target protein that disrupts drug binding *in vitro* leads to drug resistance when expressed in cells is considered the gold standard for drug target identification.

A similar strategy for target identification of cytostatic and cytotoxic compounds takes advantage of genome-wide overexpression and deletion libraries in model organisms such as *S. cerevisiae*. The premise of this approach is that modulation of the cellular level of the target protein will affect the cells' sensitivity to the drug (14). Overexpression should lead to drug resistance as a higher amount of drug should be needed to inhibit the target protein's function to the same extent as in wildtype cells, whereas deletion of one genomic copy of the target should reduce that protein's cellular level and make the cells more sensitive to the drug (22-23). In *S.*

cerevisiae, this method has been improved by genetic barcoding of each deletion and overexpression strain such that the entire library can be grown together in one culture rather than using dozens of multi-well plates (19, 24-26). The drug-treated pool is then analyzed by microarray to quantify each strain's sensitivity or resistance to the compound. This method also reduces error rate caused by differences in seeding, growth conditions, and other unintended well-to-well and plate-to-plate variability.

Similarly to the classical genetic method, overexpression and deletion of many genes in addition to the direct binding target can cause increased resistance or sensitivity to a given drug through a variety of mechanisms (27-28). However, beyond those genes that function generally in the cellular stress response, many of the identified genes will encode for proteins that function in the same pathway or cellular process as the target protein, thus providing valuable information relevant to the drug's mechanism of action. Once candidate targets are identified, the proteins can be tested for direct binding to the drug. Genome-wide overexpression and RNAi knockdown can be similarly performed in mammalian cells for drug mechanism of action studies, although its usefulness for target identification is not yet established (29-30). One caveat to this global fitness profiling method is the potentially large number of genes that may influence sensitivity to a given drug, as well as the fact that the direct target of the drug may not be among the top candidate genes identified or may not affect drug sensitivity at all (26). Moreover, acute inhibition or allosteric modulation of a protein's activity via a small molecule can have dramatically different effects than changing its expression level or knocking it out completely (31). Often, data generated from multiple such methods is necessary to further hone in on the most likely direct targets, which still must be validated biochemically for direct binding (26).

Just as overexpression or knockdown/deletion of specific genes will alter the cellular sensitivity to growth inhibition by a given compound, human cancer cell lines naturally have differential sensitivity to treatment with a specific drug due to their varied origins and genetic makeup. Moreover, this differential sensitivity or resistance to drug treatment between cancer cell lines is drug dependent, meaning a cell line that is more sensitive than average to one drug may be more resistant than average to another drug with a different mechanism of action. Therefore, it has been proposed that a drug's sensitivity/resistance profile to a panel of cancer cell lines will be similar to the profile of another drug with the same target. Although this idea has been explored thoroughly with the NCI-60 panel of cancer cell lines, with some success, it is limited because of the necessity to have already profiled the panel with additional drugs with the same target or similar mechanism of action (32-37).

Among the non-fitness based approaches to help elucidate drug targets, perhaps the most commonly used is genome-wide expression profiling using DNA microarrays. Acute inhibition of a given protein is expected to produce a specific gene expression signature, which may overlap extensively with the signatures produced by inhibition of proteins from the same complex or that function in the same pathway or process (38-39). Moreover, inhibition of the same protein with a different drug or via RNAi knockdown of the transcript encoding the protein may also produce a very similar expression signature. Therefore, comparison of the gene expression signature of a drug with those produced by treatment with other drugs with known targets as well as by genome-wide RNAi studies should yield candidate protein targets for the drug of interest. The popularity and success of the expression profiling approach is evident by the creation of the Connectivity Map, an online repository of expression profiles for thousands of RNAi and drug treatments to which any novel compound can be compared (40-41).

Beyond expression profiling, a limited number of other non-fitness drug target identification methods have been proposed. One method compares the similarity in the side effect profiles of drugs to determine those that likely share at least one major target or off-target (42). Although this approach successfully identified new targets for several clinically used drugs, it is limited to those compounds for which extensive human usage data exists. Moreover, the method is also of no use for drugs with novel targets since it is dependent on the existence of prior clinically used drugs with the same target. However, this method may be most useful for identifying unintended off-targets that are shared with other drugs. Such off-targets may be responsible for side effects, and their determination may allow for the design or screening of suitable derivatives with reduced binding to the off-target(s) while retaining potency for its intended target(s). Moreover, identification of off-targets could also be useful for drug repurposing if the off-targets are the desired targets for other indications.

High-throughput microscopic profiling and automated image analysis of drug-induced cellular phenotypes has also been proposed as a method for drug target identification. This sort of profiling has allowed for grouping of drugs of similar mechanism and targets, and in a small number of cases helped propose novel targets for uncharacterized or semi-characterized compounds, using both high-throughput immunofluorescence or live cell imaging (43-47). Other computational strategies for drug target identification utilize docking, ligand chemical similarity, and/or protein (drug binding pocket) structural similarity to propose new targets for compounds (48-60). While many new protein-ligand interactions have been discovered using these varying methods, almost all were dependent on previously known profiles for drugs with the same target(s) or known interactions for the drug of interest or similar compounds. Given that currently there are only highly selective and potent inhibitors of a small fraction of the

proteome, uncovering novel protein-ligand interactions for the remainder of proteins using such approaches is unlikely. Therefore, despite the numerous drug target identification methods already available, better universal methods that will work for any drug regardless of structure, mechanism of action, or biological effects are sorely needed.

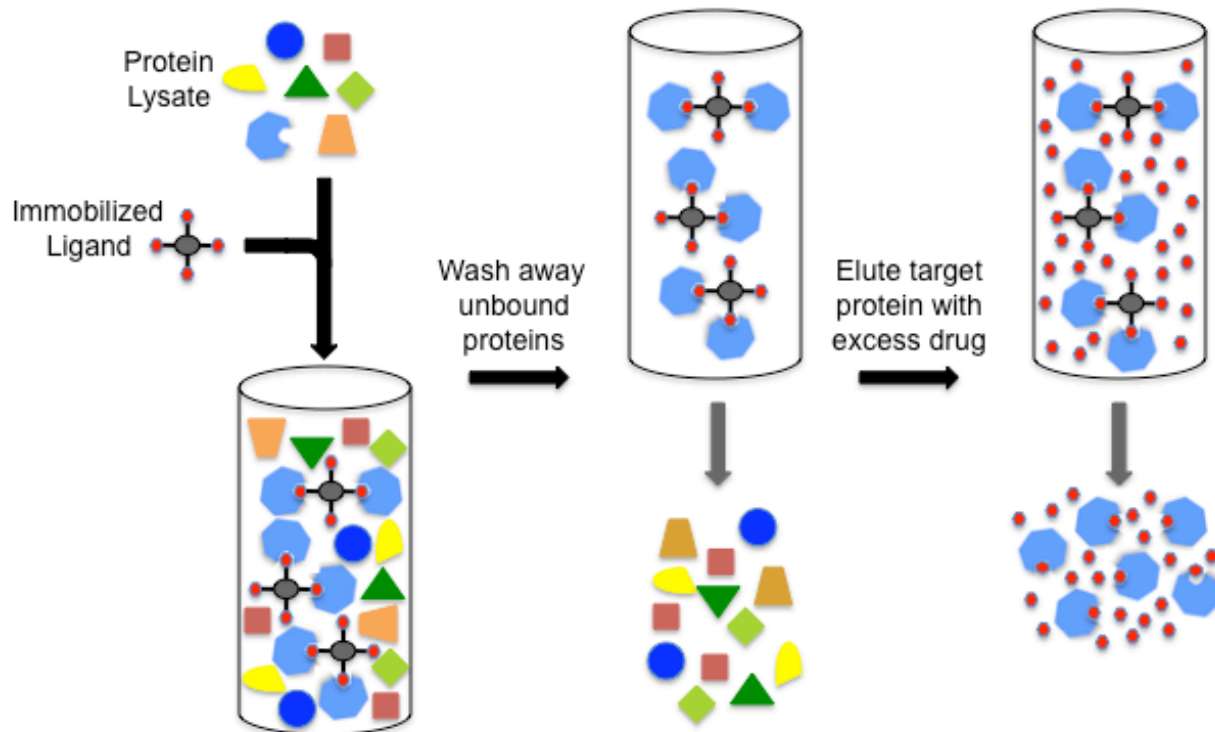


Figure 1.1. Drug target identification by affinity chromatography. A small molecule ligand immobilized to a solid support (e.g. sepharose beads) is incubated with protein lysate to bind target proteins. Subsequently, the beads are washed several times to remove all non-specific and weakly bound proteins, and the tightly bound target proteins are eluted for identification.

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CHAPTER 2

Small Molecule Target Identification using Drug Affinity Responsive Target Stability (DARTS)

Abstract

Identifying the molecular targets for the beneficial and/or detrimental effects of small molecule drugs is an important and currently unmet challenge. We have developed a method, drug affinity responsive target stability (DARTS), which takes advantage of a reduction in the protease susceptibility of the target protein upon drug binding. DARTS is universally applicable because it requires no modification of the drug and is independent of the mechanism of drug action. We demonstrate use of DARTS to identify known small molecule–protein interactions and to reveal the eukaryotic translation initiation machinery as a molecular target for the anti-aging plant natural product resveratrol. We envisage that DARTS will also be useful in global mapping of protein-metabolite interaction networks and in label-free screening of unlimited varieties of compounds for development as molecular probes and imaging agents.

Introduction

Development of effective and safe therapies is the holy grail of medicine. For small-molecule drugs, which comprise most of today's medicines, a key challenge remains the identification of the molecular targets underlying drug therapeutic effects and/or adverse side effects. For small molecules discovered in phenotypic screens, which are increasingly popular in

chemical genetics studies, identifying the biological (and potential therapeutic) targets, along with the off-targets, is a largely ad hoc affair; a systematic, widely applicable and robust approach is badly needed (1,2). To overcome the limitations of current methodologies, we sought to develop a simple, universally applicable target identification approach that analyzes direct drug binding to targets. Given that a protein might become less susceptible to proteolysis when it is drug-bound than when it is drug-free (3,4), we hypothesized that this phenomenon could be exploited for target identification. This would allow the protein target of a drug to be revealed, without requiring modification or immobilization of the small molecule. Because our method, termed DARTS (drug affinity responsive target stability), is not limited by synthetic chemistry and is independent of any biological effects of the drug (save its binding to the target protein), it can potentially be used to identify the target for any small molecule.

Materials and Methods

Reagents. Recombinant human FKBP12 was purchased from R&D Systems (Cat. #3777-FK). The recombinant human FRAP1 (mTOR) fragment corresponding to amino acids 1360-2549 (163.9 kDa, ~70% purity) was obtained from Invitrogen (#PV4753). The proteases subtilisin Carlsberg (#P5380) and thermolysin (#88303) were purchased from Sigma. Stock solutions of each protease were made, aliquoted, and stored at -20°C. New aliquots were used for each proteolysis experiment. For Western blotting, anti-EF1A1 (Abcam #37969), anti-GAPDH (Ambion #4300), and anti-FLAG (Sigma) antibodies and the streptavidin-horseradish peroxidase conjugate (Streptavidin-HRP) from the Transcend Chemiluminescent Non-Radioactive Translation Detection System (Promega) were used. Coomassie and silver staining were

performed using SimplyBlue and the SilverQuest staining kit from Invitrogen. Protein concentration was measured using the BCA protein assay kit (Pierce).

Plasmids. The bicistronic translation reporter construct pcDNA/REN/HCV/FF was described previously (5). pcDNA/FF/EMCV/REN was generated by subcloning the XhoI-BamHI fragment from pKS/FF/EMCV/REN 6 into pcDNA3.1(-) using the same restriction sites. For IVT, pcDNA3.1-hTOR1968C was generated by PCR cloning of mTOR coding region 5902-7650 (corresponding to hTOR aa 1968-2549) into pcDNA3.1 using the EcoRI and KpnI sites; the PCR template was a cDNA mixture that was synthesized by reverse transcription of total RNA isolated from HEK293 cells. pcDNA3.1-hTOR1968C/S2035T was generated similarly except using pBJ5-FLAG-FRAP/S2035T 7 as PCR template. pcDNA3.1-FLAG-hTOR was generated by ligating a NotI-KpnI fragment (containing FLAG + mTOR coding region 1-5901) from pBJ5-FLAG-FRAP•S2035T 7 and a KpnI fragment with PCR'ed hTOR coding region 5902-7650 into pcDNA3.1 using the NotI and KpnI sites. All constructs were verified by DNA sequencing.

DARTS with pure proteins. For Figure 2.2, 40 ng/ μ L (~3 μ M) recombinant FKBP12 was incubated with rapamycin, FK506, wortmannin (100 μ M each), or DMSO solvent control for 2 hr at 4°C, followed by digestion with subtilisin at room temperature. Proteolysis 1, 1:100 (wt:wt) subtilisin:FKBP12 for 3 hours; Proteolysis 2, 1:10 (wt:wt) subtilisin:FKBP12 for 30 min. For Figure 2.3, 200 ng of recombinant FRAP1 (mTOR) was incubated with E4 or DMSO solvent control for 30 min at 4°C, followed by digestion with 20 ng thermolysin for 1 hr at room temperature.

DARTS with complex protein mixtures. For Figures 2.4A and 2.5A, intact Jurkat cells were treated with DB from 100 pg/mL to 1 µg/mL or DMSO control for 30 min. Cells were lysed (without washing, in these experiments) with M-PER (Pierce) supplemented with protease and phosphatase inhibitors (1 mM β-glycerophosphate, 2.5 mM Sodium Pyrophosphate, 1 mM Sodium Vanadate, and 2 mM Sodium Fluoride). After clarifying by centrifugation (14,000 rpm, 15 min), lysates were diluted to the same final volume and protein concentration with M-PER and proteolysed in reaction buffer [50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM CaCl₂]. All steps were performed on ice or at 4°C to help prevent premature protein degradation. Each sample was then quickly warmed to room temperature and proteolysed with 1 µg thermolysin for every 15 µg lysate for 10 min. To stop proteolysis, 0.5 M EDTA (pH 8.0) was added to each sample at a 1:10 ratio, mixed well, and placed on ice. The experiment in Figure 2.5B was performed similarly except that DMSO or DB was added to the lysates of untreated Jurkat cells.

For Figure 2.6A, A549 cells were treated with indicated concentrations of rapamycin or DMSO control for 30 min and washed in PBS once before lysis in Triton X-100 lysis buffer [50 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 200 mM NaCl, 10% glycerol, 1 mM DTT, Roche protease inhibitor cocktail, and phosphatase inhibitors (10 mM Na-pyrophosphate, 50 mM NaF, and 0.1 mM orthovanadate)]. Protein concentration was determined by BCA Protein Assay kit (Pierce). Fifty-two microgram of cell lysate was used for DARTS experiment in a total of 10 µL. All steps were performed on ice or at 4°C to help prevent premature protein degradation. Each sample was then quickly warmed to room temperature and immediately proteolysed with 100 ng thermolysin for every 52 µg of lysate for the indicated time. To stop the proteolysis

reaction, 1 μ L of 0.5 M EDTA (pH 8.0) was added to each sample, mixed well, and placed on ice.

For Figure 2.6B, murine RAW264.7 macrophages were treated with LPS (200 ng/mL) for 18 hours to induce COX2 expression. Cells were washed with cold PBS, and lysed with M-PER supplemented with Roche protease inhibitor cocktail and phosphatase inhibitors. Protein concentration was determined using the Bradford Protein Assay kit (BIO-RAD). Whole cell lysates were diluted with proteolysis reaction buffer [50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM CaCl₂]. All steps were performed on ice to prevent premature protein degradation. Lysates (24 μ g) were incubated with ethyl alcohol vehicle control, or with celecoxib (Panacea Biotech Ltd., New Delhi, India) at the indicated concentrations for 2 hours at 9 °C, in triplicate. Untreated lysates diluted to the same final volume were used as controls. Samples were proteolysed with 1 μ g thermolysin for every 20 μ g lysate at 25°C for 10 min. To stop the proteolysis, samples were shifted to 4°C and EDTA was added to a final concentration of 50 mM.

For Figure 2.6C, yeast cells expressing a His-tagged F-box protein were cultured to mid-log phase (1.0×10^7 cells/mL) and treated with Smer3, an inhibitor of the SCF ubiquitin E3 ligase Met30 (8) at indicated concentrations for 45 min. Then cells were pelleted, washed once with water, and lysed in Triton-lysis buffer with FastPrep-24 (MP Biomedicals). Protein concentration of the lysate was measured using BCA Protein Assay kit (Pierce). For DARTS, 54 μ g of lysate was used in 10 μ L per reaction, and 20 ng of thermolysin was used for each reaction.

For DARTS using yeast cell lysates incubated *in vitro* with resveratrol (Figure 2.9A), *S. cerevisiae* BY4742 cells were suspended in yeast extraction buffer (40 mM HEPES/KOH pH

7.5, 350 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.1% Tween-20) supplemented with Roche complete protease inhibitor cocktail. Cell pellets were broken with glass beads for 2 X 40 s at 4°C in a FastPrep-24 (MP Biomedicals). Whole cell lysates were collected after centrifugation (1500 rpm, 10 min). Lysates were incubated with ethanol control or 1 mM resveratrol for 30 min at room temperature. Samples were then divided into two aliquots, which underwent proteolysis with thermolysin or mock proteolysis.

Mass spectrometry sample preparation and analysis. Bands from 1D SDS-PAGE gels were cut out and prepared for mass spec analysis as described previously (9,10). Briefly, each band was destained by washing twice in 200 µl 50 mM NH_4HCO_3 , 50% acetonitrile for 15 min with slow vortexing, followed by dehydrating with 100 µl 100% acetonitrile and drying by speedvac. Reduction was performed with 30 µl 10 mM DTT, 10 mM TCEP for 30 min at 56°C. The bands were then washed with 100 µl 50 mM NH_4HCO_3 , 50% acetonitrile and dehydrated with 100 µl 100% acetonitrile. Alkylation was performed with 100 µl 100 mM iodoacetamide for 30 min in the dark, followed by washing twice in 200 µl 50 mM NH_4HCO_3 , 50% acetonitrile for 2 min with slow vortexing. The bands were then dehydrated with 200 µl 100% acetonitrile and dried by speedvac.

Each band was rehydrated in 30 µl 50 mM NH_4HCO_3 pH 8.0 containing 20 ng/µl trypsin on ice for 15 min. Remaining trypsin solution was replaced with 30 µl 50 mM NH_4HCO_3 pH 8.0. In-gel tryptic digestion was then performed overnight at 37°C. Digestion was halted with 5 µl 5% aqueous TFA. Peptides were extracted by shaking the solution for 15 min, saving the solution, and replacing with 30 µl 50% acetonitrile, 0.1% TFA and shaking for 15 min twice. The extracted peptides were then concentrated to 25 µl by speedvac.

Tryptic peptides were analyzed by LC/MS/MS on a Thermo LTQ-Orbitrap mass spectrometer with an Eksigent LC pump. The peptides were loaded onto a C18 reverse-phase column at a flow rate of 3 μ l/min. Mobile phase A was 0.1% formic acid and 2% ACN in water; mobile phase B was 0.1% formic acid and 20% water in ACN. Peptides were eluted from the column at a flow rate of 220 nl/min using a linear gradient from 5% B to 50% B over 60 min, then to 95% B over 5 min, and finally keeping constant 95% B for 5 min. Spectra were acquired in data-dependent mode (using dynamic exclusion of 30 sec for fragmented peptides) with the Orbitrap used for MS scans and LTQ for MS/MS scans.

For quantitative comparison of protein and peptide abundances, MS spectra were analyzed using the differential workflow of the Rosetta Elucidator software system (Rosetta Inpharmatics) (11). Annotation was performed using PeptideTeller and ProteinTeller with a minimum probability of 0.99 and predicted error of 0.0. Protein ratio data were generated with a P-value cutoff of 0.01 for all proteins with a peptide number > 1. Intensity scaling was performed against the mean of feature signals, with 10% of outliers removed from the baseline dataset.

***In vivo* translation assays.** HEK293 cells in DMEM + 10% FBS were pre-treated with 50 μ M resveratrol for 1 hr, and transfected with bicistronic translation reporter plasmids using LipoD293 DNA in vitro transfection reagent (SignaGen). 2 hr post-transfection, cells were washed with PBS, seeded in 96-well format and treated with resveratrol or vehicle control. Reporter activity was measured 33 hr post-transfection using the Dual-Glo luciferase assay system (Promega) with an Analyst HT plate reader (Molecular Devices).

We analyzed the effect of resveratrol on different luciferase assay systems using in vitro translated firefly luciferase, and found that the effect of resveratrol on firefly luciferase reaction is kit-dependent. For example, in the Bright-Glo kit (Promega # E2610), firefly luciferase readout is significantly decreased in the presence of resveratrol, as shown by 12. On the contrary, in the Dual-Glo kit (Promega # E2940), resveratrol has little or no inhibition on firefly luciferase readout. Our data in Figure 2.10 were based on the Dual-Glo kit, in which the luciferase reaction is not affected by resveratrol. Also, there was no resveratrol added to the in vitro luciferase assay reactions; rather, resveratrol was used to treat cells and washed away before the cells were lysed for the luciferase assay.

Lifespan Analysis. N2 wild-type strains were maintained at 20°C on standard nematode growth medium (NGM) seeded with *E. coli* OP50 as described (13). *E. coli* RNAi strains were obtained from Open Biosystems; clones were verified by sequencing. To make NGM plates containing resveratrol or vehicle, 240 µl of a solution containing 2.5 mM resveratrol (or the equivalent amount of ethanol, for vehicle control), 20% DMSO, 20% ethanol, 10% phosphate buffered saline (PBS), and 50% ddH₂O were spread on top of the NGM plate, making a 50 µM final concentration of resveratrol in the plate. Resveratrol was dissolved in 95% ethanol and stored in 4°C.

Lifespan analysis was conducted with N2 worms at 20°C. Eggs were added to NGM plates containing OP50. At the L4 stage, worms were washed off and placed onto bacteria-less NGM plates for 60 min so that the worms can digest the remaining OP50 in their gut. Then worms were moved onto NGM plates containing *gfp*, *daf-16*, or *inf-1* RNAi with resveratrol or vehicle. Ampicillin (50 µg/ml) and isopropyl β-D-thiogalactopyranoside (IPTG) (1mM) were

used to select for the RNAi *E. coli* and to induce the expression of dsRNA, respectively. Initially, 100 worms were picked for each treatment. During their egg-laying days, the worms were transferred to new plates every day, and then every other fourth day thereafter. To assess the survival of the worms, the animals were prodded with a platinum wire every other day, and those that failed to respond were scored as dead. Worms that died as a result of bagging or vulva bursting, as well as those that crawled off the plate, were not included in the study.

DARTS using proteins generated by rabbit reticulocyte lysate *in vitro* transcription/translation (IVT) system. For Figure 2.11B, IVT was performed using Promega TnT T7 Quick Coupled Transcription/Translation System, with 0.5 µg of pcDNA3.1-hTOR1968C (encoding human mTOR aa 1968 to C-ter) or pcDNA3.1-hTOR1968C S2035T (encoding corresponding rapamycin-resistant mutant 14) vectors, and 2 µL of ε-biotin-Lys-tRNA (Transcend tRNA, Promega) in 50 µL reaction at 30°C for 3 hr. DARTS was performed using 5 µL of translated lysate in 10 µL total reaction volume in [50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM CaCl₂], with 1 µM of rapamycin, 50 nM of FKBP12, or 50 nM FKBP12 + 1 µM rapamycin (pre-incubated on ice for 30 min to allow complex formation), and incubated on ice for 30 min. Proteolysis was performed with 2 ng thermolysin at room temperature for 20 min, and stopped with 1 µL of 0.5 M EDTA pH 8.0.

For Figure 2.11C, N-terminal FLAG-tagged full-length human TOR protein was synthesized by IVT using 0.5 µg of pcDNA3.1-FLAG-hTOR vector in 50 µL reaction at 30°C for 3 hr (Promega TnT T7 Quick Coupled Transcription/Translation System). DARTS was performed using 6 µL of translated lysate in 10 µL total reaction volume in reaction buffer [50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM CaCl₂] with 1 µM of rapamycin or 1 µM of FK506,

or co-treatment with 50 nM of FKBP12 (pre-incubated on ice for 30 min to allow complex formation), and incubated on ice for 45 min. Proteolysis was performed with 1 ng thermolysin at room temperature for 40 min, and stopped with 1 μ L of 0.5 M EDTA pH 8.0.

Results

The basic strategy of DARTS is shown in Figure 2.1. Binding of drugs is proposed to stabilize target proteins, either globally or locally, e.g., in a specific conformation or by simply masking protease recognition sites, thereby reducing protease sensitivity of the target protein. This idea is analogous to several familiar concepts, from DNase resistance of DNA sites bound by transcription factors (15) to proteins protected from protease digestion through interacting with their natural ligands such as DNA (16) and carbohydrates (3). However, previous experiments used large (DNA) or high-affinity nanomolar hydrophilic (maltose) ligands, and both cases involve major conformational change in the host protein (17,18). It remained unclear whether protease susceptibility of the target protein would be different in the absence of large conformational changes, e.g., upon binding of small hydrophobic drugs. Another question is whether the strategy would be amenable to lower-affinity ligands, e.g., clinically used drugs, which encompass a wide range of binding affinities, and hits identified from chemical genetic screens, which typically are in the micromolar range.

As a proof-of-principle, we examined the well-studied immunophilin FKBP12 protein, which is the target for the nanomolar immunosuppressant drugs rapamycin and FK506 (Figure 2.2A) (19). Proteolysis of FKBP12 by the protease subtilisin was clearly decreased by the presence of rapamycin or FK506 (Figure 2.2B). This protection is selective: incubation with wortmannin, an unrelated drug that does not bind FKBP12, did not prevent proteolysis (Figure

2.2B), and as might be expected the drugs had no effect on subtilisin activity (Figure 2.2C). Since X-ray co-crystal structures showed that binding of FK506 or rapamycin does not cause a conformational change in FKBP12 (19), our results above suggest that drug binding alone is likely sufficient to stabilize the bound protein in the protease-resistant state. This result could be due to a direct change in the protein folding-unfolding equilibrium upon ligand binding (3) or other mechanisms.

Given that rapamycin and FK506 are among the most potent and specific drugs available, we decided to test if DARTS would work similarly with a much weaker inhibitor. E4 (Figure 2.3A) is a mid-micromolar kinase inhibitor of mTOR identified from a phenotype-based chemical genetic screen (8). Indeed, proteolysis of recombinant mTOR by thermolysin was decreased by E4 in a dose-dependent manner (Figure 2.3B), whereas it did not protect recombinant FKBP12 from proteolysis (Figure 2.3C).

The experiments above established that DARTS can efficiently test, screen, or verify drug-protein interactions when the protein is available in relatively pure form. For DARTS to be generally useful as a discovery tool, however, applicability to complex protein mixtures (such as cell lysates) would be desirable. To demonstrate feasibility, we performed DARTS using human Jurkat cells treated with didemnin B (DB), an anti-cancer marine natural product whose binding to EF-1 alpha had previously been well-characterized (20). Given that EF-1 alpha is a highly abundant protein, we first tested whether in the DARTS protocol DB would protect EF-1 alpha from proteolysis and result in a detectable difference. Indeed, DARTS revealed a strong protected band at ~50 kDa in the proteolysed extracts of DB-treated cells (Figure 2.4A), whereas no detectable difference was observed in the same samples that underwent mock digestion.

Examination of the protected band and the matching gel region of the control lane by mass spectrometry confirmed that EF-1 alpha was the primary protein present at higher abundance in the DB-treated sample (Figure 2.4B). This analysis does not exclude the possibility of other protected targets of lower abundance that were not evident by eye on the gel. DB concentration dependent proteolytic protection of EF-1 alpha was also observed by immunoblotting, both when intact cells were treated with DB (Figure 2.5A) and when the lysates of untreated cells were incubated with DB *in vitro* (Figure 2.5B). The generality of this approach is further supported by experiments using diverse protein-drug pairs ranging from nano- to micro-molar: mTOR-rapamycin, COX-2-celecoxib, and Smer3-Met30 (8) (Figure 2.6). Furthermore, DARTS is not enzyme specific, and much higher overall digestion efficiency can be achieved using other proteases while retaining protection of the target protein (Figure 2.7).

Next, we applied DARTS to identify a molecular target of resveratrol, a compound in red grapes and wine known for various health benefits including lifespan extension (21). Although resveratrol influences the activities of many proteins, no direct molecular target has been demonstrated. Low specific binding affinity as suspected from its modest size and structure (Figure 2.8A), poor potency, and potential requirement for the polyphenol groups for its activity have discouraged generation of affinity reagents for target identification. Also, even at saturating concentrations resveratrol inhibits yeast growth only very weakly if at all (Figure 2.8B), making it a poor candidate for target identification using fitness profiling strategies.

DARTS with resveratrol-dosed yeast cell lysates revealed two silver-stained bands running between the 15 and 20 kDa MW markers that were more intense in the resveratrol-treated lysate post-proteolysis as compared to vehicle control (Figure 2.9A). Mass spectrometry analysis of both bands showed that eIF4A, along with several ribosomal proteins, were enriched

in the resveratrol-treated sample (Table 2.1). This enrichment was confirmed by western blotting using the TAP-tagged (22) eIF4A yeast strain (Figure 2.9B). This finding suggests that resveratrol might directly bind to one or more proteins comprising the protein translation machinery. Potential direct binding was further supported by a target mutation analysis, where a Tif1 A64Q point mutant prevents protection from proteolysis by resveratrol (Figure 2.9B). While the alanine is conserved throughout fungi and animals, plants have a glutamine at this position (Figure 2.9C), and the bulkier side chain is hypothesized to protect plant eIF4A from resveratrol inhibition by minimizing self-binding.

It is interesting to note that multiple genome-wide studies in *S. cerevisiae* and *C. elegans* have found knockouts or knockdowns of eIF4A and several ribosomal proteins to have significant increases in lifespan (23). Our finding of resveratrol-mediated protection of eIF4A and ribosomal proteins by DARTS suggested that the protein translation machinery may be a molecular target of resveratrol in lifespan extension. To test this notion, we first asked whether resveratrol has a specific effect on protein translation. Using bicistronic dual-luciferase reporters to monitor cap-dependent translation (which requires initiation factors) and translation mediated by IRESs (which exhibit differing requirements for initiation factors), we found that cap-dependent translation and EMCV IRES-mediated translation, both of which require eIF4A, were inhibited in a dose-dependent manner by resveratrol, whereas translation from the eIF4A-independent, HCV IRES was unaffected (Figure 2.10A). These results indicate that resveratrol specifically inhibits eIF4A- or eIF4G-dependent translation initiation, and does not impinge on other translation initiation factors or on translation elongation.

Finally, we asked whether eIF4A is required for resveratrol's longevity effect. Whereas resveratrol lengthens the lifespan of wild-type worms (Figure 2.10B), as reported previously

(21), this longevity effect is lost in eIF4A knockdown worms (Figure 2.10B), consistent with eIF4A being a physiological target of resveratrol. Interestingly, the longevity effect of resveratrol appears to require daf-16 (Figure 2.10B), the Forkhead transcription factor that mediates lifespan extension by the insulin/IGF-1 pathway (24), reminiscent of its requirement for longevity in eIF4G knockdown animals (25). Taken together, it is plausible that resveratrol increases lifespan by direct inhibition of translation initiation, through binding to eIF4A and/or one or more ribosomal proteins in the pre-initiation complex. However, this interpretation should be taken with an important caveat since the eIF4A-knockdown worms show a significantly enhanced lifespan (beyond the extension produced by resveratrol in wild-type worms). Furthermore, it is possible that knocking down an initiation factor like eIF4A will affect the expression level of many other proteins that could be targets. Our findings also point to eIF4A (and potentially other translation factors) as a new druggable target for anti-aging therapy. Several potent eIF4A inhibitors have recently been identified (26), and it will be interesting to test them for potential longevity effects.

The utility of DARTS in complex mixtures suggests that potential drug targets can be identified using a wide range of biological systems, and the method is unlimited by the availability and coverage of knockout (or knockdown) libraries and genome arrays for model organisms. The limiting aspect of DARTS analysis is likely to be sensitivity of detection by mass spectrometry or other potential methods (as in affinity chromatography). This limitation is being increasingly alleviated with the development of more sensitive analytical tools. Nonetheless, we tested whether DARTS can be applied to a complementary unbiased platform, namely using proteins generated from cDNAs by *in vitro* transcription/translation (IVT) (Figure 2.11A).

Reticulocyte lysate IVT is a powerful technique routinely employed in studies of protein function, and is readily adaptable to express proteins in a high-throughput manner (27). As a test case, we used the human mTOR (mammalian target of rapamycin) protein, which is an important drug target (28,29) and had been identified on the basis of its association with the FKBP12-rapamycin complex (30). An IVT mTOR fragment containing the FKBP-rapamycin binding domain (14) was protected from thermolysin digestion by the presence of FKBP12-rapamycin, whereas the S2035T-substituted mTOR that abolishes rapamycin binding (14) was not protected (Figure 2.11B). IVT TOR proteins were labeled with biotin-Lys in this experiment, but other forms of amino acids such as S35 could also be used. Alternatively, IVT proteins could be detected through an epitope tag without incorporation of any artificially labeled amino acids. For example, FLAG-tagged full-length mTOR protein was also a robust source of protein for the DARTS method (Figure 2.11C). These results further demonstrate the versatility of DARTS and suggest nearly unlimited possibilities for screening cDNA libraries (31) and genome-wide collections of epitope-fused proteins (22,32) using DARTS to systematically analyze small molecule–protein interactions.

Discussion

Developing new methods for drug target identification is an area of intense interest, and both experimental and computational approaches have been developed (33,34). Previous methods for drug target identification have had substantial success, but many limitations remain. Traditionally, affinity chromatography has played a major role in the identification of the binding targets for many biologically active small molecules and natural products (35). In addition to affinity chromatography, many new methods for drug target identification have been developed,

ranging from biochemistry, to genetics, proteomics, and imaging (36-41). However, most of these methods suffer from either the inability to pinpoint direct binders or the necessity to modify each small molecule. DARTS therefore represents a significant breakthrough in the field.

Like affinity chromatography, DARTS relies on the affinity between a drug molecule and its protein target, and thereby is able to pinpoint direct binding partner(s) of the drug. The key advantage of DARTS, however, is that because it does not require labeled ligands and instead uses “native” (i.e., unmodified) small molecules for binding, it is not limited by chemistry and can potentially be used to identify binding targets for any small molecule. Additionally, unlike cell-based methods, DARTS is completely independent of any effects of the drug on the system, and is therefore compatible with any mechanism of action, making it useful for any small molecule of interest. Moreover, DARTS can be performed using any cell or tissue type from any organism, and is thus not limited by the availability and coverage of knockout (or knockdown) libraries and genome arrays for model organisms.

Once identified, potential drug targets can be confirmed by functional studies, and kinetics and affinities of the interactions can be measured using a variety of analytical methods. While biophysical methods (i.e., surface plasmon resonance, isothermal titration calorimetry, etc.) are traditionally used to analyze direct binding, DARTS proves to be a fast and robust method to determine direct binding of a small molecule (or metabolite) without requiring large amounts of pure protein, and is even amenable to using whole cell lysates.

Despite its many advantages, several potential limitations of the DARTS method exist. First, the binding affinity of the drug to its target may be a limiting factor. To date, our experiments suggest that DARTS is effective for molecules with inhibitory concentrations across

many orders of magnitude, up to high-micromolar. Second, a potential fundamental limitation for DARTS is that a protein's susceptibility to proteolysis is determined by its conformational energy landscape, and it has been demonstrated that a small number of evolutionarily selected proteins (e.g., stress proteins) are quite refractory to protease digestion (42). Third, drug binding may change the protease susceptibility of non-target proteins, such as those that interact with or are part of complexes containing the target. However, this result could be an advantage of DARTS as well, insofar as it would provide information about protein complexes that are dissociated (or formed) upon drug binding. Drug binding *in vivo* might also increase proteolytic susceptibility of the target protein (43). This would also help identify target proteins of the small molecule being analyzed. A small-molecule effector that destabilizes a protein *in vitro* could, of course, also be identified by DARTS by its increased degradation in the presence versus absence of compound.

An extrinsic limiting aspect of DARTS analysis is likely to be sensitivity of detection by mass spectrometry (as in affinity chromatography). While DB-mediated protection of EF-1 alpha was visualized by eye on a stained gel in Figure 2.4A, this will not necessarily be the case with many target proteins of lower abundance. Quantitative imaging or densitometry could prove useful with DARTS to assist in finding more subtle differences in protein abundance. Furthermore, proteomic techniques including 2D gels (9), DIGE (44), and gel-free approaches like MudPIT (45) would likely provide even greater sensitivity in conjunction with DARTS. Finally, the use of cDNA libraries to express proteins in cell culture or by IVT, as demonstrated in Figure 2.11, also provides a viable alternative.

While we rely on *in vitro* proteolysis using exogenous proteases for DARTS, protein stability *in vivo*, on the other hand, is a much more complicated problem. Since degradation of

proteins inside the cell is predominantly carried out by supramolecular machines known as the proteasomes and aggresomes, and is elaborately controlled by post-translational modifications such as phosphorylation and ubiquitination, protein stability *in vivo* is for the most part unpredictable. Indeed, *in vivo* stability of proteins upon drug/ligand binding is very much idiosyncratic in the literature; drug binding has been shown to both increase and decrease proteolytic susceptibility of the target protein in cells (4,43,46,47). For instance, whereas unstable FRB domain and FKBP12 mutants are stabilized by the presence of ligands (4,46) and topoisomerase-1 is destabilized by camptothecin (43), binding of estrogen receptor ligands each affects receptor stability differently (48). In any event, this information would be useful in conjunction with DARTS for elucidating the molecular mechanisms of action of drugs.

Beyond drugs, we envisage that DARTS will also be useful for global mapping of protein-metabolite interaction networks and in elucidating potential protein targets for small molecules found in food or dietary supplements. DARTS may also be useful in identifying a wide range of small molecules that can be developed into a new genre of molecular imaging agents. Pharmaceutical agents almost always interact with the active sites of enzymes or the ligand binding sites of receptors. The design of most small-molecule molecular imaging probes usually begins with modification of the structure of known drugs. However, enzyme active sites and ligand binding sites represent only a very small percentage of the tertiary structures of target proteins. Small molecules that bind tightly and specifically to sites other than the active site or the ligand binding site on a protein, and are detectable by DARTS, would provide initial hits from which probes that can stoichiometrically measure protein concentrations by fluorescence, positron emission tomography, single photon emission tomography and other molecular imaging

technologies could be developed through conventional medicinal chemistry or secondary chemical library procedures.

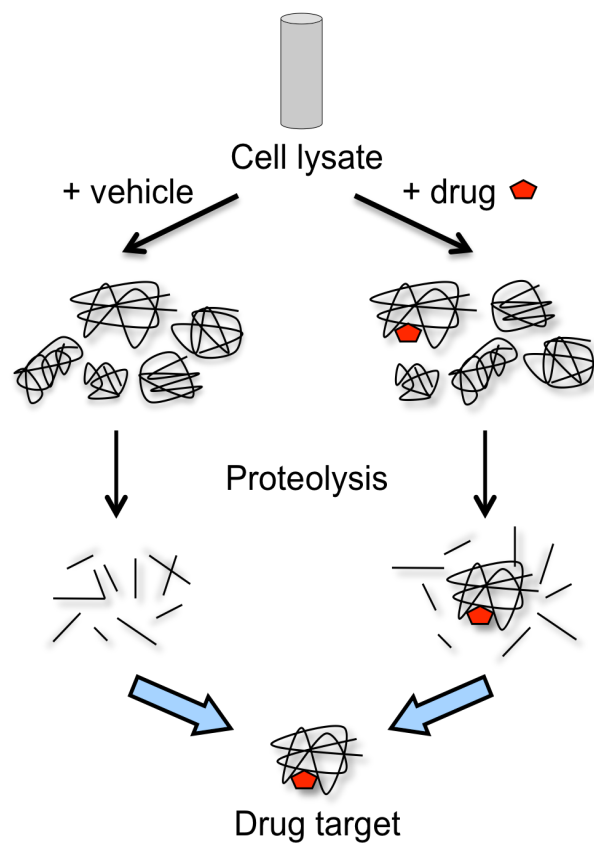


Figure 2.1. Scheme of DARTS. Cell lysates are split into two samples that are treated with the drug of interest or an equivalent amount of solvent as a control. After a short incubation to allow binding, each sample is treated with proteases to digest away unbound proteins. Proteins resistant to proteolysis in the drug-treated sample in comparison to the control sample represent putative targets.

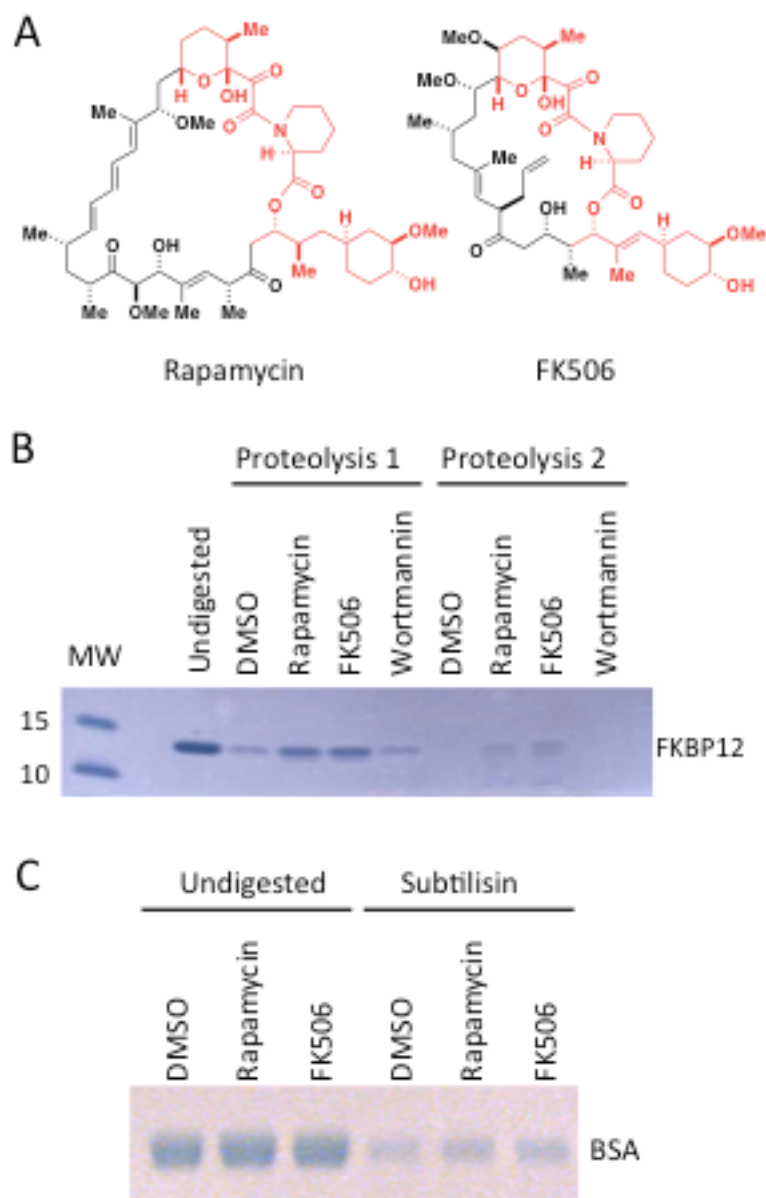


Figure 2.2. DARTS proof-of-principle using FKBP12. (A) Structures of rapamycin and FK506. (B) Recombinant human FKBP12 was incubated with the indicated drugs or DMSO and digested with subtilisin. (C) Bovine serum albumin (BSA) was incubated with the indicated drugs or DMSO and digested with subtilisin.

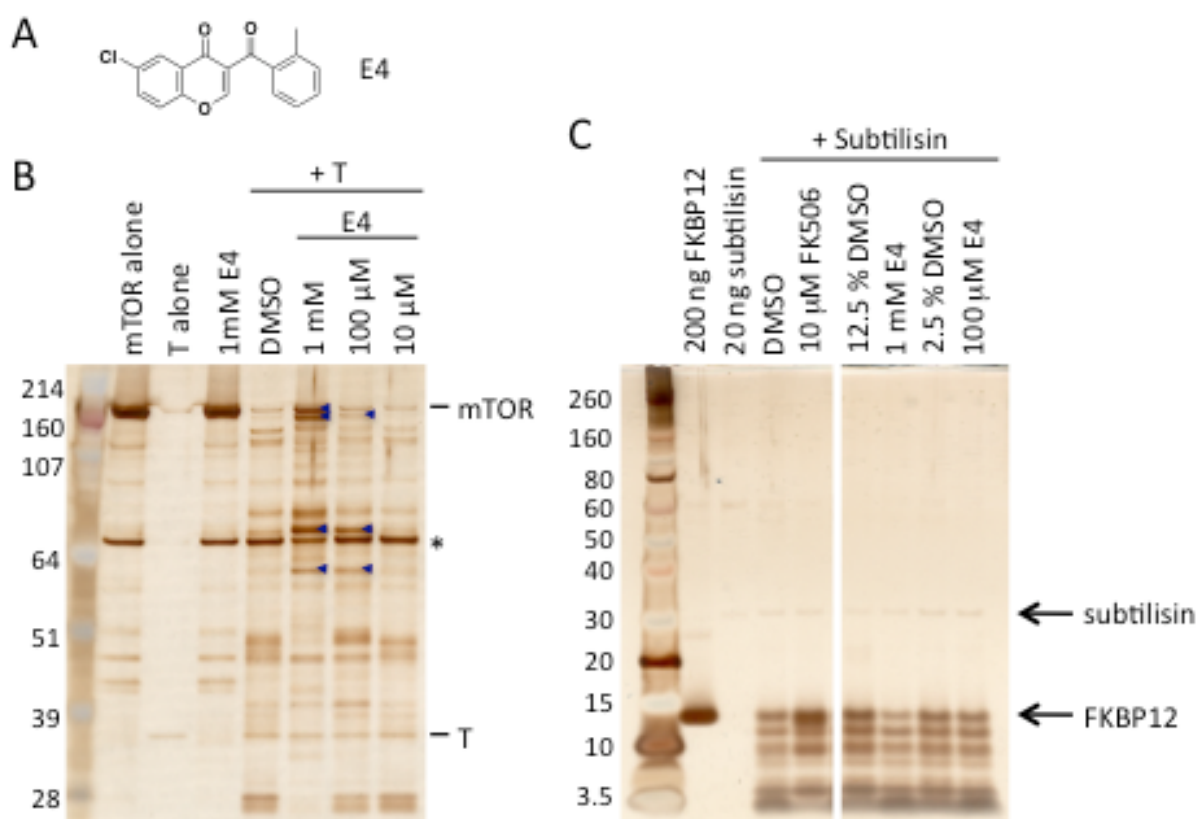


Figure 2.3. DARTS proof-of-principle using mTOR. (A) Structure of E4 (B) Recombinant human TOR was incubated with a micromolar mTOR kinase inhibitor (E4) and digested with thermolysin. Purple arrow, recombinant human TOR fragments protected from thermolysin proteolysis; *, nonspecific band; T, Thermolysin. (C) Recombinant human FKBP12 was incubated with the micromolar mTOR kinase inhibitor E4, FK506, or DMSO and digested with subtilisin.

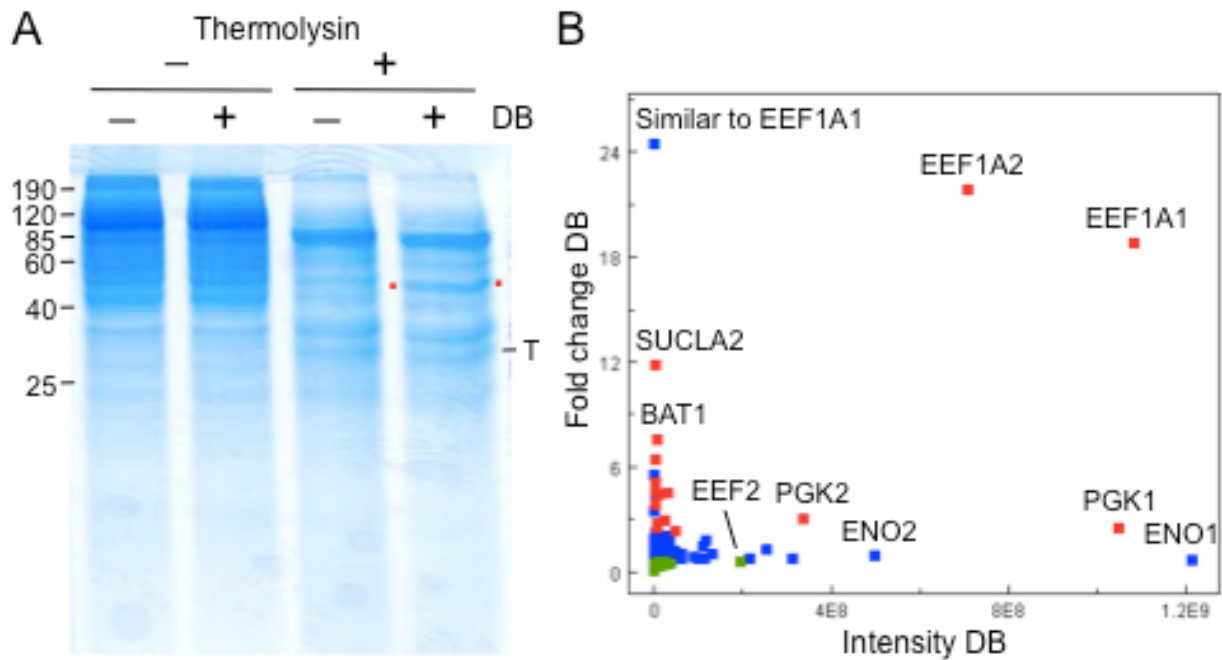


Figure 2.4. DARTS using whole cell lysate, gel staining, and mass spectrometry. (A) Intact Jurkat cells were treated with DB (1 μ g/mL) or DMSO, and lysates were subjected to thermolysin digestion and SimplyBlue staining. Red dots indicate the sole protein band protected from digestion. (B) Enrichment of EF-1 α isoforms in the protected band from A revealed by mass spectrometry analysis. Red, protein enriched >2-fold with P value <0.001; green, protein depleted >2-fold with P value <0.001; blue, unchanged protein.

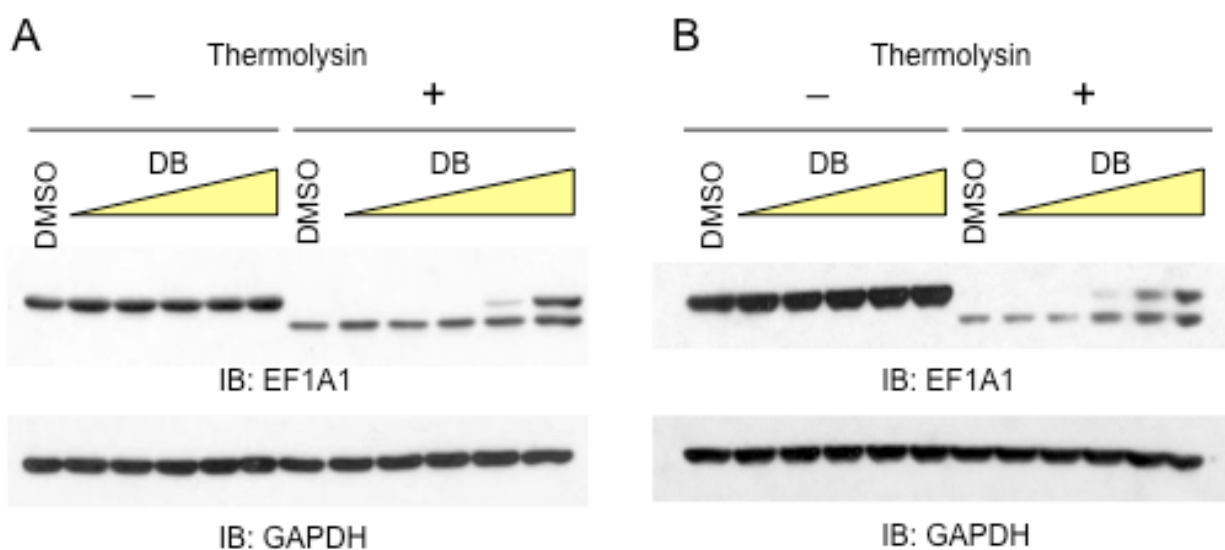


Figure 2.5. DARTS using whole cell lysate and western blotting. (A) Intact Jurkat cells were treated with DMSO or DB (0.1, 1, 10, 100, or 1000 ng/mL), and lysates were subjected to thermolysin digestion and western blotting. (B) Jurkat cell lysates were incubated with DMSO or DB (1, 10, 100, 300, or 1000 ng/mL) and subjected to thermolysin digestion and western blotting.

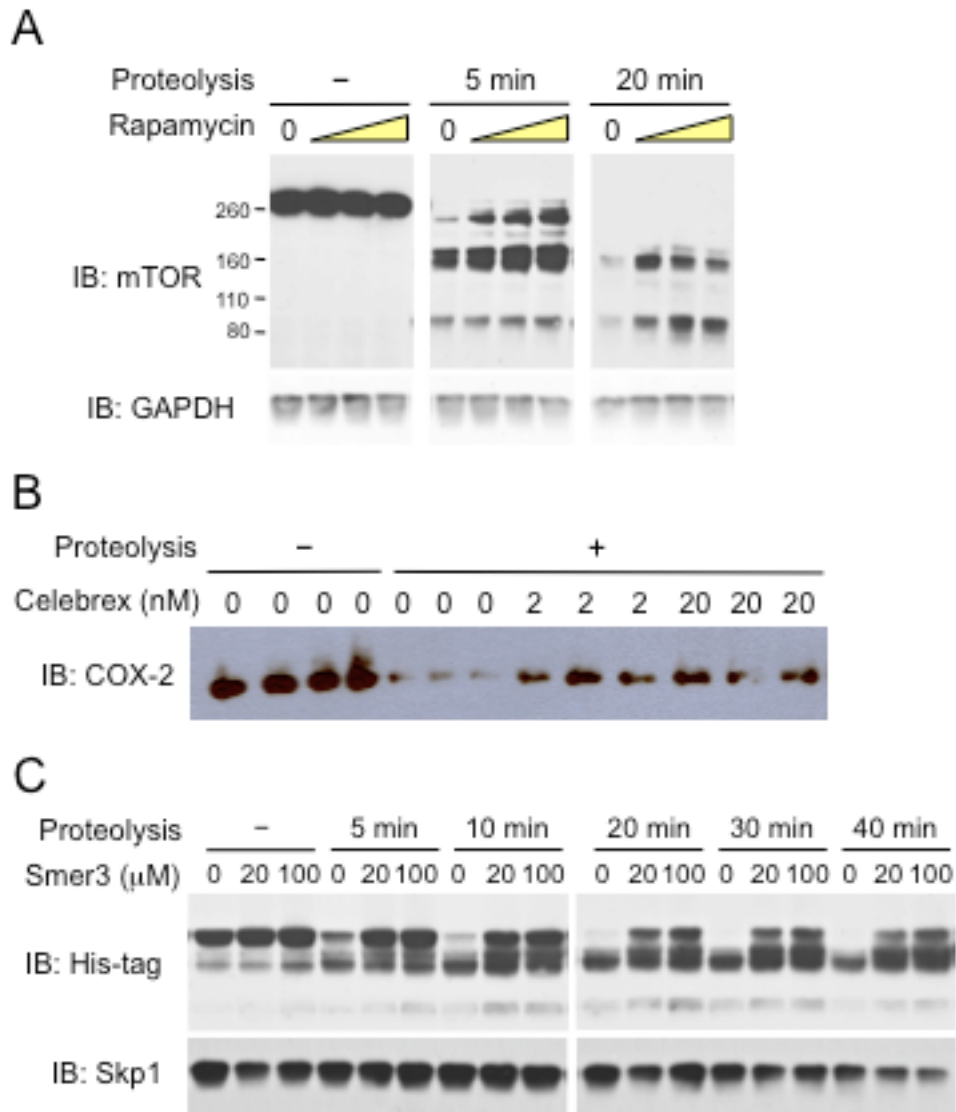


Figure 2.6. DARTS using whole cell lysates and additional compounds. (A) A549 cells were treated with DMSO or rapamycin (10, 100, or 1000 ng/mL), and the lysates were subjected to thermolysin digestion. (B) Raw264.7 cells were activated with LPS, and lysates were incubated with ethanol or Celebrex followed by digestion with thermolysin. (C) Yeast cells expressing His-tagged Met30 were treated with DMSO or Smer3, and the lysates were subjected to thermolysin digestion.

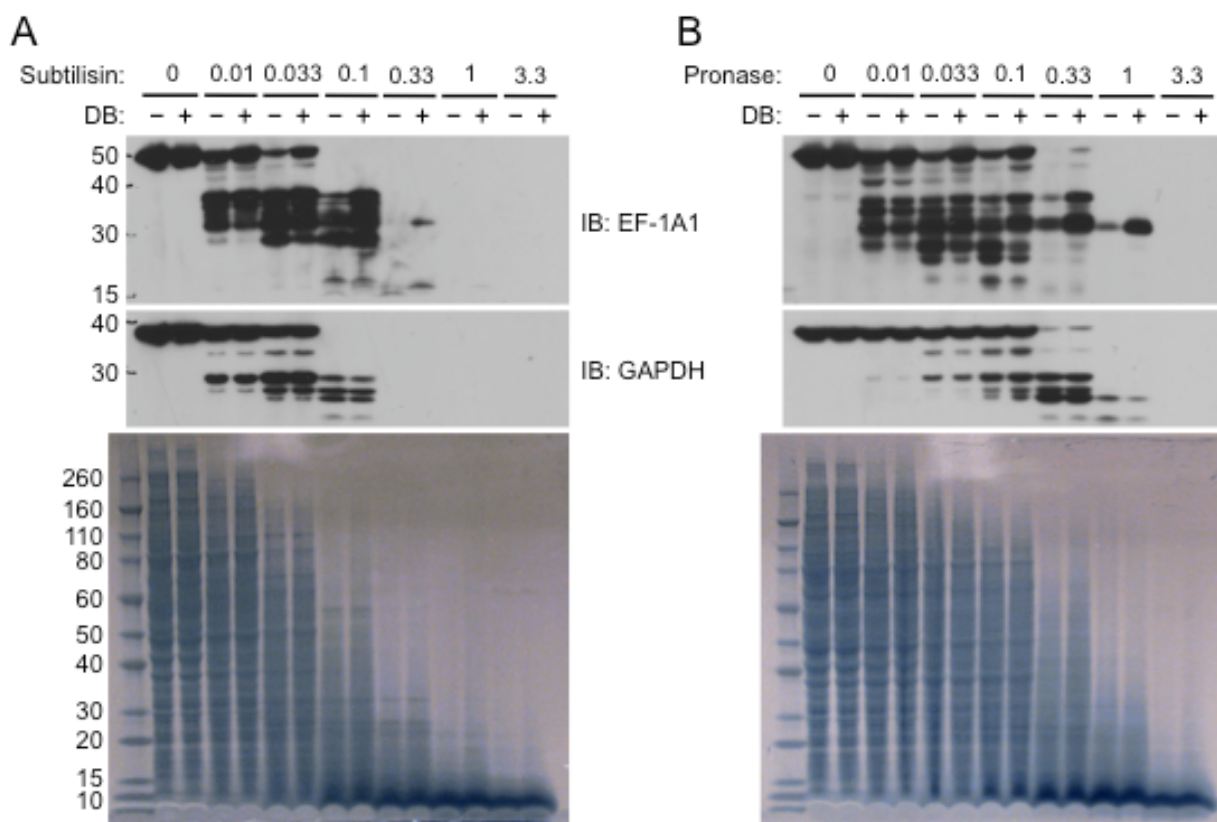


Figure 2.7. DARTS using subtilisin and Pronase. (A) Jurkat cell lysates were incubated with DMSO or 1 μ g/mL DB, digested with varying amounts of subtilisin, and analyzed by western blotting and SimplyBlue staining. (B) Experiment performed as in A except digestion was performed with Pronase instead of subtilisin.

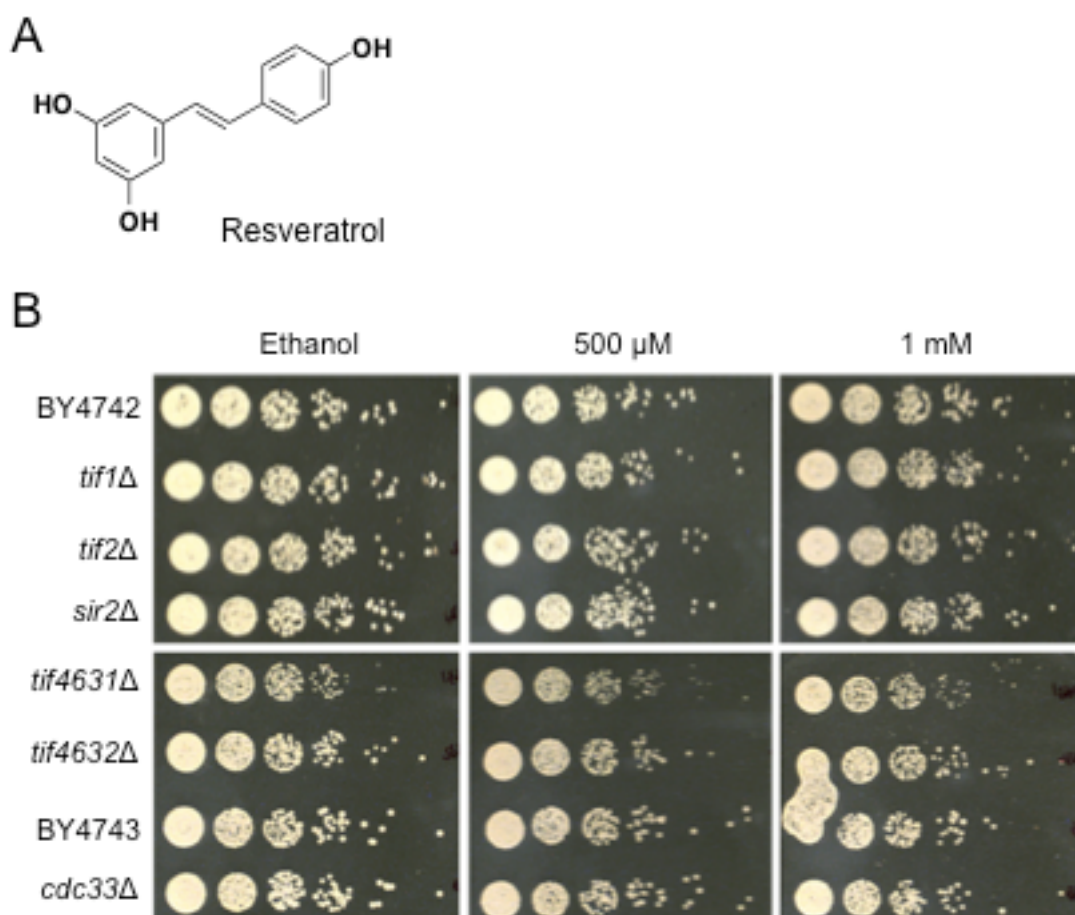


Figure 2.8. Resveratrol does not inhibit yeast growth. (A) Structure of resveratrol. (B) Resveratrol shows no detectable growth inhibition of wildtype or selected deletion strains of haploid or diploid *S. cerevisiae*.

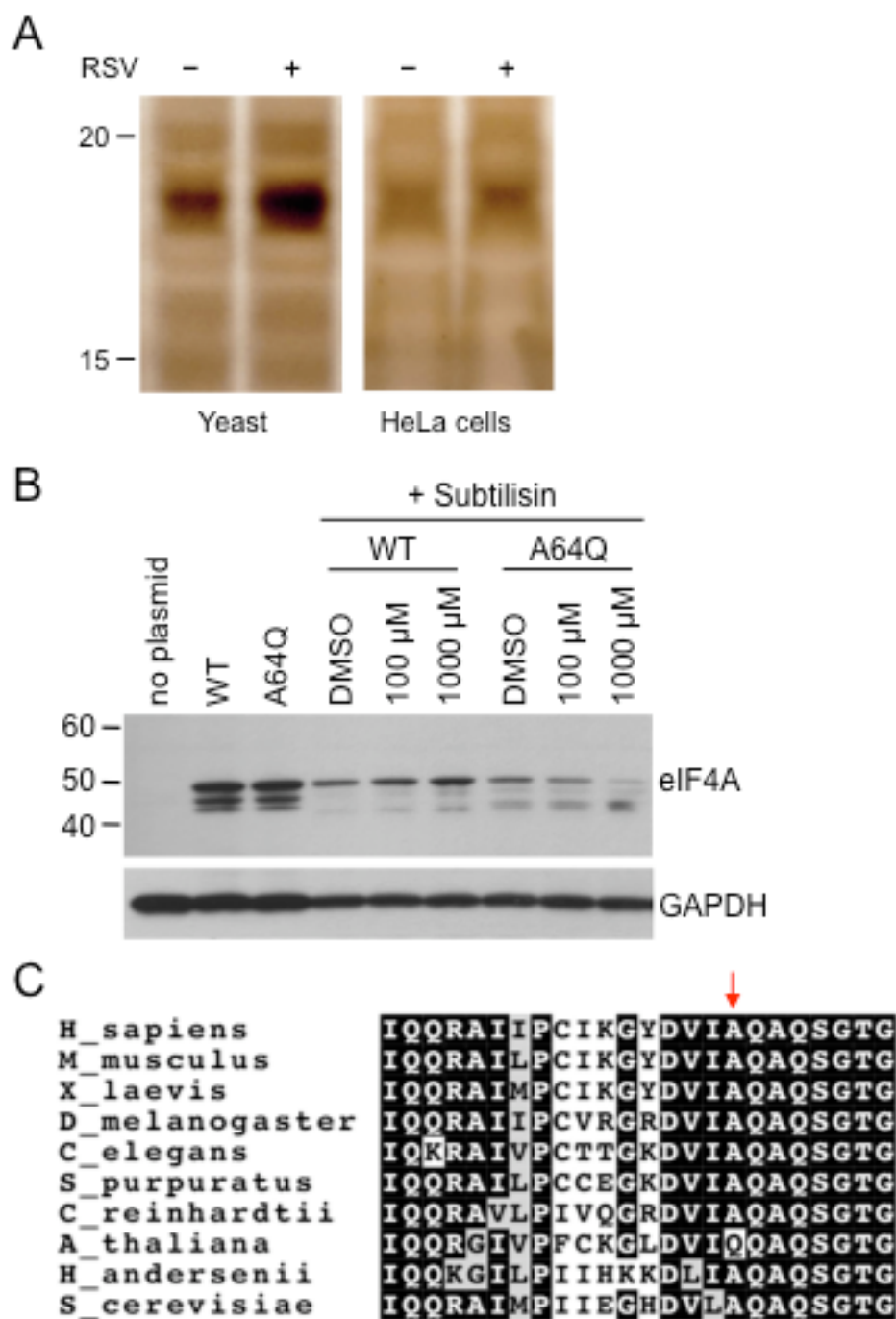


Figure 2.9. Resveratrol target analysis with DARTS. (A) Resveratrol protects 18-kDa and 20-kDa bands in yeast and HeLa cells. (B) Resveratrol protects wildtype eIF4A but not the A64Q mutant eIF4A in DARTS. (C) Amino acid conservation analysis of eIF4A in metazoans and *Arabidopsis thaliana*.

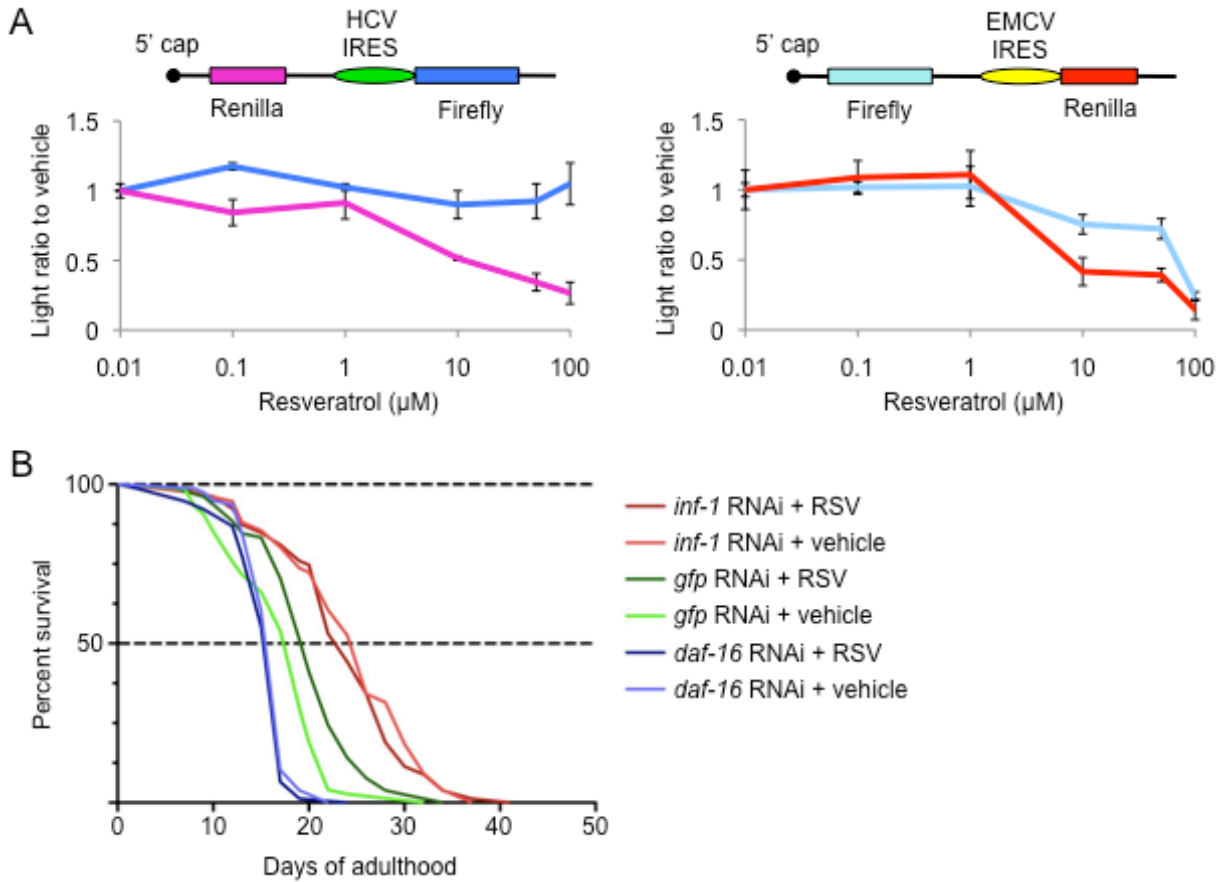


Figure 2.10. Resveratrol target analysis with DARTS. (A) Resveratrol inhibits eIF4A-dependent translation in HEK 293 cells as assayed by bicistronic translation reporters. The EMCV IRES requires the eIF4A and eIF4G subunits of eIF4F, whereas the HCV IRES does not (55). (B) The translation initiation factor eIF4A is required for longevity in resveratrol-treated animals. Resveratrol (50 μM) lengthens the lifespan of wild-type N2 worms fed control (*gfp*) RNAi (green), but not worms fed eIF4A (*inf-1*) RNAi (red) or *daf-16* RNAi (blue). *gfp* (RNAi), $m_{Veh} = 19$ (n = 74), $m_{RSV} = 20$ (n = 78), ***, $P = 0.0006$; *inf-1* (RNAi), $m_{Veh} = 26$ (n = 76), $m_{RSV} = 24$ (n = 79), $P = 0.4687$; *daf-16* (RNAi), $m_{Veh} = 17$ (n = 78), $m_{RSV} = 17$ (n = 76), $P = 0.3305$. m , mean lifespan (days of adulthood); n, number of animals tested.

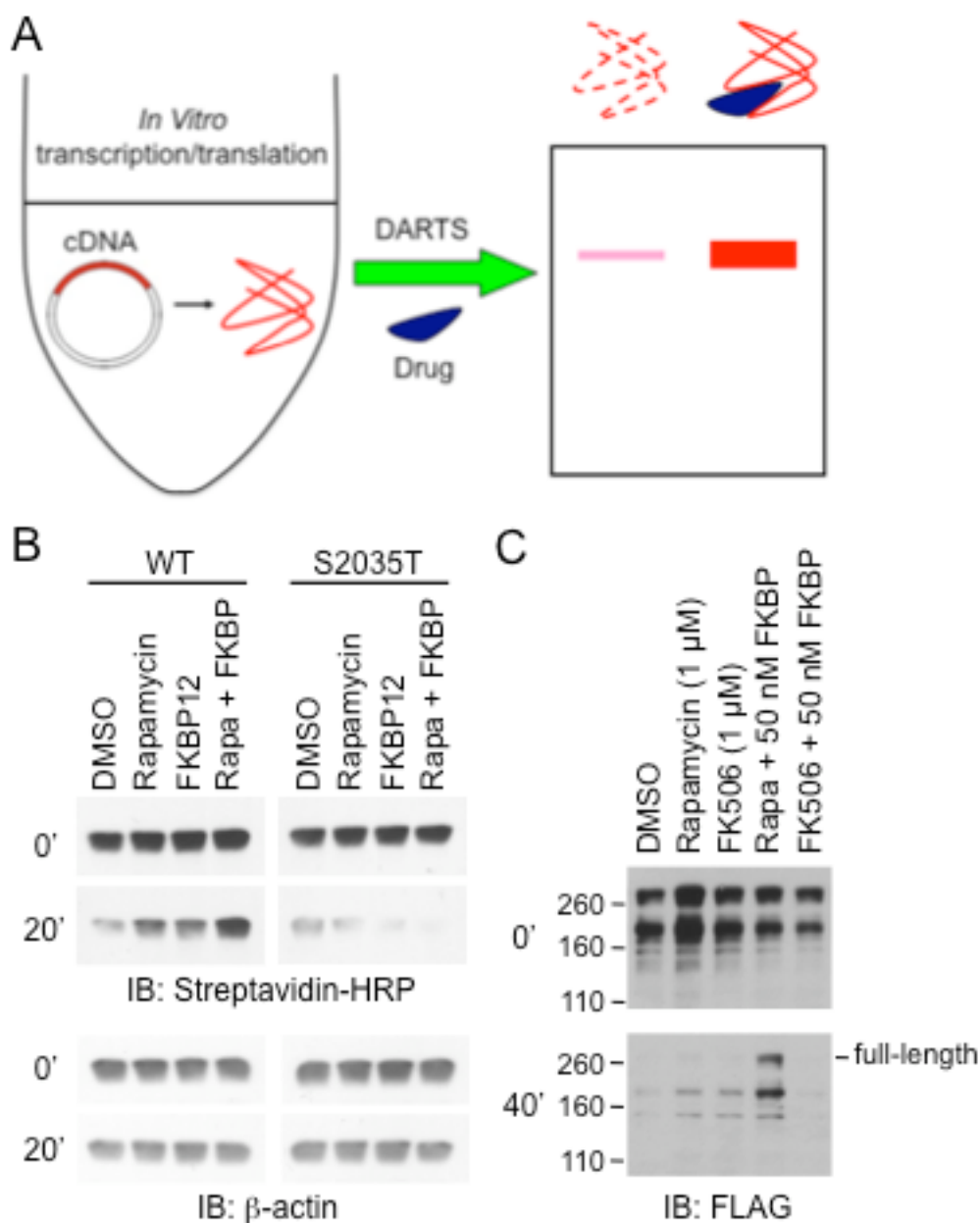


Figure 2.11. DARTS using cDNAs. (A) Plasmid cDNA is used to program *in vitro* transcription/translation (IVT) for DARTS. (B) FKBP12-rapamycin protects wildtype but not the mutant translated mTOR fragment in DARTS. Streptavidin-HRP was used to detect biotin-Lys incorporated into the translation products. β -actin was less susceptible to thermolysin under the condition and served as a loading indicator. (C) DARTS with IVT FLAG-tagged mTOR.

Table 2.1. Proteins enriched in resveratrol DARTS sample (p-value cutoff <0.01).

<u>Protein</u>	<u>Fold Change</u>	<u>P-value</u>	<u>Accession Number</u>	<u>Band</u>
60S ribosomal protein L26-B	11.107	1.35E-04	P53221	Lower
60S ribosomal protein L26-A	9.181	8.12E-27	P05743	Lower
60S ribosomal protein L24	6.05	1.24E-07	P04449, P24000	Upper
40S ribosomal protein S23	4.84	1.05E-04	P32827	Upper
40S ribosomal protein S26	4.701	0.002	P39938, P39939	Upper
60S ribosomal protein L35	3.774	7.27E-18	P39741	Lower
40S ribosomal protein S15	3.677	5.53E-06	Q01855	Lower
60S ribosomal protein L25	3.564	3.45E-21	P04456	Lower
Histone H2A	3.002	3.19E-04	P04911, P04912	Lower
40S ribosomal protein S16	2.906	2.17E-19	P40213	Lower
60S ribosomal protein L21-A	2.777	0.007	Q02753	Upper
60S ribosomal protein L25	2.713	9.60E-05	P04456	Upper
60S ribosomal protein L27-A	2.554	2.37E-04	P0C2H6, P0C2H7	Upper
Ornithine aminotransferase	2.531	9.05E-04	P07991	Lower
40S ribosomal protein S26-B	2.429	4.32E-10	P39938, P39939	Lower
40S ribosomal protein S18	2.412	7.16E-06	P35271	Lower
60S ribosomal protein L34	2.383	1.89E-06	P40525, P87262	Lower
Histone H2B	2.229	5.42E-04	P02293, P02294	Lower
40S ribosomal protein S12	2.07	5.42E-04	P48589	Lower
40S ribosomal protein S19	1.965	3.62E-08	P07280, P07281	Lower
ATP-dependent RNA helicase eIF4A	1.961	0.007	P10081	Upper
60S ribosomal protein L28	1.804	1.72E-04	P02406	Upper
60S ribosomal protein L14-A	1.614	5.05E-04	P36105	Lower
40S ribosomal protein S24	1.481	0.007	P26782	Lower

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CHAPTER 3

Small Molecule Binding Domain Mapping with Drug Affinity Responsive Target Stability (DARTS)

Abstract

For the majority of small molecule compounds with biological activity derived from interaction with specific proteins, knowledge of the binding domain is important to understand how the compound modulates the protein's function. Although a variety of methods exist to determine the protein binding domains of small molecules, they mostly require labeling or tagging the compounds or specialized biochemical instrumentation and purified proteins. DARTS was developed as a method for small molecule target identification based upon drug-induced protease resistance of target proteins, which can be monitored on a proteome-wide scale. Here we demonstrate that DARTS is also useful for determining the binding domains of small molecules. Using DARTS for domain mapping is advantageous because it uses the native compounds, does not require advanced biochemical or biophysical instrumentation, and can be performed using the purified target protein or complex protein mixtures such as cell lysates.

Introduction

The majority of eukaryotic proteins consist of multiple, often independently folded domains whose structures and functions vary widely (1). Although some ligands may make

direct contacts with more than one protein domain, most small molecule compounds bind to only a single domain of their target proteins. Given that the different domains of multi-domain proteins have unique roles, determination of which domains are bound by small molecule compounds is critical for elucidation of their mechanisms of action.

Mapping the binding domain of a small molecule ligand is frequently one of the first tasks performed after target identification. Often the methodology utilized for target identification can also be used for binding domain determination. For example, classical affinity chromatography uses immobilized or derivatized (such as biotin-tagged) small molecules to fish out the target proteins from cell or tissue lysates (2). Once specific binding proteins are isolated, individual domains of the target proteins can be cloned and expressed in cells or *in vitro* and analyzed for their ability to bind, or the target protein can be partially proteolysed into smaller fragments prior to affinity chromatography to determine which fragments retain the ability to bind (3). Similarly, photoactivatable and other reactive activity-based probes that become covalently attached to their target proteins could also be used with cloned domains or proteolysed fragments to determine their binding domains (4). Moreover, it is often straightforward to determine the exact residues to which these reactive probes attach, providing even more precise data about where the small molecules bind (5,6).

Although valuable, these approaches require the use of chemically modified ligands, which are not always available or feasible (2). Alternatively, several methods exist to determine the binding domain that use the native ligand and purified target protein. X-ray co-crystal structural determination of the protein-ligand complex is able to provide precise atomic level information about the binding site and which residues make direct contacts with the ligand (7). Hydrogen-deuterium exchange in combination with proteolysis and peptide analysis by mass

spectrometry can provide information about the regions of a protein whose stability are most altered upon ligand binding, which are likely to be at or near the binding domain (8). Similarly, NMR chemical shift analysis and distance measurements of the target protein in the presence versus absence of the small molecule can reveal specific residues within the protein that are most affected by ligand binding, including those in direct contact with the ligand (9). Alternatively, NMR analysis can be performed using individual domains of the target protein rather than the full-length protein to determine which domain is bound by the small molecule (10). While powerful, these methods are limited by the need for purified proteins, expensive instrumentation, and the technical expertise required to perform them successfully.

Developed initially for small molecule target identification, the DARTS method identifies target proteins based upon ligand binding-induced stabilization of target protein structure resulting in resistance to proteolysis (2,11). Resistance to proteolysis is specific to target proteins while digestion of non-target proteins is unaltered, allowing for target identification using both the native, unmodified small molecules and complex protein mixtures such as whole cell lysates. Previously we provided preliminary evidence that DARTS may be useful for binding domain mapping for two separate drug-protein pairs using *in vitro* translated protein fragments. First, for the well-studied natural product drug rapamycin, we demonstrated that the FKBP12-rapamycin complex could protect from digestion a C-terminal fragment of mTOR (residues 1968-2549) that contains the FKBP12-rapamycin binding domain (11). Subsequently, we determined that SMER3 protects the F-box region (residues 1-290) of Met30 from proteolysis, but not the WD-40 repeat region of Met30 (residues 291-641) or the F-box region of the closely related Cdc4 protein (12). In this paper we further validate the utility of DARTS for binding domain mapping using additional protein-drug pairs and show that it can be

performed using the native, full-length proteins in whole cell lysates, eliminating the need to clone and express the individual domains of the target proteins.

Materials and Methods

Reagents. Antibodies used were as follows: EF1A1 (residues 200-300) from Abcam (ab37969; lot #837511 and 266206), EF1A1 (residues 400-450) from Aviva Systems Biology (ARP48166; lot QC18064), EF1A2 (residues 1-215) from GeneTex (GTX102326S; lot #40100), EF1A2 (residues 300-400) from Abcam (ab60283; lot GR6868-1), eEF1A (C-terminal) from Cell Signaling (Cat. #2551; lot #1), EF-1A clone CBP-KK1 (whole protein) from Millipore (Cat. #05-235; lot DAM1745276), GAPDH (Ambion Cat. #AM4300; lot #1011015), eIF4A (F52) from Cell Signaling (Cat. #2425, lot #1), eIF4A (C32B4) from Cell Signaling (Cat. #2013, lot #2), and Flag (M2) from Sigma (F3165). EF1A1 was cloned from HEK293 cDNA with an N-terminal Flag-tag between the XbaI and BamHI restriction sites into the pcDNA 3.1- vector using the following primers:

GCTCTAGAGCCAAAATGGACTACAAAGACGATGACGACAAGGGAAAGGAAAAGAC
TCATATCA and CGGGATCCTCATTTAGCCTTCTGAGCTTTC.

DARTS All DARTS experiments were performed essentially as described (13). Jurkat cells were collected in mid log phase growth and lysed with M-PER (Pierce) containing 1x Complete Mini Protease Inhibitor (Roche) and phosphatase inhibitors (1 mM β -glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, and 2 mM sodium fluoride). HEK293 cells were transfected with pcDNA-Flag-EF1A1 using Xfect transfection reagent (Clontech)

according to the manufacturer's instructions. Two days after transfection cells were harvested by trypsin treatment and lysed with M-PER as for Jurkat cells.

Results

Our preliminary domain mapping experiments with rapamycin and SMER3 used thermolysin as the sole protease. However, thermolysin has limited usage in DARTS because many proteins are extremely resistant to digestion by thermolysin, which led us to try additional proteases (2). Ultimately we chose to use Pronase, a commercially available mixture of multiple proteases with broad substrate cleavage sites, in many of our subsequent experiments. One difference between thermolysin and Pronase is the number and pattern of protein fragments that are generated during digestion, with Pronase digestion generally producing many more fragments of varied sizes (11 and unpublished results). As expected, when analyzed by western blotting these digestion fragment patterns also depend on the antibody used for their detection, as different antibodies will bind to separate small peptide epitopes of the protein which are present in different fragments.

We reasoned that the digestion fragment patterns generated during DARTS might provide information about the three-dimensional structure of the full-length protein, especially in regards to the interaction between a small molecule and its protein target. Specifically, we hypothesized that compound binding would preferentially protect its binding domain within the target protein in comparison to other regions not directly bound by the drug. As an initial test we chose the marine natural product didemnin B (DB), which strongly binds the eukaryotic translation elongation factor 1 alpha (eEF1A) and produces a robust protective effect in DARTS (11,14). Although DB provides superb protection of eEF1A from thermolysin digestion,

thermolysin was not useful for determining the binding domain of DB within eEF1A. This is demonstrated in Figure 3.1 where cell lysates incubated with DB or DMSO as a control were digested with thermolysin over a three-hour period to determine how the proteolytic protection changed over time. Whereas protection of the full-length (50 kDa) protein or a 45-kDa fragment was evident at each time point up to two hours, no smaller fragments of eEF1A were evident by western blotting at any time point.

In contrast to thermolysin, digestion with other proteases including subtilisin and Pronase resulted in many fragments of eEF1A ranging in size from 15-45 kDa (Figure 2.7). We therefore decided to analyze how DB would alter the Pronase digestion fragment patterns of eEF1A in more detail. To do so, cell lysates with and without DB were digested with increasing amounts of Pronase, followed by western blotting with six different antibodies that recognize various regions of eEF1A. Interestingly, even though all six antibodies could detect protection of full-length eEF1A by DB, the antibodies recognized diverse patterns of fragments (Figure 3.2). Only two antibodies, GTX102326 and ab37969, were able to detect protected fragments of eEF1A as small as 30 kDa. Another two antibodies, ab60283 and CBP-KK1, were able to detect a 40 kDa protected fragment of eEF1A. One of the remaining two antibodies, ARP48166, was able to detect a protected fragment of eEF1A that was slightly smaller than full-length (~48 kDa), while the final antibody, Cell Signaling 2551, detected no fragments of eEF1A whatsoever.

When the ability of these antibodies to detect different protected eEF1A fragments was analyzed with respect to the location of their immunizing peptides within eEF1A, a clear pattern emerged (Figure 3.3). The two antibodies that could detect the 30-kDa fragment bound within the first 300 amino acids of eEF1A. On the other hand, the two antibodies that could only detect full-length or nearly full-length eEF1A bound at or near the C-terminus (residues 400-462).

Finally, of the two antibodies that could detect the 40-kDa fragment but not the 30-kDa fragment, one is known to bind between amino acids 300 – 400. The other antibody, CBP-KK1, is a monoclonal antibody whose binding epitope is unknown since it was created using full-length eEF1A. However, based on its ability to recognize the 40-kDa fragment but not the 30-kDa fragment, it likely also binds between residues 300 – 400.

The analysis of DB-protected eEF1A fragments upon digestion with Pronase suggested that DB binds within the first 300 amino acids of eEF1A (Figure 3.3). We propose that although DB can protect full-length eEF1A from low Pronase concentrations (1:4800 and 1:2400), higher amounts of Pronase can overcome this and allow digestion of eEF1A to proceed primarily from the C-terminal end (Figure 3.2). At intermediate Pronase concentrations (1:1200 and 1:600), a 40-kDa fragment that is protected from digestion remains, which has lost the C-terminal portion of eEF1A. Finally, upon digestion with a high Pronase concentration (1:300), there is further digestion from the C-terminal end of eEF1A, which leaves the 30-kDa fragment of eEF1A that is very refractory to degradation in the presence of DB. The use of even higher amounts of Pronase than shown in Figure 3.2 will eventually result in complete digestion of the 30-kDa fragment even in the presence of DB, and no smaller protected fragments become visible (results not shown).

To determine whether any degradation from the N-terminus of eEF1A occurred, we performed DARTS using an N-terminal Flag-tagged EF1A1 construct. Even with a high Pronase concentration (1:300), protection of the 30-kDa fragment visible with the ab37969 antibody was evident by western blotting with an anti-Flag antibody, demonstrating that DB protects the entire N-terminus of eEF1A from degradation (Figure 3.4). Mass spectrometry analysis of the protected 30-kDa fragment also verified that it spans the first 300 residues of eEF1A (Table 3.1).

Taken together, these results demonstrate that DB binding preferentially protects a 30-kDa portion of eEF1A spanning approximately the first 300 residues. Moreover, it suggests that even once the remaining C-terminal portion is removed by protease degradation, DB binding is retained given its ability to continue to protect the region from digestion.

The protected region of eEF1A contains two separate domains: the Ras-like GTPase superfamily domain and the EF1alpha II domain (Fig. 3.3). Notably, GTP binding to eEF1A is required for DB to bind (14). Moreover, molecular dynamics simulations predicted that DB binds to a pocket located between the GTPase and EF1alpha II domains that is formed upon GTP binding, with DB making direct contacts to multiple residues in both domains (15). However, until now no experimental data existed to support or refute this prediction. Our DARTS results provide the first direct evidence demonstrating that DB does bind to and stabilize both domains 1 and 2 of eEF1A.

To further validate that DARTS is generally applicable for domain mapping, we sought to test additional small molecule inhibitors beyond DB. The eukaryotic translation initiation factor 4A (eIF4A) is a 48-kDa protein of the DEAD box helicase family that consists of two domains of similar size, which together utilize ATP binding to facilitate RNA duplex unwinding in the 5' UTRs of mRNAs to facilitate translation initiation (Reviewed by 16,17). Given the existence of multiple small molecule inhibitors of eIF4A with different binding domains, eIF4A was chosen as a test protein (18). The first inhibitor of eIF4A discovered was the marine sponge-derived heteroaromatic macrolide pateamine A, which was shown to bind the N-terminal domain of eIF4A by affinity chromatography (19,20). Another eIF4A inhibitor is hippuristanol, a polyoxygenated steroid derived from the coral *Isis hippuris* that was found to potently and specifically inhibit cap-dependent translation through binding to the C-terminal domain of eIF4A

(10). We found that hippuristanol strongly protects many smaller fragments of eIF4A that are visible by western blotting with an antibody (C32B4) that binds within the C-terminal domain of eIF4A (Figure 3.5A). However, western blotting with an antibody that binds within the N-terminal domain of eIF4A (F52) does not show any significantly protected fragments in the presence of hippuristanol (Figure 3.5A). On the other hand, DARTS using des-methyl, des-amino pateamine A, a synthetic analog of pateamine A that retains potent inhibition of eIF4A, strongly protects an approximately 15-kDa band recognizable with the F52 antibody (Figure 3.5B). These results further demonstrate that DARTS can be used for mapping the small molecule binding domains within multi-domain proteins and validate binding of pateamine A and hippuristanol to the N-terminal and C-terminal domain of eIF4A, respectively.

Although our work highlights the ability to easily determine the binding domains of proteins using DARTS and western blotting, it must be noted that some antibodies may give misleading results. For example, we were unable to detect significant enrichment of eEF1A using shotgun (MudPIT) mass spectrometry (results not shown) in both thermolysin and Pronase DARTS experiments for which western blotting showed clear protection of eEF1A by DB (2,13). Upon using a newer lot of the same eEF1A antibody used in our initial experiments (ab37969), we unexpectedly found many smaller eEF1A fragments that were not detected with the earlier lot (Figure 3.6). These fragments were not protected from digestion by DB and were larger than the 10-kDa cutoff for removal from the DARTS sample via dialysis or spin column filtration. Therefore, although DB clearly protected eEF1A from proteolysis, the presence of these smaller fragments in the MudPIT samples prevented eEF1A from being detected as significantly enriched in the DB sample.

Discussion

The results presented in this chapter demonstrate that DARTS is useful not only for target identification and validation but also for determining the binding domain of a small molecule within its target protein. Prior results showed that expressed fragments of mTOR and Met30 that contain the binding domains for rapamycin and Smer3 could each be protected just as well as the full length protein. Here we built on these results by demonstrating that expression of cloned fragments of the target protein was not necessary for domain mapping. Utilizing the ability of Pronase to digest proteins into many different fragments, we were able to show that the fragments containing the binding domain will continue to be protected whereas other fragments lacking the binding domain are not. Therefore, domain mapping can be performed with DARTS and whole cell lysates so long as antibodies that recognize each domain of the target protein are available. Alternatively, domain mapping could be performed with DARTS using the purified target protein, gel staining, and mass spectrometry to identify the protected fragments. The ability of DARTS to determine the binding domains of small molecules is highly advantageous because it uses the native compounds, can be performed with whole cell lysates or purified proteins, and does not require any specialized biophysical equipment. Its simplicity and ability to be performed quickly will allow for its widespread adoption as a go-to method for binding domain mapping.

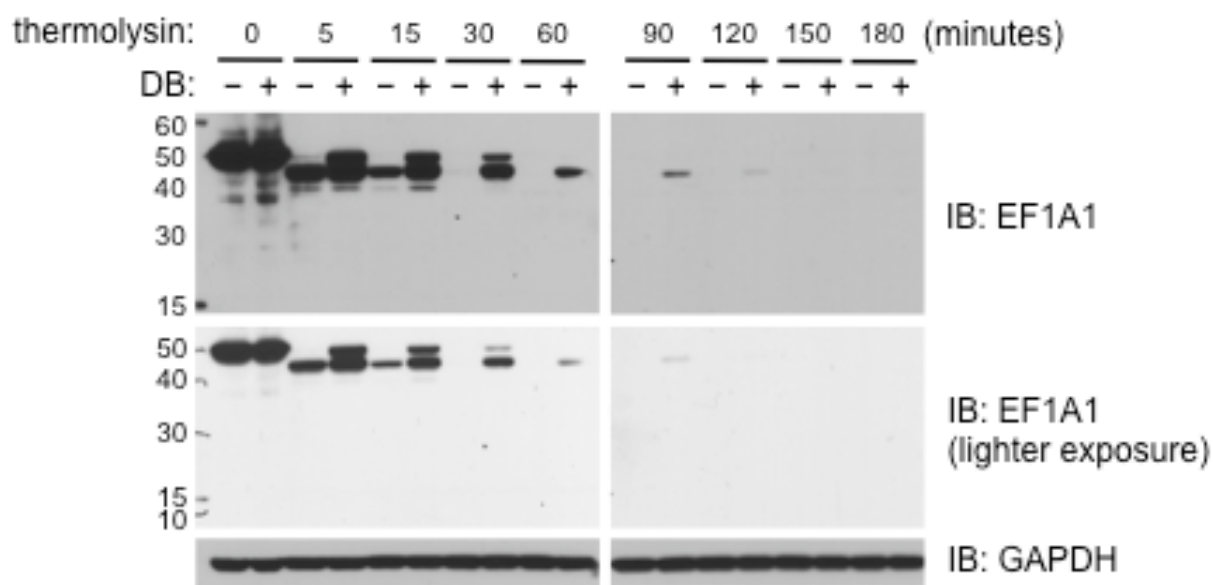


Figure 3.1. DARTS using a time course of thermolysin digestion. Jurkat cell lysates were incubated with 1 μ g/mL DB or 1% DMSO, followed by digestion with thermolysin at a 1:20 ratio. Aliquots of each digestion reaction were removed and stopped at the indicated time points for western blotting analysis with anti-EF1A1 antibody (ab37969).

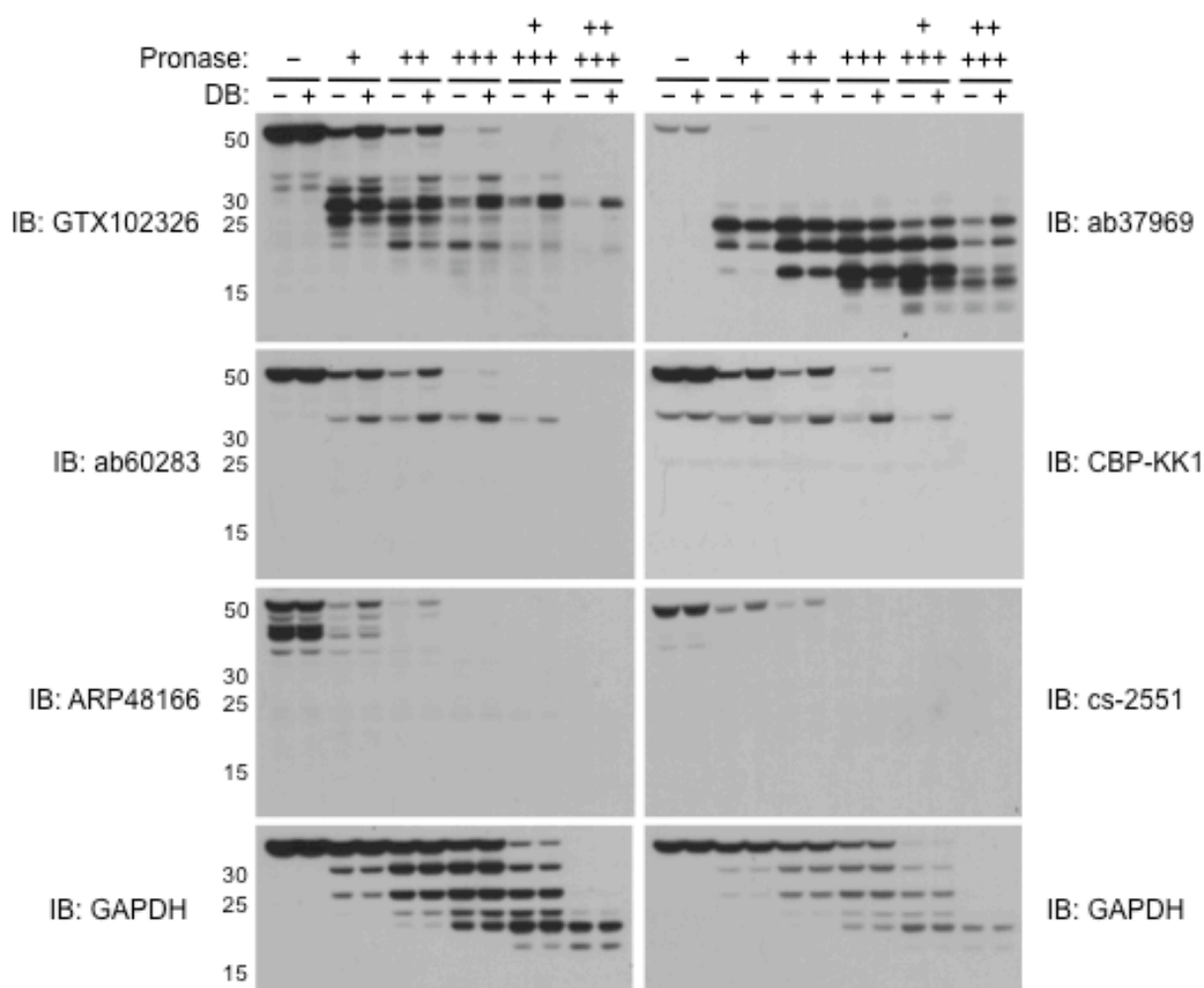


Figure 3.2. DARTS using a Pronase titration. Jurkat cell lysates were incubated with 1 $\mu\text{g/mL}$ DB or 1% DMSO, followed by digestion of aliquots of each sample with Pronase at ratios of 1:4800, 1:2400, 1:1200, 1:600, or 1:300 for 30 minutes. Western blotting was performed using six separate eEF1A antibodies on six different membranes to ensure that the chemiluminescent signal was solely from the indicated eEF1A antibody. Immunoblotting for GAPDH was performed on each membrane as well to ensure consistent loading and transfer of all samples (GAPDH data shown for one membrane only).

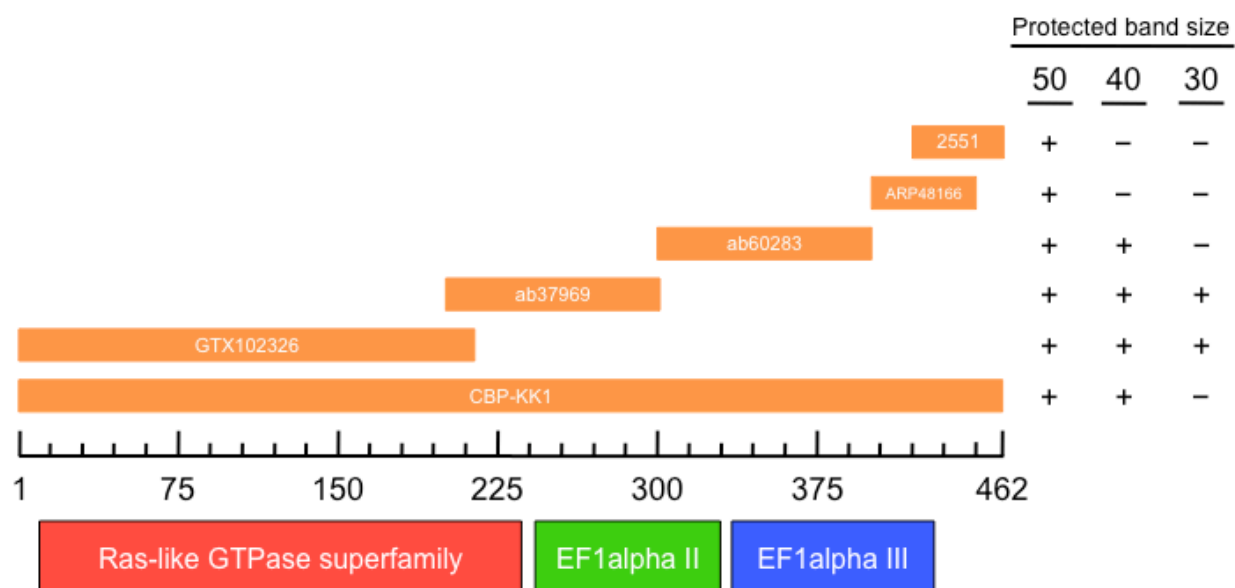


Figure 3.3. Mapping the binding domain of DB to eEF1A with DARTS. The orange bars indicate the region of eEF1A that was used as an antigen to create each antibody. Matching these regions with the ability of each antibody to recognize protected fragments of eEF1A indicates that the 30-kDa fragment spans approximately the first 300 residues of eEF1A.

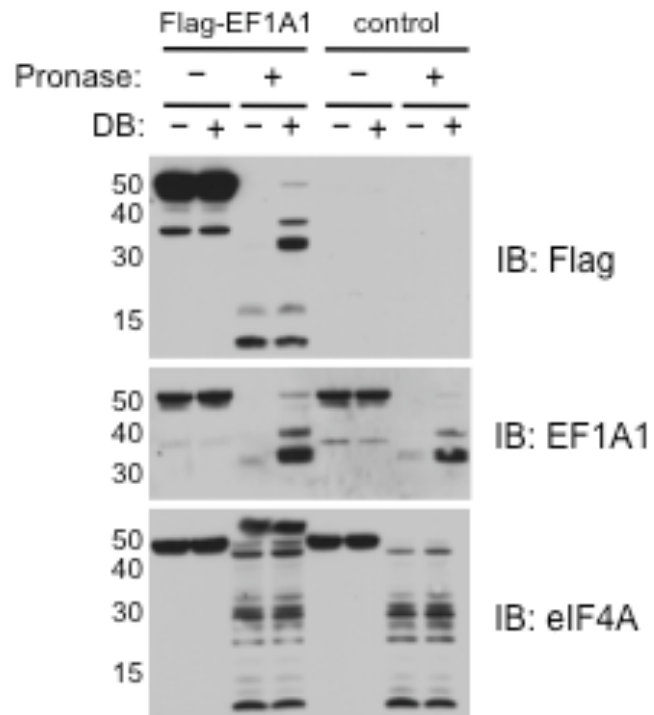


Figure 3.4. DARTS using DB and N-terminal Flag-tagged EF1A1. Lysate from HEK293 cells expressing Flag-EF1A1 and non-transfected HEK293 cell lysate were incubated with 10 $\mu\text{g/mL}$ DB or 1% DMSO, followed by digestion with Pronase at a 1:300 ratio for 30 minutes.

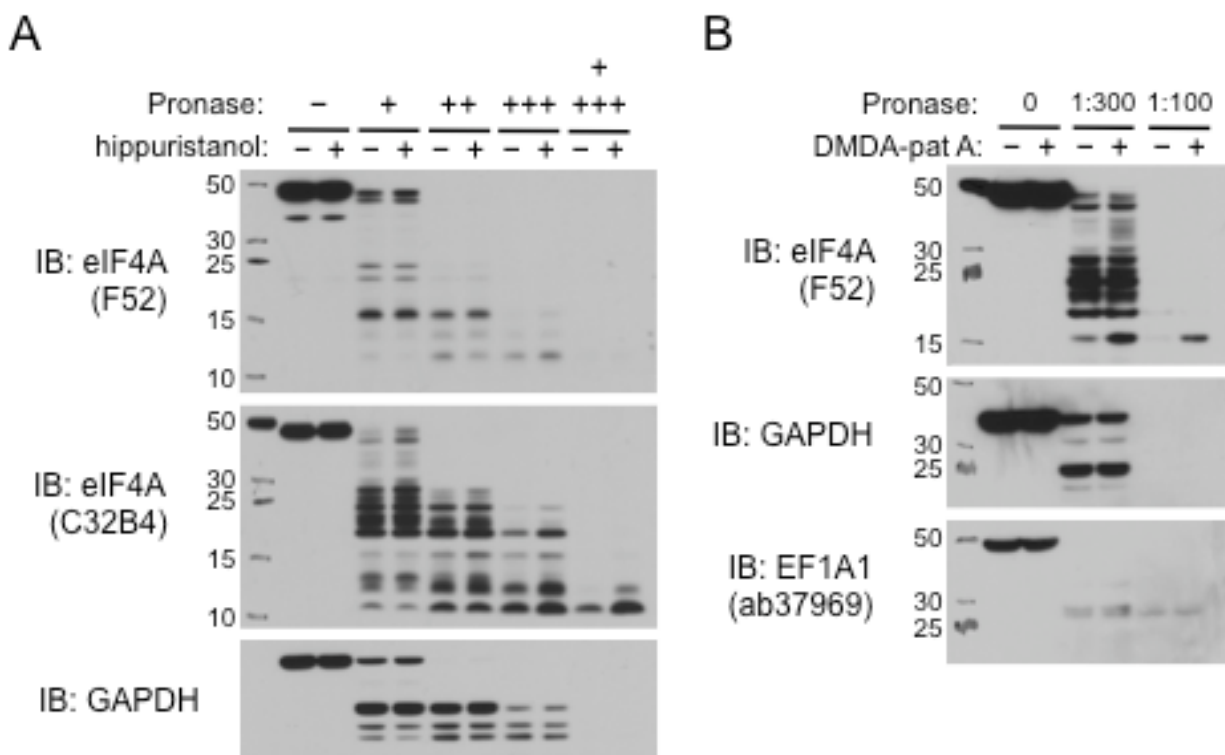


Figure 3.5. Mapping the binding domain of hippuristanol and DMDA-pateamine A to eIF4A with DARTS. (A) Jurkat cell lysates were incubated with 10 μ M hippuristanol or 1% DMSO, followed by digestion of aliquots of each sample with Pronase at ratios of 1:800, 1:400, 1:200, or 1:100 for 30 minutes. (B) Jurkat cell lysates were incubated with 10 μ M DMDA-pateamine A or 1% DMSO, followed by digestion of aliquots of each sample with Pronase at a 1:300 or 1:100 ratio for 30 minutes.

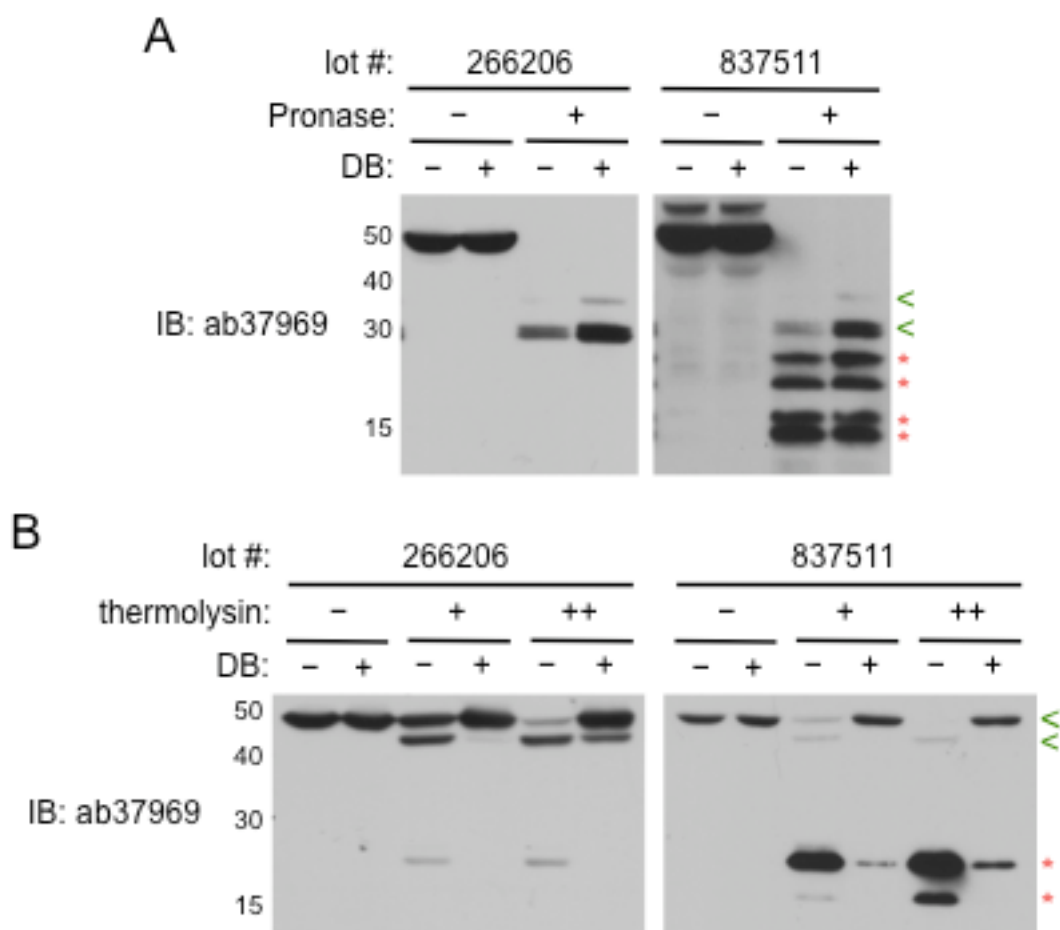


Figure 3.6. Comparison of results with different lots of the same antibody. (A) Jurkat cell lysates were incubated with 1 $\mu\text{g/mL}$ DB or 1% DMSO, followed by digestion with Pronase at a 1:300 ratio for 30 minutes. The membrane was first probed with EF1A1 antibody from lot 266206, then re-probed with EF1A1 antibody from lot 837511. **(B)** Lysates from Jurkat cells treated with 1 $\mu\text{g/mL}$ DB or DMSO for 40 minutes were digested with thermolysin at a 1:15 ratio for 10 or 30 minutes. Western blotting was performed as in A.

Table 3.1. Peptides identified by mass spectrometry in 30-kDa protected fragment of eEF1A.

<u>Peptide</u>	<u>Residues</u>
THINIVVIGHVDSGK	6-20
STTTGHLIYK	21-30
YYVTIIDAPGHR	85-96
EHALLAYTLGVK	135-146
MDSTEPPYSQK	155-165
LPLQDVYK	248-255
IGGIGTVPVGR	256-266
VETGVLK	267-273
PGMVVTFAPVNVTTTEVK	274-290

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CHAPTER 4

An Advanced Gel-Free DARTS Approach Identifies a New Target of Didemnin B

Abstract

Naturally occurring small molecules (natural products) include many toxic compounds produced by microorganisms as a defense mechanism or to inhibit the growth of competing organisms. Often characterized by extremely complex chemical structures, these natural products typically inhibit critical cellular processes by binding to essential and highly connected protein targets such as microtubules, cytoskeletal components, and proteins involved in polypeptide synthesis, folding, and degradation (1). The cyclic depsipeptide didemnin B (DB) is an intriguing marine natural product with potent cytostatic, cytotoxic, and immunosuppressive activity against a wide range of cell types. Although characterized as an inhibitor of protein synthesis due to its binding and inhibition of eukaryotic elongation factor 1 alpha (eEF1A), evidence suggests that additional protein targets of didemnin B exist to explain its myriad activities (2-5). Here we used an innovative gel-free DARTS (Drug Affinity Responsive Target Stability) platform to discover novel protein targets of DB. We show that didemnin B binds and inhibits the eukaryotic TRiC/CCT chaperonin, an essential 1-MDa complex required for the proper folding of up to 10% of all eukaryotic proteins, including actin and tubulin (6). Importantly, our evidence also suggests that TRiC inhibition contributes to the cytotoxicity of didemnin B. DB is the first non-ATP mimetic small molecule found to bind to and modulate the

activity of TRiC, and thus the didemnin natural products may serve as a starting point for making more potent and specific inhibitors for investigating this fundamental cellular complex.

Introduction

Didemnin B is among the best studied of the didemnin class of marine natural products found in several species of tunicates. DB in particular displays potent cytostatic, cytotoxic, and immunosuppressive activities in a range of *in vitro* and *in vivo* assays, which led it to be the first marine peptide drug to undergo clinical trials for cancer (7). Although promising, DB ultimately did not pass any phase II trials due to lack of efficacy and dose-limiting toxicities (8). However, lack of insight into the mechanism of action of DB and the other didemnins has hampered their development into therapeutics.

DARTS is a powerful method for drug target identification because it identifies target proteins based solely on drug binding yet uses the native, unmodified compounds (9,10). However, the first generation DARTS scheme to identify targets *de novo* involved visualizing protected bands on a stained SDS-PAGE gel. This approach is limited due to the low resolution and loading capacity of SDS-PAGE gels, making identification of lower abundance protein targets unlikely. To overcome this, we sought to utilize a more sensitive non gel-based method for identifying protected proteins that does not require them to be seen by eye. In this chapter we present the use of a gel-free proteome fractionation technology with DARTS and the identification of TRiC as a novel target of didemnin B.

Materials and Methods

Didemnin B target ID using DARTS and Protomap. Jurkat cells (ATCC) were grown at a starting density of 5×10^5 cells/mL for 5.5 hr in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin solution (Invitrogen 15140-122), and 4 mM L-Glutamate (final concentration). 130 mL cells were collected by centrifugation at 500 g for 10 min at 4°C and the cell pellet lysed with 1 mL M-PER lysis buffer (Pierce #78505) containing Complete Mini protease inhibitor (Roche #11836153001) and phosphatase inhibitors (1 mM β -glycerophosphate, 2.5 mM sodium pyrophosphate, 2 mM NaF, and 1 mM sodium vanadate). The lysates were clarified by centrifugation at 18,000 g for 10 min at 4°C and the supernatant transferred to a new pre-chilled tube. 0.9 mL lysates was mixed with 0.1 mL ice-cold 10x TNC buffer (0.5 M Tris-HCl pH 8, 0.5 M NaCl, 0.1 M CaCl_2) for DARTS.

BCA analysis determined the concentration to be 5 $\mu\text{g}/\mu\text{L}$. Two 300 μL samples were brought to room temperature and incubated with either 10 $\mu\text{g}/\text{mL}$ DB or 1% DMSO for 1 hr in a thermomixer at 600 rpm. Then, 20 μL was removed from each sample for use as a non-digestion control, followed by digestion of the remaining 280 μL samples with 4.66 μg Pronase (1:300 Pronase-to-protein ratio) for 30 min. The digestions were stopped by adding 14 μL 20x protease inhibitor solution and placing on ice. 12 μL of each digested and undigested control samples were separated by SDS-PAGE on a 4-12% Bis-Tris gel with MES running buffer (Invitrogen), followed by transfer to a 0.45 μm PVDF membrane (Millipore IPVH00010). Total protein and protein digestion levels were verified to be even by western blotting for GAPDH (Ambion #4300), and protection of EF1A1 verified using Abcam #ab37969.

115 μL of each digested sample was desalted with 7K MWCO Zeba Spin Columns (Pierce #89882) according to manufacturer's instructions, followed by mixing 112 μL of each

sample with 8 μ L 1 M DTT and 30 μ L of 5x acetate sample buffer (Protein Discovery #42302) and heating to 50°C for 10 minutes. After cooling to room temperature, the samples were fractionated by molecular weight in separate lanes of a 5% Tris-Acetate cartridge (Protein Discovery #42104) using the Gelfree 8100 system. The proteins that were collected in fraction 1 of each sample (representing those smaller than ~55 kDa) were concentrated by speedvac to 132 μ L, followed by mixing with 3 μ L 1 M DTT and 15 μ L of 5x acetate sample buffer and heating to 50°C for 10 minutes. After cooling to room temperature, these samples were fractionated in separate lanes of a Gelfree 8100 12% Tris-Acetate cartridge (Protein Discovery #42102).

All fractions were prepared for mass spectrometry analysis using a modified Filter-Aided Sample Preparation (FASP) protocol (11). FASP was performed by first mixing each fraction with 200 μ L 8 M urea (prepared in 0.1 M Tris-HCl pH 8.5) in individual 10K NMWL ULTRAFREE-MC spin columns (Millipore UFC3LG00) and centrifuging the columns at 14,000 g until nearly dry (5-10 μ L remained). Then the columns were washed with 200 μ L 8 M urea (pH 8.5) and centrifuged again. Each column was then treated with 100 μ L 50 mM iodoacetamide (prepared in 8 M urea, pH 8.5), mixed for 1 minute at 600 rpm in a Thermomixer, and incubated at room temperature in the dark for 10 minutes. The columns were then centrifuged as before and all flow-through discarded. The columns were then washed three times, each with 100 μ L 8 M urea (prepared in 0.1 M Tris-HCl pH 8.0), by centrifuging at 14,000 g until nearly dry each time. The filter columns were then transferred to clean collection tubes and the flow-through discarded. The total volume in each filter column was then brought to 40 μ L with 8 M urea (pH 8.0), followed by mixing the columns for 1 minute at 600 rpm in a Thermomixer. 120 μ L 50 mM ammonium bicarbonate containing 1.25 ng/ μ L sequencing grade trypsin (prepared on ice) was then added into each column and mixed for 1 minute at 600 rpm.

Digestion proceeded overnight at 37°C. The next day the columns were centrifuged for 20 minutes at 14,000 g. Then, 50 µl 0.5M NaCl was added onto each column and the centrifugation repeated. Finally, the eluates containing the digested peptides were acidified with 5 µL 5% trifluoroacetic acid.

Prior to LC-MS/MS analysis, each trypsin-digested fraction was desalted using C18 UltraMicro TIP Columns (The Nest Group STU SS18V) according to the manufacturer's instructions. Desalted fractions were concentrated to about 5 µL by speedvac, then brought up to 25 µL with 2% acetonitrile/0.1% trifluoroacetic acid. LC-MS/MS analysis of each fraction was performed on a Thermo LTQ-Orbitrap mass spectrometer with an Eksigent LC pump. The peptides were loaded onto a C18 reversed-phase column at a flow rate of 3 µL/min. Mobile phase A was 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitrile. Peptides were eluted from the column at a flow rate of 300 nL/min by using a gradient from 2% B to 15% B for 12 min, 15% B to 45% B over 84 min, 45% B to 70% B for 4 min, 70% B to 90% B for 2 min, and finally keeping constant at 90% B for 6 min. The column was then re-equilibrated using a gradient from 90% B to 2% B over 2 minutes and keeping constant at 2% B for 10 minutes. Spectra were acquired in data-dependent mode (using dynamic exclusion of 30 sec for fragmented peptides) with the Orbitrap used for MS scans and LTQ for MS/MS scans.

Analysis of the mass spectrometry data was performed by searching the ms2 files (extracted from the raw data files using RawXtract version 1.9) against the human Uniprot database (May 2010 download) using ProLuCID version 1.0, with carbamidomethylation of cysteines as a static modification. Search results were then filtered using DTASelect (version 2.0.25) with a false positive rate of 5% (results were very similar with a 1% false positive rate). Finally, the data from all fractions of each sample were compared using Protomap (12) to find

proteins protected from digestion by DB. Protection was defined as having at least 3x as many spectra in the DB sample versus the DMSO sample. Only proteins with > 20 total spectral counts between the two samples were considered to reduce the likelihood of false positives.

Validation of CCT protection by DB. Jurkat cells were grown as above for 5.5 hr. 150 mL cells were spun down and the cell pellet lysed with 1.05 mL M-PER lysis buffer (protease and phosphatase inhibitors). The lysates were clarified by centrifugation at 18,000 g for 10 min at 4°C and the supernatant transferred to a new pre-chilled tube. 110 µL ice cold 10x TNC buffer was added to the lysates, followed by BCA analysis. Two 0.5 mL aliquots of lysates were then prepared and warmed to room temperature. One aliquot was treated with 5 µL DMSO and the other with 5 µL 1mg/mL DB for a final concentration of 10 µg/µL. Both samples were mixed for 1.25 hr at room temperature in a thermomixer at 650 rpm. Subsequently, 80 µL aliquots of both samples were digested with Pronase for 30 min at room temperature using Pronase-to-protein ratios of 1:4800, 1:2400, 1:1200, 1:600, and 1:300. Digestions were stopped by adding 8 µL 20x protease inhibitor solution and placing on ice. Immunoblotting was performed for TRiC complex members using the following antibodies: anti-TCP1a (Abcam ab92587), anti-TCP1b (Abcam ab56717), anti-CCT3 (Abcam ab91473), anti-CCT5 (Sigma HPA005958), anti-CCT6a (Santa Cruz 13896), and anti-CCT7 (Abcam ab56713).

Purification of TRiC from bovine testes. The following is a hybrid, modified procedure from two published protocols (13,14). Fresh bovine testes were obtained locally from castrated bulls, washed with 200 mL ice-cold homogenization buffer (250 mM sucrose, 100 mM NaCl, 2 mM EDTA, 1 mM DTT, 50 mM HEPES-KOH, pH 7.4), cut into approximately 5 g pieces, washed

once more with 100 mL ice-cold homogenization buffer (HB), flash frozen in liquid nitrogen, and stored at -80°C . All pieces from a single testis (80 – 90 g) were used for each purification, and all purification steps were performed in the cold room.

The pieces of 1 testis were thawed and minced in 150 mL HB containing protease inhibitors (3 Roche Complete protease inhibitor tablets, 2 $\mu\text{g/mL}$ leupeptin, and 0.5 $\mu\text{g/mL}$ aprotinin) in a prechilled blender. The testes suspension was then homogenized, 40 mL at a time, with 15 strokes in a 45-mL Potter-Elvehjem tissue grinder. Next, the homogenate was clarified by a 30-minute centrifugation step at 16,000 g, followed by an hour centrifugation step at 130,000 g. The supernatant was then collected and filtered through 3 layers of cheesecloth.

Ammonium sulfate precipitation was then performed using the filtered supernatant. First, a 30% saturation cut was performed, followed by a 20-minute centrifugation step at 16,000 g. Then, a 50% saturation cut was performed on the supernatant, followed by a 30-minute centrifugation step at 16,000 g to pellet TRiC. The pellet was resuspended in 15 mL column buffer (5% glycerol, 5 mM MgCl_2 , 0.5 mM EDTA, 1 mM DTT, 20 mM HEPES-KOH, pH 7.4, and Roche Complete protease inhibitor) + 200 mM NaCl, followed by a 20-minute centrifugation step at 20,000 g to clarify the suspension containing TRiC.

The clarified suspension was then layered over two 2-mL cushions of 1M sucrose in column buffer (CB) + 200 mM NaCl in 13 mL ultracentrifuge tubes and centrifuged at 250,000 g for 3 hours. The supernatant was then removed and the sucrose layer and pellet containing TRiC was resuspended in 40 mL CB + 200 mM NaCl. TRiC was then purified by heparin affinity chromatography. A 5-mL GE HiTrap Heparin column was equilibrated with 20 mL CB + 200 mM NaCl and the TRiC-containing solution loaded using a flow rate of 2 mL/minute. Elution was then performed with a 100-mL linear gradient from 200 mM NaCl to 1 M NaCl. 3-mL

fractions were collected and analyzed for TRiC by western blotting. The 7 fractions containing the majority of TRiC (approximately 0.5 – 0.7 M NaCl) were pooled, layered over 2-mL cushions of storage buffer (20 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.5 mM EDTA, and 50% glycerol) in 13 mL ultracentrifuge tubes, and centrifuged at 265,000 g for 5 hours. The TRiC-containing storage solution was collected from the bottom of the tubes by piercing with a needle and draining into a 15-mL tube, aliquoted, snap-frozen in liquid nitrogen, and stored at –80°C. BCA analysis determined the protein concentration to be about 9 mg/mL. A Novex Blue NativePAGE Bis-Tris gel (Life Technologies) was used to validate isolation of the intact TRiC complex.

Proteinase K digestion of purified TRiC. TRiC was diluted to 0.35 μ M (0.35 μ g/ μ L) in 1x TRiC assay buffer (20 mM HEPES-KOH pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol) containing 1 μ M ATP. The TRiC solution was then split into two: one aliquot was treated with 100 μ M DB and the other with 1% DMSO. The samples were then mixed and incubated at room temperature while shaking at 650 rpm. 25 μ L aliquots of each sample were then digested at room temperature for 5 minutes with 0.5, 1, or 2 μ g/mL Proteinase K. Digestions were stopped by mixing in 2.5 μ L 100x protease inhibitor solution (Sigma P8340) and transferring to ice. The digested samples were then mixed with 7.5 μ L 5x SDS loading buffer, heated to 70°C for 10 minutes, cooled to room temperature, and separated by SDS-PAGE using 4-12% Bis-Tris gels and MOPS buffer (Life Technologies). Proteins were then transferred to Millipore Immobilon PVDF membranes and western blotting performed using anti-CCT6a (Santa Cruz 13896), anti-CCT7 (Abcam ab56713), and anti-CCT5 (Sigma HPA005958) antibodies.

Actin folding assay with purified TRiC. TRiC was diluted to 0.8 μM (0.8 $\mu\text{g}/\mu\text{L}$) in 1x TRiC assay buffer lacking ATP. The sample was centrifuged at 14,000 g for 10 min at room temperature to pellet any aggregates and the supernatant collected. The TRiC solution was then split into two samples, which were treated with 100 μM DB or 1% DMSO. Folding of co-purified actin was then initiated by the addition of 1 mM ATP to each sample. The folding reaction proceeded by incubation at room temperature for 60 min and was then stopped by transferring the tubes to ice. The samples were then immediately loaded onto NativePAGE gels and ran at 100 V for 140 min. NativePAGE gels were prepared fresh each day and contained 1x Tris-Glycine buffer (25 mM Tris, 192 mM Glycine, pH 8.3), 7.5% acrylamide, 0.1% Triton-X100, 0.5 mM CaCl_2 , 0.2 mM DTT, and 0.2 mM ATP to stabilize G-actin (15). The NativePAGE running buffer also contained 1x Tris-Glycine buffer, 0.5 mM CaCl_2 , 0.2 mM DTT, and 0.2 mM ATP. Proteins were then transferred to Millipore Immobilon PVDF membranes and western blotting performed using an anti-beta-actin antibody (Sigma a1978) that can bind to native G-actin. Densitometry was performed using ImageJ.

Measuring DB cytotoxicity against TRiC knockdown and control siRNA cells. A549 cells were seeded at 10^5 cells/mL in DMEM containing 10% FBS and grown overnight. The cells were then transfected with 50 nM CCT6a siRNA, TCP1a siRNA, and non-targeting siRNA #1 ON-TARGETplus SMARTpools (Dharmacon) using Dharmafect Reagent 1 according to the manufacturer's instructions. The following day the cells were collected with trypsin, seeded into 96-well plates at 2,000 cells per well in 100 μL DMEM containing 10% FBS, and grown overnight. Additional cells were seeded into wells of a 12-well plate for collection on days 3 and

4 for western blotting analysis to verify knockdown efficiency. At 48 hours post-transfection the knockdown cells in the 96-well plates were treated in triplicate with a 2-fold dose curve of DB from 0.25 – 64 ng/mL. Control wells were treated with an equivalent concentration of DMSO. After 2 days treatment the cells were analyzed by CellTiter-Glo (Promega). Cell growth/viability (ATP level) was normalized to the DMSO treated wells, which were considered to be 100%.

DB induced activation of immediate early signaling pathways in TRiC knockdown and control siRNA cells. A549 cells were seeded and transfected with CCT6a and non-targeting siRNA #1 as above except using 25 nM siRNA concentrations. The next day the cells were collected with trypsin and seeded into a 12-well plate at 60,000 cells per well in 1 mL DMEM containing 10% FBS and grown overnight. At 48 hours post-transfection one well of each knockdown was treated with DMSO or 1, 5, 20, or 50 ng/mL DB. The remaining wells were left untreated. Following 2 hours treatment the cells were washed once with PBS and lysed on ice with 1% Triton lysis buffer containing protease and phosphatase inhibitors (same as for M-PER). The lysates were clarified by centrifugation at 14,000 g for 10 min at 4°C, treated with 5x SDS-PAGE loading buffer, and heated to 70°C for 10 min. Gel electrophoresis, transfer, and western blotting were as above and with the following additional antibodies: GAPDH (Ambion AM4300), phospho-cJun (Cell Signaling 9261), cJun (Cell Signaling 9165), phospho-p38 MAPK (Cell Signaling 9211), p38 MAPK (Cell Signaling 9212), and Survivin (Cell Signaling 2808). Analysis of immediate early signaling events over time with CCT6a knockdown versus non-targeting control siRNA cells was performed as above using 25 nM siRNA.

TRiC ATPase assay. ATPase activity was measured using the Malachite Green Phosphate Assay Kit from BioAssay Systems (cat. no POMG-25H) according to the manufacturer's instructions. All steps were performed at room temperature. For each experiment (repeated in triplicate using different aliquots of TRiC), 270 μ L 1 mg/mL TRiC was prepared in 1x TRiC assay buffer without ATP. 120 μ L was aliquoted into two tubes, followed by the addition of 1.2 μ L DMSO to one sample and 1.2 μ L 10 mg/mL DB to the other (the final concentrations were 1% DMSO and 100 μ g/mL DB). The ATPase reaction was initiated by adding 2.4 μ L 50 mM ATP to each (1 mM ATP final). At each time point, 20 μ L was removed from each sample, added into a single well of a 96-well plate containing 60 μ L 1x TRiC assay buffer, and mixed with 20 μ L malachite green solution to stop the ATPase reaction. The 96-well plate was then incubated at room temperature and the OD at 620 nm read for each well 30 minutes after addition of malachite green. Raw OD values were adjusted against wells containing only 250 μ M ATP. Wells with TRiC only (no ATP) had negligible OD values. The levels of released phosphate were determined using the standard curve generated using the phosphate standard solution provided with the kit.

Results

To date, only one protein target of DB has been discovered and validated. The eukaryotic elongation factor 1 alpha (eEF1A) was found to bind DB by affinity chromatography, an interaction that was subsequently shown to require GTP (2). Consistent with its binding to eEF1A, DB inhibits protein synthesis at the translocation step during polypeptide elongation *in vitro* and *in vivo* (16,17). However, this inhibition likely contributes only to the cytostatic effect of DB, and the mechanisms by which DB exerts its cytotoxic and immunosuppressive effects are

unknown (3,4,5). One possibility is that other unknown targets are responsible for these activities. To discover additional protein targets of DB besides eEF1A, we combined an advanced gel-free proteome fractionation technology with our small molecule target identification method, DARTS (9). In this approach, Jurkat cell lysates were incubated with DB or DMSO as a solvent control, digested with Pronase, and in-solution fractionated by molecular weight using the Gelfree 8100 system from Protein Discovery. Each fraction was then analyzed by liquid chromatography-mass spectrometry (LC-MS) to identify and quantify the proteins present in each sample as well as to analyze their digestion patterns. For data analysis, we utilized the Protein Topography and Migration Analysis Platform (Protomap) (12).

As expected, we observed a very strong protective effect on EF1A1 by DB, whereas the digestion patterns of control proteins such as GAPDH were unchanged, both by western blotting (Figure 4.1A) and by Protomap analysis (Figure 4.1B). In addition to EF1A1, we discovered that 10 other proteins were also highly protected (3-fold or more) by DB (Table 4.1). Notably, this included five of the eight subunits of the TRiC/CCT chaperonin complex, with a sixth (CCT2) enriched over 2-fold (Table 4.1 and Figure 4.2). To validate this finding, we performed an additional DARTS experiment with various Pronase concentrations and analyzed the samples by western blotting. In agreement with the Protomap data, CCT1, CCT2, CCT3, CCT5, CCT6a, and CCT7 were all protected from Pronase digestion by DB (Figure 4.3). Interesting, CCT1 (TCP1a) and CCT6a appear to be most strongly protected, both by western blotting and by mass spectrometry (Figures 4.2 and 4.3 and Table 4.1).

Although DARTS showed that DB is able to bind the TRiC complex in cell lysates, it was unclear if this is a direct interaction or whether other cellular components or cofactors are necessary. Therefore, we purified TRiC from bovine testes for use in biochemical assays with

DB (Figure 4.4A). The integrity and activity of TRiC was validated by Native-PAGE (Figure 4.4B) and the ability of ATP to protect TRiC from Proteinase K digestion (Figure 4.4C). We found that DB can also protect purified TRiC from Proteinase K digestion, but only in the presence of ATP, the hydrolysis of which is required for folding of TRiC substrates (Figure 4.5). Additionally, DB inhibits the folding of actin by purified TRiC *in vitro* (Figure 4.6A), although it does not have a significant effect on the ATPase activity of TRiC (Figure 4.6B). These results further suggest that DB binds directly to TRiC and modulates its function.

To determine whether TRiC may be involved in the cellular mechanism of action of DB, we tested the effect of TRiC inhibition by genetic means. RNAi-mediated depletion of a single TRiC subunit, which has been reported to decrease the amount of the entire TRiC complex, was reported to induce caspase-dependent apoptosis (18,19), consistent with DB's effect in the cell. Furthermore, we found that reducing TRiC levels, e.g., by RNAi of the CCT6a subunit, led to elevated cellular resistance to DB treatment (Figure 4.7). Although much remains to be known about the mechanism by which the didemnins exert their cytotoxicity, early activation of p38 MAPK and c-Jun N-terminal kinase (JNK) has been reported to be important for the activity of dehydrodidemnin B (aka Aplidine), which is in agreement with previous reports and our own observations for DB (20,21 and Wu et al, submitted). Consistently, RNAi depletion of TRiC also led to activation of JNK as measured by phosphorylation of c-Jun (Figure 4.8A). Phosphorylation of p38 MAPK was lowered (Figure 4.8A), presumably due to feedback inhibition of the pathway resulting from activation at an earlier time point; a similar response is seen in DB treatment, which causes a rapid and strong but transient increase in p38 MAPK activation (Wu et al., submitted). In addition, depletion of TRiC lowered cellular levels of Survivin, which is also seen with Aplidin or DB treatment (Figure 4.8A and B) (21). On the

other hand, the strong early activation of JNK and p38 MAPK upon DB treatment was slightly diminished in TRiC-depleted cells versus control RNAi cells, suggesting that binding of DB to TRiC is necessary for activation of these signaling pathways (Figure 4.8B). Taken together, these results suggest that TRiC may be important for the cytotoxic effect of DB.

Discussion

While DB displays broad growth inhibitory activity against a wide range of cancer cell types, activated lymphocytes and B-cell and T-cell malignancies in particular are extremely susceptible to its apoptosis inducing activity (22,23 and Wu et al., submitted). Interestingly, lymphocyte development and function has been shown to be drastically affected by loss of Prefoldin, a co-chaperone complex that is critical for transferring newly translated cytoskeletal proteins including actin from the ribosome to TRiC for proper folding (24). It is tempting to speculate that inhibition of TRiC may at least partially explain the extreme sensitivity of lymphocytes to DB treatment.

Given the existence of at least several dozen naturally occurring or synthetic didemnins compounds for which structure-activity relationships exist in regards to cytotoxicity and inhibition of macromolecular synthesis, it may be possible to determine the contribution of TRiC inhibition to the didemnins' cellular effects (4). As some of the existing didemnin species may bind to and inhibit TRiC more potently than eEF1A, they could also serve as useful small molecule probes for studying TRiC function. Moreover, as Aplidin is still undergoing clinical trials for various cancers and was given orphan drug status for acute lymphoblastic leukemia and multiple myeloma in Europe, it will be important to determine whether TRiC inhibition is partially responsible for its anticancer activity (7). The other putative novel DB targets identified

in this study (Table 1) may also be important for one or more of DB's biological activities, but this will require further studies to first validate their binding. In conclusion, this chapter shows the successful identification of novel drug targets using a sensitive gel-free proteomics approach with DARTS that should facilitate many additional target identification projects.

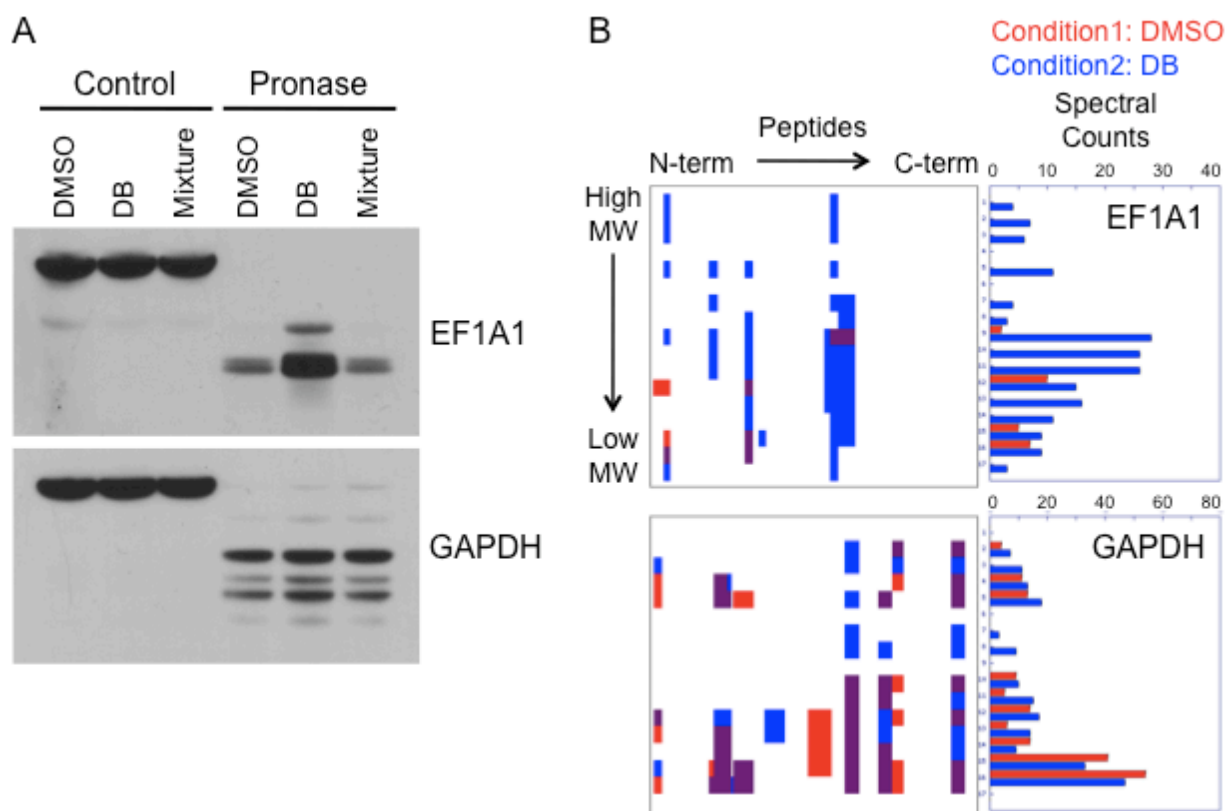


Figure 4.1. Didemnin B DARTS and Protomap analysis. (A) Jurkat cell lysates were incubated with 1% DMSO, 10 $\mu\text{g/mL}$ DB, or a mixture of five different compounds (excluding DB), followed by digestion with Pronase at a 1:300 ratio. Western blotting was performed to verify protection of DB's known target, EF1A1, and to ensure that proteolysis was even between each sample (using GAPDH as a control). (B) Protomap analysis comparing the DB and DMSO DARTS samples from A demonstrating protection of EF1A1 from digestion. The digestion pattern of GAPDH, on the other hand, is not affected by DB. The fractions for each sample are presented from high to low molecular weight (top to bottom) and the peptides identified for each protein are shown from N-terminus to C-terminus (left to right). Total spectral counts for all peptides from each protein found in each fraction are represented by the bar chart on the right. Purple areas on the left represent peptides that were identified in both the DMSO (red) and DB (blue) fractions.

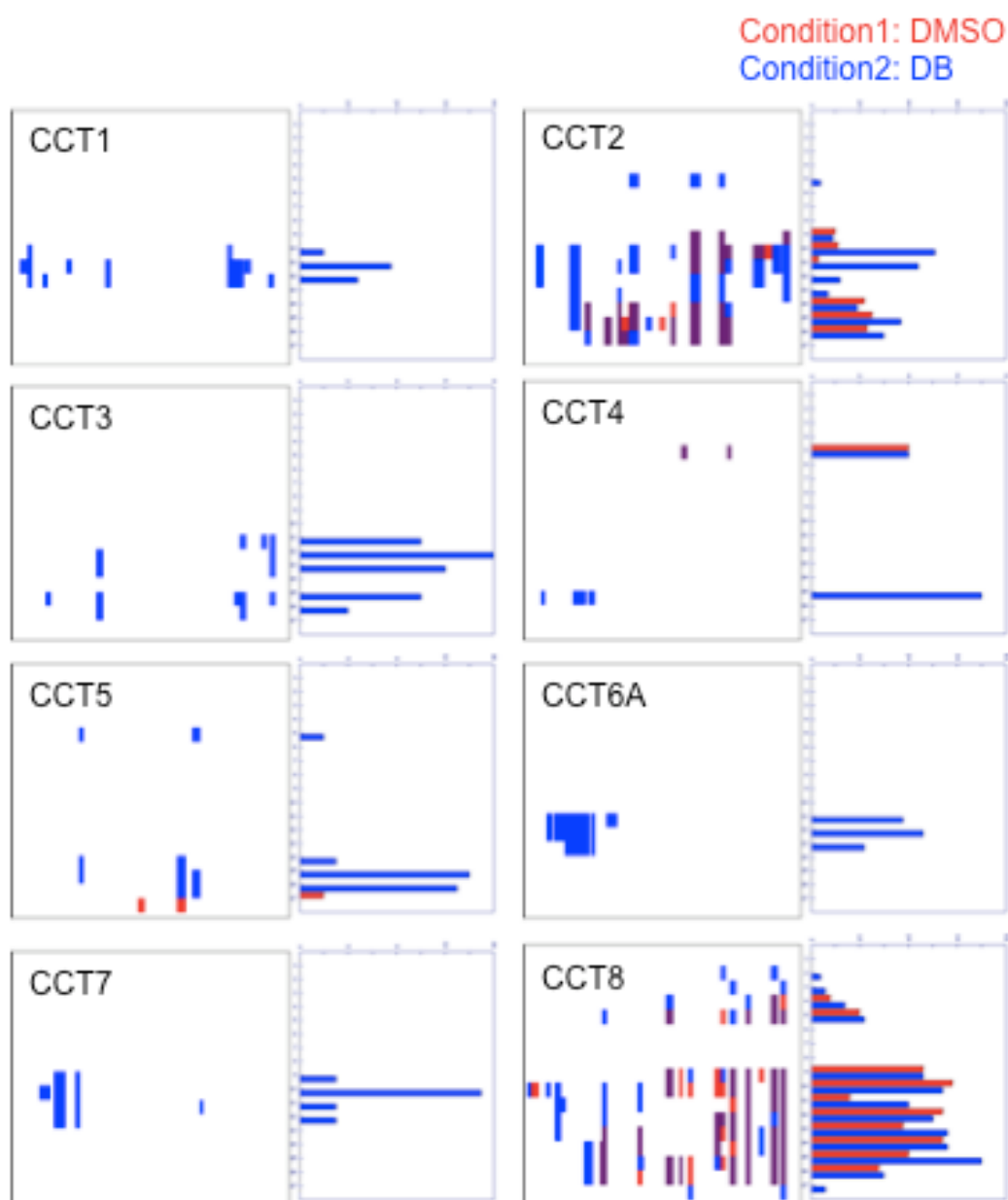


Figure 4.2. Protomap analysis of TCP1 complex members. Digestion patterns of all 8 TRiC subunits in DMSO and DB samples from Figure 4.1. All subunits except CCT4 and CCT8 show substantial protection from digestion by DB.

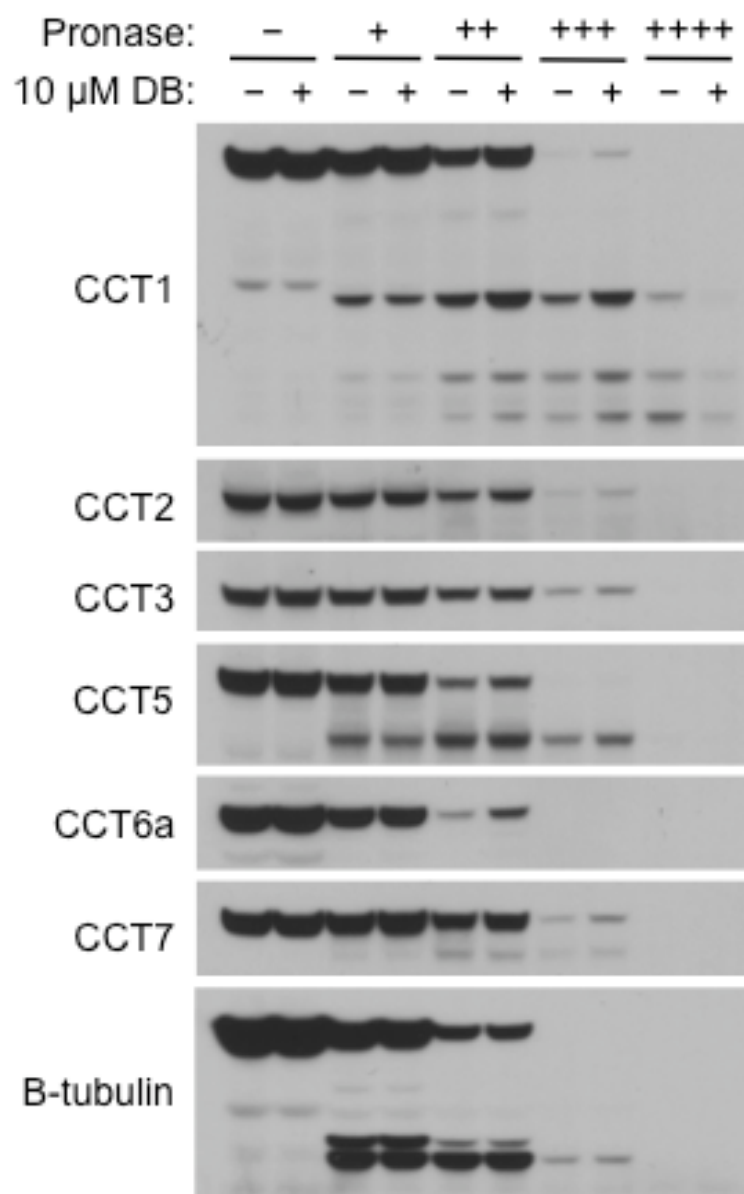


Figure 4.3. Protection of TCP1 complex members by DB. DARTS and western blotting shows protection of individual TRiC subunits by DB. Notably, CCT1 and CCT6a appear to be protected more strongly than the other subunits.

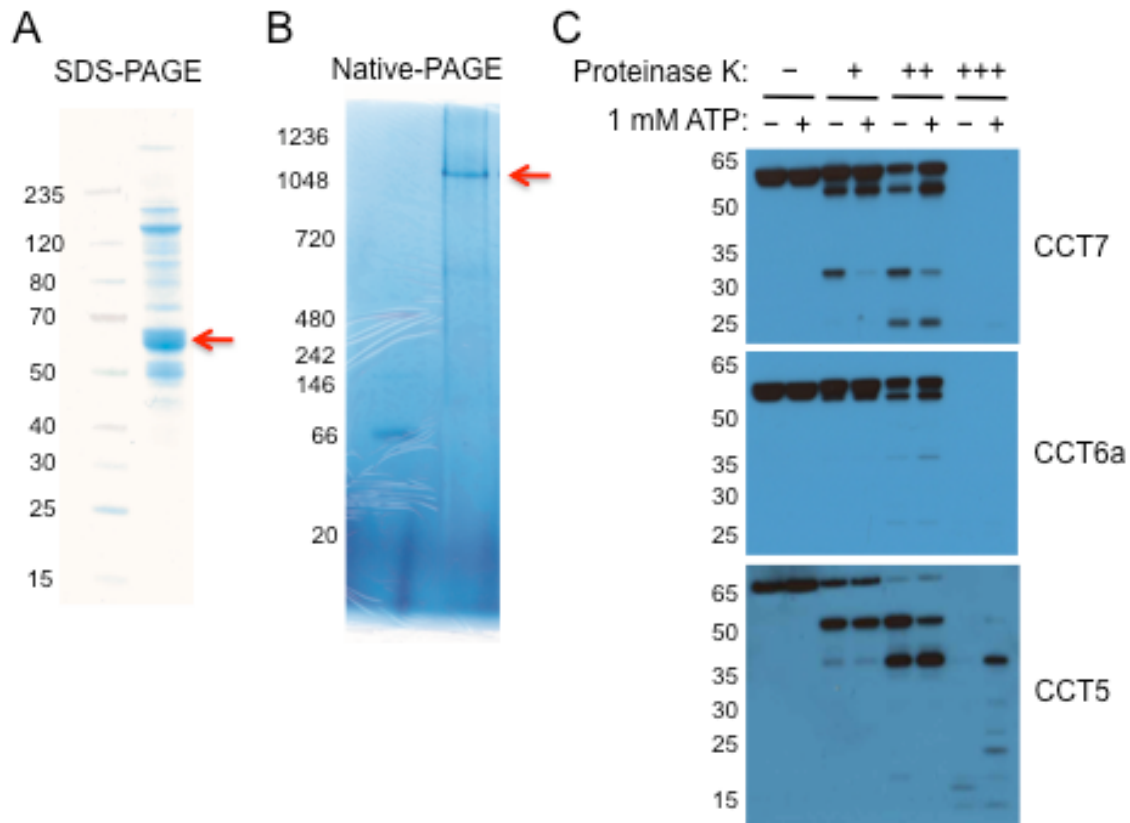


Figure 4.4. Analysis of TRiC purified from bovine testes. (A) Analysis of purified TRiC by SDS-PAGE and SimplyBlue staining shows the strongest bands around 60 kDa, consistent with the molecular weight of the individual TRiC subunits. (B) Analysis of purified TRiC by Blue Native-PAGE shows a single, strong band around 1 MDa, consistent with the size of the intact TRiC complex. (C) ATP provides protection of purified TRiC from proteinase K digestion. Protection could also be seen by staining the membrane with Ponceau (results not shown), but is much more clear by western blotting for individual subunits.

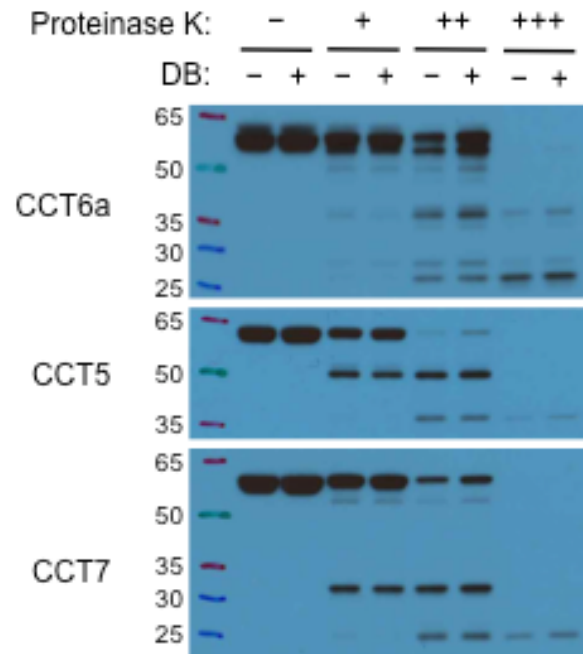


Figure 4.5. DB protects purified TRiC from digestion by Proteinase K. Limited proteolysis of TRiC was performed in the presence of DB or DMSO. Protection of TRiC by DB could be seen when 1 μ M ATP was included, but little or no protection was seen with no ATP or 10 or 100 μ M ATP (results not shown). Notably, CCT6a appears to be protected more strongly than CCT5 or CCT7, consistent with DARTS results using Jurkat cell lysates (Figures 4.2 and 4.3), whereas 1 mM ATP protects CCT5 and CCT7 more strongly than CCT6a (Figure 4.4).

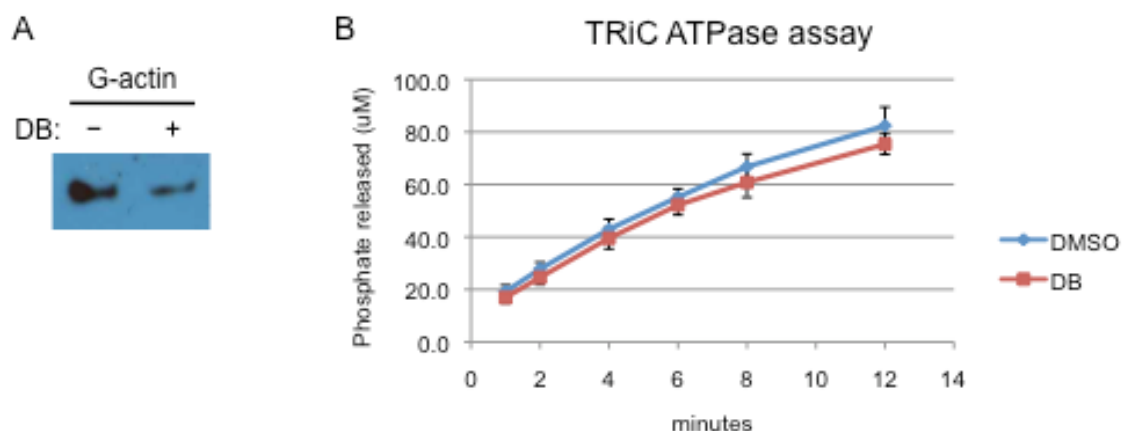


Figure 4.6. Analysis of the effect of DB on TRiC activity. (A) Representative image of inhibition of beta-actin folding by DB. The average amount of folded beta-actin detected in the presence versus absence of DB was 35% $\pm$ 5% (performed in triplicate). (B) DB does not significantly inhibit the ATPase activity of TRiC as measured using the malachite green assay (performed in triplicate).

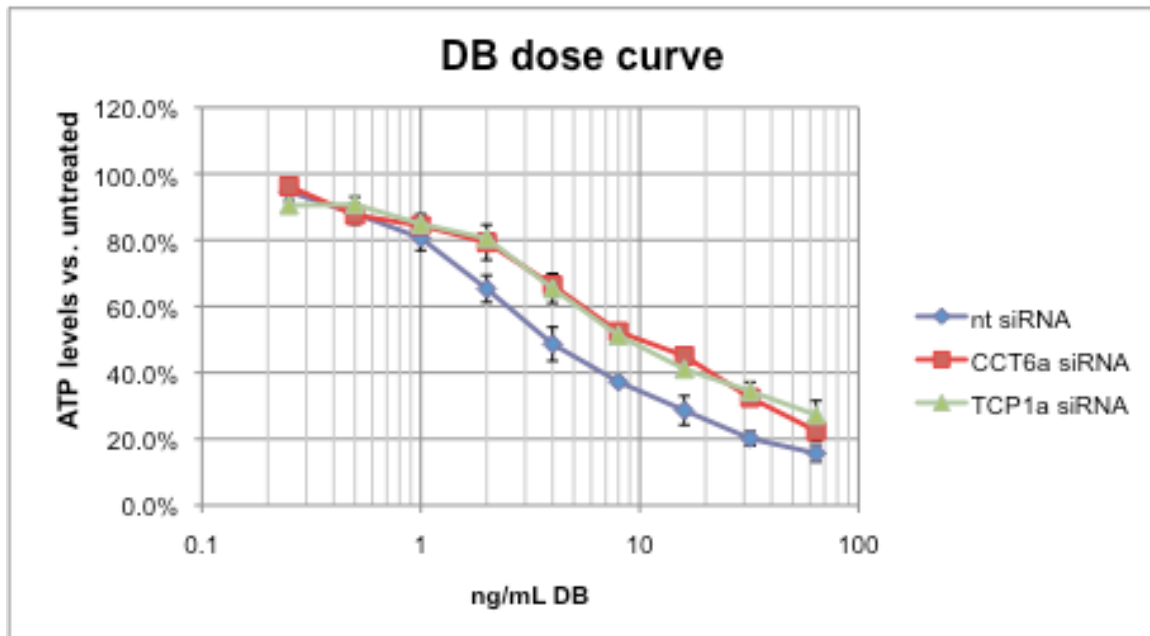


Figure 4.7. TRiC knockdown provides resistance to DB treatment. RNAi depletion of TRiC was performed in A549 cells, followed by treatment with a 2-fold dose curve of DB. After 2 days treatment, cell growth and viability was measured by CellTiter-Glo. Values represent percentage luminescence of each sample versus that of DMSO treated control samples (set to 100%).

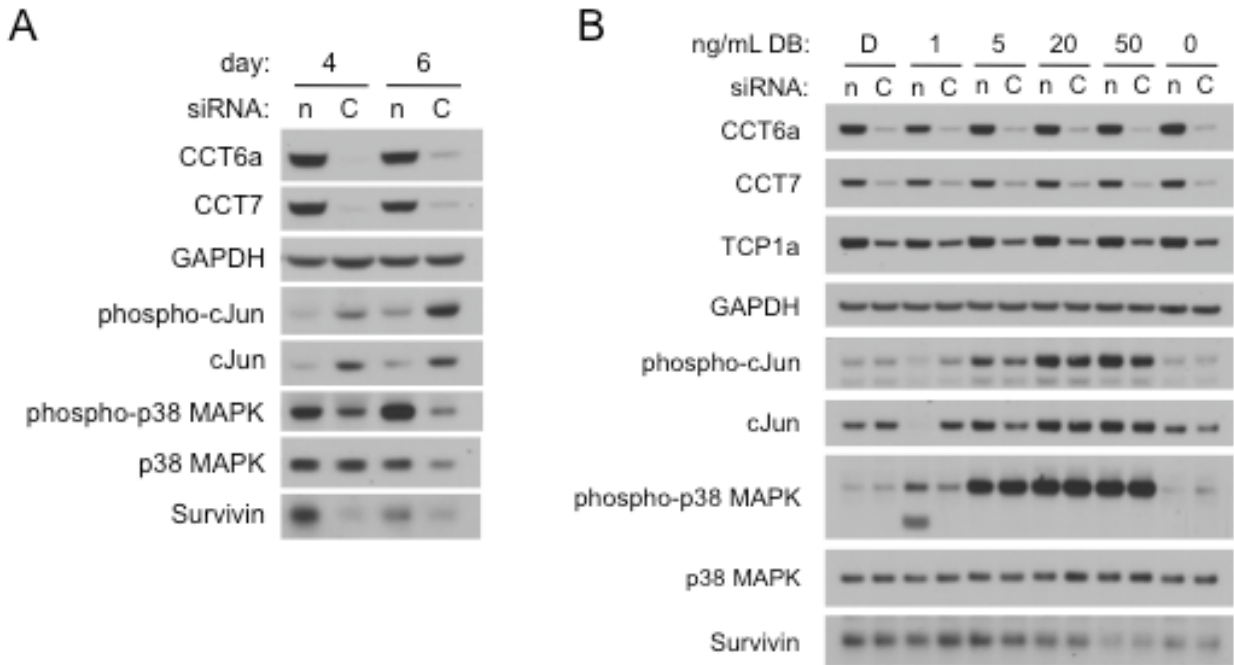


Figure 4.8. RNAi knockdown of TRiC in A549 cells affects DB-induced immediate early signaling events. (A) CCT6a siRNA (C) and non-targeting siRNA control (n) cells were collected 4 and 6 days post-transfection to analyze effects on the p38 MAPK and JNK pathways. (B) CCT6a siRNA (C) and non-targeting siRNA control (n) cells were treated for 2 hours with DMSO (D) or increasing concentrations of DB 2 days post-transfection to analyze effects on the p38 MAPK and JNK pathways.

Table 4.1. Proteins protected by DB in Protomap DARTS.

Protein	Spectra		Enrichment
	DMSO	DB	
CCT6A	0	53	
TCP1	0	36	
PLS3	0	27	
CCT3	0	26	
CCT7	0	24	
CCT5	2	32	16
UBE2L3	2	24	12
PPA1	3	30	10
EF1A1	28	225	8.04
ANP32E	5	22	4.4
RPS2	5	20	4

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CHAPTER 5

Conclusion

The data presented in this dissertation collectively demonstrate the utility of Drug Affinity Responsive Target Stability (DARTS) for identifying and studying protein-ligand interactions (1-3). We first established that small molecule binding renders its target protein protease resistant using a number of test cases: rapamycin and FK506 with FKBP12, rapamycin and E4 with mTOR, didemnin B with eEF1A, celecoxib with COX-2, and Smer3 with Met30 (Chapter 2). Importantly, this phenomenon of drug binding-induced protease resistance occurs regardless of whether the target protein is purified or it is part of a complex solution such as whole cell lysate. Moreover, ligand-mediated protease resistance is specific to the target protein, as proteolysis of non-target proteins is not altered by the presence of the ligand.

While DARTS can be used to individually test or validate candidate target proteins, its primary utility is as a discovery tool for uncovering novel protein-ligand interactions in an unbiased fashion. We first demonstrated this by performing mass spectrometry analysis of protein bands protected from proteolysis by didemnin B and resveratrol, which were visualized by SDS-PAGE and gel staining (Chapter 2). Although this analysis was only able to identify DB's known target eEF1A as being protected, it provided a valuable example of the power of DARTS to quickly and easily identify small molecule targets in an unbiased manner. For resveratrol, DARTS identified the protein translation initiation complex as a likely novel target, which was previously unknown. Although we could not determine which exact proteins are

directly bound by resveratrol due to the large number of subunits that were protected, we did provide evidence that the eIF4A/4G-containing complex is a likely physiological target by a cell-based translation assay and lifespan analysis in *C. elegans*.

In addition to target identification, we found that DARTS can also be used for mapping the small molecule binding domains within target proteins (Chapter 3). Upon digestion with increasing amounts of Pronase, proteins will generally be chopped into increasingly smaller fragments. We found that in addition to protection of the full-length target protein, small molecules are also capable of providing protection to fragments of the target protein that contain the binding domain. First, we demonstrated that proteolysis of eEF1A primarily proceeds from the C-terminus, and in the presence of DB multiple fragments as small as 30-kDa are highly protected. Analysis of this 30-kDa band by western blotting with various eEF1A antibodies and by mass spectrometry demonstrated that this fragment contains domains 1 and 2 of eEF1A, which were previously predicted to encompass the binding domain of DB by docking analysis (4). Importantly, this was the first experimental demonstration that this region of eEF1A does contain the DB binding domain. Moreover, we demonstrated that N-terminal fragments of eIF4A are protected by des-methyl, des-amino pantoic acid whereas C-terminal fragments of eIF4A are protected by hippuristanol; both of these results are consistent with the known binding domains of the two compounds (5,6).

Although our initial use of gel-based approaches with DARTS proved successful, their limited sensitivity may hinder finding novel targets using DARTS, particularly if those targets are not highly abundant proteins. Therefore, we implemented a gel-free proteomics approach with DARTS to improve the sensitivity of detecting proteins protected from digestion (Chapter 4). By fractionating Pronase-digested samples prior to mass spectrometry analysis, we were able

to identify both the known target of didemnin B (eEF1A) as well as several potential novel targets that were missed by the less sensitive gel-based approach (Chapter 2) and by affinity chromatography (7). For validation we decided to further test the TRiC complex since six out of its eight subunits were protected. After validating that DB protects the TRiC complex in cell lysates by western blotting in an independent DARTS experiment, we used the purified TRiC complex for further analysis. Not only did DB also protect purified TRiC from proteolysis, it inhibited folding of its most well studied substrate beta-actin *in vitro*. RNAi knockdown of TRiC in A549 lung cancer cells also affected immediate-early signaling pathways induced by DB and provided some resistance to DB treatment. Together these results suggest that TRiC may be an important physiological target of DB.

As modern medicine struggles with how to prevent, treat, and cure complex diseases such as cancer, diabetes, and neurodegeneration, a detailed understanding of the molecular mechanisms of drug action will become even more important. The success rate of targeted therapies continues to be relatively low, both because of a lack of knowing how best to target a complex biological system as well as because of harmful side effects from off-targets of the drugs used. This has led many to return to phenotypic screening to find compounds that are active in a more physiological setting that better predicts for beneficial effects in humans. Discovering the molecular targets of small molecule drugs is vital, both for uncovering additional targets of designer compounds as well as to discover the targets of compounds discovered through phenotypic screens. Such knowledge not only helps us to develop safer, more effective therapies but can also uncover novel biological connections and protein functions. Therefore, target identification methods such as DARTS will continue to be invaluable for the future of pharmacology and medicine.

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APPENDIX 1

Target identification using drug affinity responsive target stability (DARTS)

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Identifying the molecular targets for the beneficial or detrimental effects of small-molecule drugs is an important and currently unmet challenge. We have developed a method, drug affinity responsive target stability (DARTS), which takes advantage of a reduction in the protease susceptibility of the target protein upon drug binding. DARTS is universally applicable because it requires no modification of the drug and is independent of the mechanism of drug action. We demonstrate use of DARTS to identify known small-molecule–protein interactions and to reveal the eukaryotic translation initiation machinery as a molecular target for the longevity-enhancing plant natural product resveratrol. We envisage that DARTS will also be useful in global mapping of protein–metabolite interaction networks and in label-free screening of unlimited varieties of compounds for development as molecular imaging agents.

aging | label-free | proteomics | small molecules

Development of effective and safe therapies is the holy grail of medicine. For small-molecule drugs, which comprise most of today's medicines, a key challenge remains the identification of the molecular targets underlying drug therapeutic effects and/or adverse side effects. For small molecules discovered in phenotypic screens, which are increasingly popular in chemical genetics studies, identifying the biological (and potential therapeutic) targets, along with the off-targets, is a largely ad hoc affair; a systematic, widely applicable and robust approach is badly needed (1, 2). Current affinity-based target identification techniques are limited by the necessity to modify each drug individually (without losing bioactivity), whereas indirect, non-affinity-based approaches depend on the drug's ability to induce specific biochemical or cellular readouts (3, 4) (supporting information (SI) Text).

To overcome these limitations, we sought to develop a simple, universally applicable target identification approach that analyzes direct drug binding to targets. Given that a protein might become less susceptible to proteolysis when it is drug-bound than when it is drug-free (5–7), we hypothesized that this phenomenon could be exploited for target identification. This would allow the protein target of a drug to be revealed, without requiring modification or immobilization of the small molecule. Because our method, termed DARTS (drug affinity responsive target stability), is not limited by synthetic chemistry and is independent of any biological effects of the drug (save its binding to the target protein), it can potentially be used to identify the target for any small molecule.

Results

DARTS Strategy and Proof-of-Concept. The basic strategy of DARTS is shown in Fig. 1*A*. Binding of drugs is proposed to stabilize target proteins, either globally or locally, e.g., in a specific conformation or by simply masking protease recognition sites, thereby reducing protease sensitivity of the target protein. This idea is analogous to several familiar concepts, from DNase resistance of DNA sites bound by transcription factors (8) to

proteins protected from protease digestion through interacting with their natural ligands such as DNA (9) and carbohydrates (5). However, previous experiments used large (DNA) or high-affinity nanomolar hydrophilic (maltose) ligands, and both cases involve major conformational change in the host protein (10, 11). It remained unclear whether protease susceptibility of the target protein would be different in the absence of large conformational changes, e.g., upon binding of small hydrophobic drugs. Another question is whether the strategy would be amenable to lower-affinity ligands, e.g., clinically used drugs, which encompass a wide range of binding affinities, and hits identified from chemical genetic screens, which typically are in the micromolar range.

As a proof-of-principle, we examined the well-studied immunophilin FKBP12, which is the target for the nanomolar immunosuppressant drugs rapamycin and FK506 (12). Proteolysis of FKBP12 by the protease subtilisin was clearly decreased by the presence of rapamycin or FK506 (Fig. 1*B*). This protection is selective: Incubation with wortmannin, a drug that does not bind FKBP12, did not prevent proteolysis (Fig. 1*B*), and the drugs had no effect on subtilisin activity (Fig. S1*A*). Because X-ray cocrystal structures showed that binding of FK506 or rapamycin does not cause a conformational change in FKBP12 (12), our results above suggest that drug binding alone is likely sufficient to stabilize the bound protein in the protease-resistant state. This result could be due to a direct change in the protein folding–unfolding equilibrium upon ligand binding (5).

Given that rapamycin and FK506 are among the most potent and specific drugs available, we decided to test whether DARTS would work similarly with a much weaker inhibitor. E4 is a mid-micromolar kinase inhibitor of mTOR identified from a phenotype-based chemical genetic screen. Indeed, proteolysis of mTOR by thermolysin was decreased by E4 in a dose-dependent manner (Fig. 1*C* and Fig. S1*B*).

DARTS Using Complex Protein Mixtures. The experiments above established that DARTS can efficiently test, screen, or verify drug–protein interactions when the protein is available in relatively pure form. For DARTS to be generally useful as a discovery tool, however, applicability to complex protein mixtures (such as cell

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The authors declare no conflict of interest.

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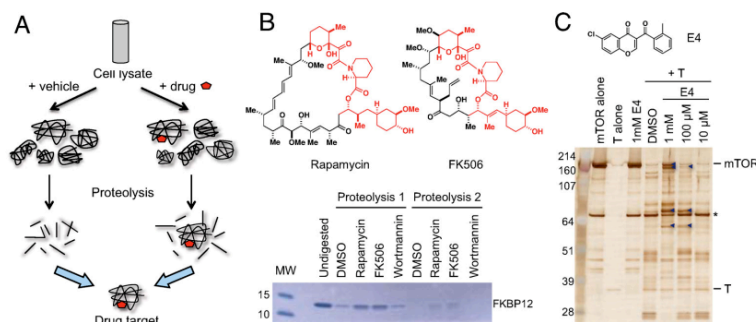


Fig. 1. The DARTS method for drug target identification. (A) Scheme of DARTS. (B) Proof of principle. Recombinant human FKBP12 was incubated with indicated drugs and digested with subtilisin. (C) DARTS with a micromolar mTOR kinase inhibitor (E4). Purple arrow, recombinant human TOR fragments protected from thermolysin proteolysis; *, nonspecific band.

lysates) would be desirable. To demonstrate feasibility, we performed DARTS using human Jurkat cells treated with didemnin B (DB), an anticancer marine natural product whose binding to EF-1 α had previously been well characterized (13). Given that EF-1 α is a highly abundant protein, we first tested whether in the DARTS protocol DB would protect EF-1 α from proteolysis and result in a detectable difference. Indeed, DARTS revealed a strong protected band at ≈ 50 kDa in the proteolysed extracts of DB-treated cells (Fig. 2A), whereas no detectable difference was observed in the same samples that underwent mock digestion.

Examination of the protected band and the matching gel region of the control lane by mass spectrometry confirmed that EF-1 α was the primary protein present at higher abundance in the DB-treated sample (Fig. 2B). This analysis does not exclude the possibility of other protected targets of lower abundance that were not evident by eye on the gel. DB-concentration-dependent proteolytic protection of EF-1 α was also observed by immunoblotting, both when intact cells were treated with DB (Fig. 2C) and when the lysates of untreated cells were incubated with DB in vitro (Fig. S2). The generality of this approach is further supported by experiments using diverse protein–drug pairs ranging from nano- to micromolar: mTOR–rapamycin, COX-2–celecoxib, and SCF E3 ubiquitin ligase-inhibitor (Fig. S3). Furthermore, DARTS is not enzyme specific, and much higher overall digestion efficiency can be achieved by using other proteases while retaining protection of the target protein (SI Text and Fig. S4).

Identification of a Molecular Target for Resveratrol Using DARTS.

Next, we applied DARTS to identify a molecular target of resveratrol, a compound in red grapes and wine known for various health benefits including lifespan extension (14). Although resveratrol influences the activities of many proteins, no direct molecular target has been demonstrated. Low specific binding affinity as suspected from its modest size and structure (Fig. 3A), poor potency, and potential requirement of the polyphenol groups for its activity have discouraged generation of affinity reagents for target identification. Also, even at saturating concentrations, resveratrol inhibits yeast growth only very weakly if at all (SI Text), making it a poor candidate for target identification using fitness profiling strategies.

DARTS with resveratrol-dosed yeast cell lysates revealed two silver-stained bands between the 15- and 20-kDa MW markers that were more intense in the resveratrol-treated lysate postproteolysis compared with vehicle control (Fig. 3B). Mass spectrometry analysis of both bands showed that eIF4A, along with several ribosomal proteins, were enriched in the resveratrol-treated sample (Table S1 and Dataset S1). This enrichment was confirmed by Western blot analysis using the TAP-tagged (15) eIF4A yeast strain (Fig. S5). This finding suggests that resveratrol might directly bind to one or more proteins comprising the protein translation machinery. Potential direct binding was further supported by a target mutation analysis, where a Tif1 A64Q point mutant confers resistance to resveratrol (Fig. 3C). Although the alanine is conserved throughout fungi and animals, plants have a glutamine at this position, and the

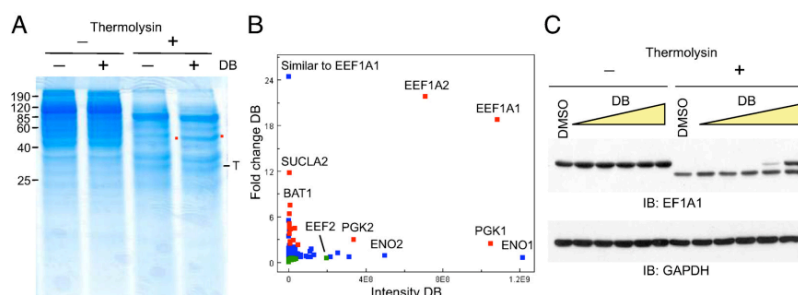


Fig. 2. DARTS using whole-cell lysate. (A) Intact Jurkat cells were treated with DB (1 μ g/mL), and lysates were subjected to thermolysin digestion and Coomassie (SimplyBlue)-staining. (B) Enrichment of EF-1 α isoforms in the protected band from A revealed by mass spectrometry analysis (SI Text and Fig. S6). Red, protein enriched >2 -fold with P value <0.001 ; green, protein depleted >2 -fold with P value <0.001 ; blue, unchanged protein. (C) DARTS detection via immunoblotting. GAPDH was resistant to thermolysin under the condition and served as a loading indicator.

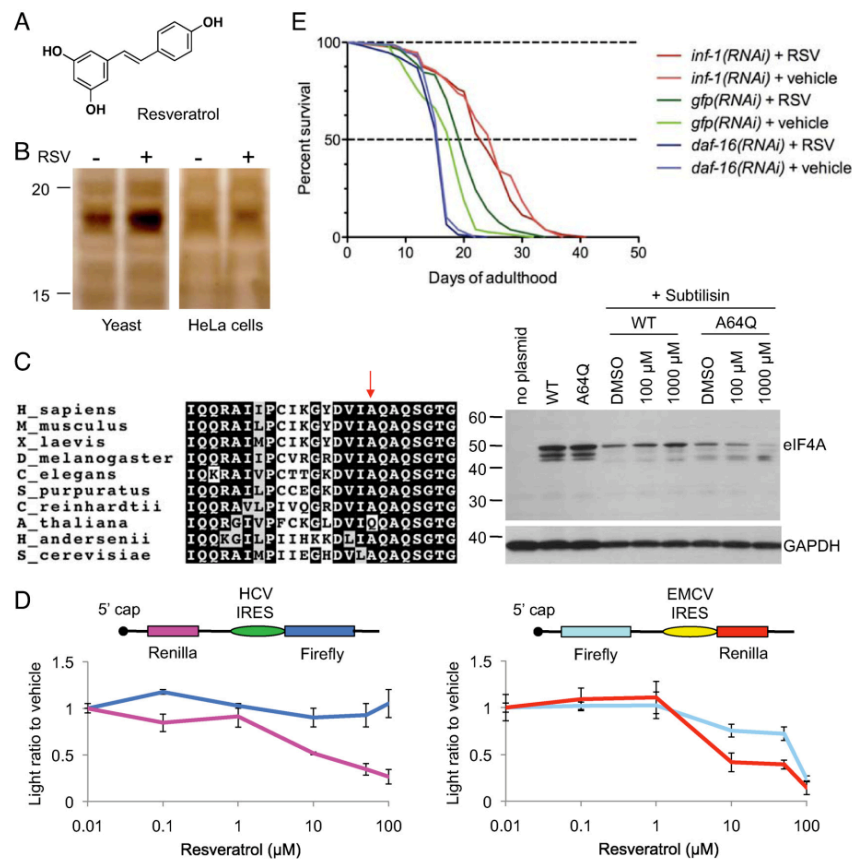


Fig. 3. DARTS identifies a molecular target of resveratrol. (A) Chemical structure of resveratrol. (B) Yeast cell lysates and human HeLa cell lysates were each treated with resveratrol in vitro, followed by thermolysin digestion and silver staining. Protected bands of similar size were detected. (C) Resveratrol protects the wild-type eIF4A, but not the A64Q-substituted eIF4A mutant protein, from proteolysis. (D) Resveratrol inhibits eIF4A-dependent translation in HEK 293 cells as assayed by bicistronic translation reporters. The EMCV IRES requires the eIF4A and eIF4G subunits of eIF4F, whereas the HCV IRES does not (55). (E) eIF4A is required for longevity in resveratrol-treated animals. Resveratrol (50 μ M) lengthens the lifespan of wild-type N2 worms fed control (*gfp*) RNAi (green), but not worms fed eIF4A (*inf-1*) RNAi (red) or *daf-16* RNAi (blue). *gfp(RNAi)*, $m_{veh} = 19$ ($n = 74$), $m_{RSV} = 20$ ($n = 78$), $***, P = 0.0006$; *inf-1(RNAi)*, $m_{veh} = 26$ ($n = 76$), $m_{RSV} = 24$ ($n = 79$), $P = 0.4687$; *daf-16(RNAi)*, $m_{veh} = 17$ ($n = 78$), $m_{RSV} = 17$ ($n = 76$), $P = 0.3305$. m , mean lifespan (days of adulthood); n , number of animals tested.

bulkier side chain is hypothesized to protect plant eIF4A from resveratrol inhibition by minimizing self binding.

The molecular mechanisms underlying resveratrol's lifespan effect have been controversial (16, 17), and whether Sir2 serves as a direct target for resveratrol is an interesting problem that is being pursued. On the other hand, it is interesting to note that multiple genome-wide studies in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* have found knockouts or knockdowns of eIF4A and several ribosomal proteins to have significant increases in lifespan (18). Our finding of resveratrol-mediated protection of eIF4A and ribosomal proteins by DARTS suggested that the protein translation machinery may be a molecular target of resveratrol in lifespan extension. To test this notion, we first asked whether resveratrol has a specific effect on protein translation. Using bicistronic dual-luciferase reporters to monitor cap-dependent translation (which requires initiation factors) and translation mediated by IRESs (which exhibit differing requirements for initiation factors), we

found that cap-dependent translation and EMCV IRES-mediated translation, both of which require eIF4A, were inhibited in a dose-dependent manner by resveratrol, whereas translation from the eIF4A-independent, HCV IRES was unaffected (Fig. 3D). These results indicate that resveratrol specifically inhibits eIF4A- or eIF4G-dependent translation initiation and does not impinge on other translation initiation factors or on translation elongation.

Finally, we asked whether eIF4A is required for resveratrol's longevity effect. Whereas resveratrol lengthens the lifespan of wild-type worms (Fig. 3E), as reported previously (14), this longevity effect is lost in eIF4A knockdown worms (Fig. 3E), consistent with eIF4A being a physiological target of resveratrol. Interestingly, the longevity effect of resveratrol appears to require *daf-16* (Fig. 3E), the Forkhead transcription factor that mediates lifespan extension by the insulin/IGF-1 pathway (19), reminiscent of its requirement for longevity in eIF4G knockdown animals (20). Taken together, it is plausible that resveratrol increases lifespan by

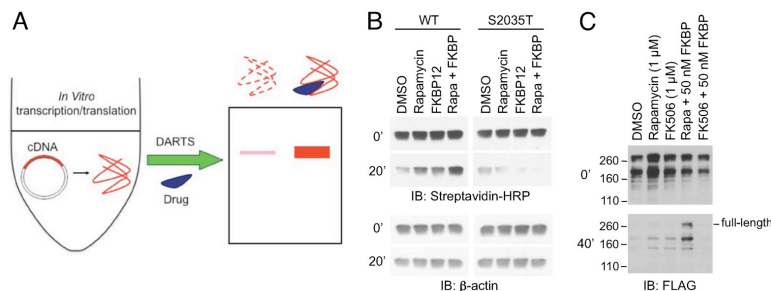


Fig. 4. DARTS using cDNAs. (A) Plasmid cDNA is used to program IVT for DARTS. (B) FKBP12-rapamycin protects translated mTOR fragment in DARTS. Streptavidin-HRP was used to detect biotin-Lys incorporated into the translation product. β -Actin was less susceptible to thermolysin under the condition and served as loading indicator. (C) DARTS with IVT FLAG-tagged mTOR.

direct inhibition of translation initiation, through binding to eIF4A and/or one or more ribosomal proteins in the preinitiation complex. However, this interpretation should be taken with an important caveat because the eIF4A-knockdown worms show a significantly enhanced lifespan (beyond the extension produced by resveratrol in wild-type worms). Furthermore, it is possible that knocking down an initiation factor like eIF4A will affect the expression level of many other proteins that could be targets. Our findings also point to eIF4A (and possibly other translation factors) as a previously uncharacterized druggable target for antiaging therapy. Several potent eIF4A inhibitors have recently been identified (21), and it will be interesting to test them for potential longevity effects.

DARTS Using Proteins Generated from cDNA. The utility of DARTS in complex mixtures suggests that potential drug targets can be identified by using a wide range of biological systems, and the method is unlimited by the availability and coverage of knockout (or knockdown) libraries and genome arrays for model organisms. The limiting aspect of DARTS analysis is likely to be sensitivity of detection by mass spectrometry or other potential methods (as in affinity chromatography). This limitation is being increasingly alleviated with the development of more sensitive analytical tools. Nonetheless, we tested whether DARTS can be applied to a complementary unbiased platform, namely using proteins generated from cDNAs by in vitro transcription/translation (IVT) (Fig. 4A).

Reticulocyte lysate IVT is a powerful technique routinely used in studies of protein function and is readily adaptable to express proteins in a high-throughput manner (22). As a test case, we used the human mTOR (mammalian target of rapamycin) protein, which is an important target (23, 24) and had been identified on the basis of its association with the FKBP12-rapamycin complex (see references in ref. 25). As shown in Fig. 4B, an IVT mTOR fragment containing the FKBP-rapamycin-binding domain (25) was protected from thermolysin digestion by the presence of FKBP12-rapamycin, whereas the S2035T-substituted mTOR that abolishes rapamycin binding (25) was not protected. IVT TOR proteins were labeled with biotin-Lys in this experiment, but other forms of amino acids could also be used. Alternatively, IVT proteins could be detected through an epitope tag without incorporation of any artificially labeled amino acids. For example, FLAG-tagged full-length mTOR protein was also a robust source of protein for the DARTS method (Fig. 4C). These results further demonstrate the versatility of DARTS and suggest nearly unlimited possibilities for screening cDNA libraries (26) and genome-wide collections of epitope-fused proteins (15, 27) using DARTS to systematically analyze small-molecule–protein interactions.

Discussion

Developing new methods for drug target identification is an area of intense interest, and both experimental and computational approaches have been developed (28, 29). Previous methods for drug target identification have had substantial success, but many limitations remain. Traditionally, affinity chromatography has played a major role in the identification of the binding targets for many biologically active small molecules and natural products (SI Text). In addition to affinity chromatography, many new methods for drug target identification have been developed, ranging from biochemistry to genetics, proteomics, and imaging (30–36) (SI Text). All current target identification methods are of two main categories: affinity-based methods, which detect the direct binding of the drug to its target(s); and phenotype-based methods, which infer drug targets/pathways from the physiological responses or biochemical signatures the drugs produce.

Affinity-Based Target Identification Methods. Affinity-based methods include matrix-based affinity detection and matrix-free affinity labeling. Matrix-based affinity detection fuses the small molecule of interest to a solid support or capturable moiety such as biotin (SI Text). Such matrix-based methods must satisfy three basic conditions: (i) that the small molecule contains a derivatizable functionality, (ii) that bioactivity/binding specificity of the small molecule is unaffected by the derivatization, and (iii) that the matrix does not hinder the binding of target protein to drug. The latter two criteria cannot be predicted *a priori*. Matrix-free affinity labeling relies on the incorporation of radioisotope, photoreactive, or fluorescent labels into the small molecule of interest (SI Text) and must also satisfy criteria one and two above. In both affinity chromatography and matrix-free methods, proteins are incubated with the modified small molecule, and the binding proteins are revealed by mass spectrometry after gel electrophoresis. Genetic and other versions of matrix-based affinity chromatography, e.g., yeast three-hybrid (37) and phage display cloning (38), require tagged small molecules as well. Thus, all current affinity methods are limited to small molecules that contain derivatizable functionalities and whose bioactivity/binding is unaffected by the modification (SI Text). Because DARTS does not require labeled ligands and instead uses “native” (i.e., unmodified) small molecules for binding, it is not limited by chemistry and can potentially be used for any small molecule.

Affinity-Free Target Identification Methods. Indirect, non-affinity-based approaches, which infer drug targets/pathways from the physiological responses or biochemical signatures the drugs produce, have also been developed. For example, classical genetics relies on the isolation of drug-resistant mutations (39) or gene

dosage effects (40), and several genome-wide methods also rely on fitness changes (3, 41–44). An inherent limitation of these methods is that they are applicable only to drugs that affect cell growth/viability. Another powerful approach, genome-wide expression profiling (4, 45), on the other hand, is applicable only to drugs that induce major transcriptome changes. These genetic and large-scale “omic” profiling approaches are also primarily limited to yeast or other simple, well-characterized model organisms. Moreover, the “readout” is often far downstream from the drug target.

Advantages of DARTS. Like affinity chromatography, DARTS relies on the affinity between a drug molecule and its protein target and thereby is able to pinpoint direct binding partner(s) of the drug. The key advantage of DARTS, however, is that because it does not require labeled ligands and instead uses “native” (i.e., unmodified) small molecules for binding, it is not limited by chemistry and can potentially be used to identify binding targets for any small molecule. Additionally, unlike cell-based methods, DARTS is completely independent of any effects of the drug on the system, and is therefore compatible with any mechanism of action, making it useful for any small molecule of interest. Moreover, DARTS can be performed by using any cell or tissue type from any organism and is thus not limited by the availability and coverage of knockout (or knockdown) libraries and genome arrays for model organisms.

Once identified, potential drug targets can be confirmed by functional studies, and kinetics and affinities of the interactions can be measured by using a variety of analytical methods. Although biophysical methods (i.e., surface plasmon resonance, isothermal titration calorimetry, etc.) are traditionally used to analyze direct binding, DARTS proves to be a fast and robust method to determine direct binding of a small molecule (or metabolite) without requiring large amounts of pure protein and is even amenable to using whole-cell lysates.

Potential Limitations of the DARTS Method. First, the binding affinity of the drug to its target may be a limiting factor. To date, our experiments suggest that DARTS is effective for molecules with inhibitory concentrations across many orders of magnitude, up to high-micromolar. Second, a potential fundamental limitation for DARTS is that a protein’s susceptibility to proteolysis is determined by its conformational energy landscape, and it has been demonstrated that a small number of evolutionarily selected proteins (e.g., stress proteins) are quite refractory to protease digestion (46). Third, drug binding may change the protease susceptibility of nontarget proteins, such as those that interact with or are part of complexes containing the target. But this result could be an advantage of the DARTS approach as well, insofar as it would provide information about protein complexes that are dissociated (or formed) upon drug binding. Drug binding *in vivo* might also increase proteolytic susceptibility of the target protein (47) (see *SI Text*). This would—in the DARTS protocol—also identify target proteins of the small molecule being analyzed. A small-molecule effector that destabilizes a protein could, of course, also be identified by DARTS.

An extrinsic limiting aspect of DARTS analysis is likely to be sensitivity of detection by mass spectrometry (as in affinity chromatography). Although DB-mediated protection of EF-1 α was visualized by eye on a stained gel in Fig. 2*A*, this will not necessarily be the case with many target proteins of lower abundance. Quantitative imaging or densitometry could prove useful with DARTS to assist in finding more subtle differences in protein abundance. Furthermore, proteomic techniques including 2D gels (48), DIGE (49), and gel-free approaches like MudPIT (50) would likely provide even greater sensitivity in conjunction with DARTS. Finally, the use of cDNA libraries to express proteins in cell culture or by IVT, as demonstrated in Fig. 4, also provides viable alternatives.

Effect of *in Vivo* Protein Stability on DARTS. We rely on *in vitro* proteolysis using exogenous proteases in our DARTS method. Protein stability *in vivo* on the other hand is a much more complicated problem. Because degradation of proteins inside the cell is predominantly carried out by supramolecular machines, known as the proteasomes and aggresomes, and is elaborately controlled by posttranslational modifications such as phosphorylation and ubiquitinylation, protein stability *in vivo* is largely unpredictable. Indeed, *in vivo* stability of proteins upon drug/ligand binding is highly idiosyncratic in the literature; drug binding has been shown to both increase and decrease proteolytic susceptibility of the target protein (6, 47, 51, 52). For instance, whereas unstable FRB domain and FKBP12 mutants are stabilized by the presence of ligands (6, 51) and topoisomerase-1 is destabilized by camptothecin (47), binding of estrogen receptor ligands each affects receptor stability differently (53). In any event, this information would be useful in conjunction with DARTS for elucidating the molecular mechanisms of action of drugs.

Additional Applications of DARTS. Beyond drugs, we envisage that DARTS will also be useful for global mapping of protein–metabolite interaction networks and in elucidating potential protein targets for small molecules found in food or dietary supplements. DARTS may also be useful in identifying a wide range of small molecules that can be developed into a new genre of molecular imaging agents. Pharmaceutical agents almost always interact with the active sites of enzymes or the ligand-binding sites of receptors. The design of most small-molecule molecular imaging probes usually begins with modification of the structure of known drugs. However, enzyme active sites and ligand-binding sites represent only a very small percentage of the tertiary structures of target proteins. Small molecules that bind tightly and specifically to sites other than the active site or the ligand-binding site on a protein and are detectable by DARTS would provide initial “hits” from which probes that can stoichiometrically measure protein concentrations by fluorescence, positron emission tomography, single-photon emission tomography, and other molecular imaging technologies could be developed through conventional medicinal chemistry or secondary chemical library procedures.

Materials and Methods

Reagents and Plasmid Constructs. See *SI Text* for additional information.

DARTS with Pure Proteins. See *SI Text* for additional information.

DARTS with Complex Protein Mixtures. For Fig. 2*A* and *C*, intact Jurkat cells were treated with DB from 100 pg/mL to 1 μ g/mL or DMSO control for 30 min. Cells were lysed (without washing, in these experiments) with M-PER (Pierce) supplemented with protease and phosphatase inhibitors. After centrifugation (14,000 rpm using Beckman Coulter Microfuge 22R with F241.5P rotor, 15 min), lysates were diluted to the same final volume and protein concentration with M-PER and proteolysed in reaction buffer [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM CaCl₂]. All steps were performed on ice or at 4 °C to help prevent premature protein degradation. Each sample was then quickly warmed to room temperature and proteolysed with 1 μ g of thermolysin for every 15 μ g of lysate for 10 min. To stop proteolysis, 0.5 M EDTA (pH 8.0) was added to each sample at a 1:10 ratio, mixed well, and placed on ice.

For DARTS using yeast cell lysates incubated *in vitro* with resveratrol (Fig. 3*B*), *S. cerevisiae* BY4742 cells were used (see *SI Text* and Fig. S7).

Mass Spectrometry Analysis. Gel bands were cut out and prepared for mass spectrometry analysis with trypsin digestion as described in *SI Text*. Peptides were analyzed by LC/MS/MS on a Thermo LTQ-Orbitrap mass spectrometer with an Eksigent LC pump. For quantitative comparison of protein and peptide abundances, MS spectra were analyzed by using the differential workflow of Rosetta Eludicator (Rosetta Inpharmatics) (54). Annotation was performed using PeptideTeller and ProteinTeller (see *SI Text*).

***In Vivo* Translation Assays.** See *SI Text* for additional information.

Lifespan Analysis. *C. elegans* lifespan analysis was conducted with N2 worms at 20 °C (see *SI Text*).

DARTS Using Proteins Generated by Rabbit Reticulocyte Lysate In Vitro Transcription/Translation (IVT) System. For Fig. 4B, IVT was performed by using Promega TnT T7 Quick Coupled Transcription/Translation System, with 0.5 μ g of pcDNA3.1-hTOR1968C (encoding human mTOR amino add 1968 to C-ter) or pcDNA3.1-hTOR1968C S2035T [encoding corresponding rapamycin-resistant mutant (25)] vectors (see *SI Text*), and 2 μ L of *e*-biotin-Lys-tRNA (Transcend tRNA; Promega) in 50- μ L reaction at 30 °C for 3 h. DARTS was performed by using 5 μ L of translated lysate in a 10- μ L total reaction volume in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM CaCl₂, with 1 μ M rapamycin, 50 mM FKBP12, or 50 mM FKBP12 + 1 μ M rapamycin (preincubated on ice for 30 min to allow complex formation) and incubated on ice for 30 min. Proteolysis was performed with 2 ng of thermolysin at room temperature for 20 min, and stopped with 1 μ L of 0.5 M EDTA (pH 8.0).

For Fig. 4C, N-terminal FLAG-tagged full-length human TOR protein was

synthesized by IVT using 0.5 μ g of pcDNA3.1-FLAG-hTOR vector in a 50- μ L reaction at 30 °C for 3 h (Promega TnT T7 Quick Coupled Transcription/Translation System). DARTS was performed by using 6 μ L of translated lysate in a 10- μ L total reaction volume in reaction buffer [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM CaCl₂] with 1 μ M rapamycin or 1 μ M FK506 or cotreatment with 50 nM FKBP12 (preincubated on ice for 30 min to allow complex formation) and incubated on ice for 45 min. Proteolysis was performed with 1 ng of thermolysin at room temperature for 40 min and stopped with 1 μ L of 0.5 M EDTA (pH 8.0).

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APPENDIX 2

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REVIEW

Identification of Direct Protein Targets of Small Molecules

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ABSTRACT Small-molecule target identification is a vital and daunting task for the chemical biology community as well as for researchers interested in applying the power of chemical genetics to impact biology and medicine. To overcome this “target ID” bottleneck, new technologies are being developed that analyze protein–drug interactions, such as drug affinity responsive target stability (DARTS), which aims to discover the direct binding targets (and off targets) of small molecules on a proteome scale without requiring chemical modification of the compound. Here, we review the DARTS method, discuss why it works, and provide new perspectives for future development in this area.

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Humans have been using small molecule drugs for their medicinal, hallucinogenic, and toxic properties for at least thousands of years; the oldest materia medica might be traced back to the legendary Shen Nong (the Divine Farmer) of 2737 B.C. Until very recently, Nature was the exclusive source of all such drugs. There are literally millions of species of plants, fungi, bacteria, and animals on Earth that produce a plethora of compounds with equally diverse chemical structures and pharmacological properties, many of which are still unknown to man. Only in the last one and a half centuries, and mainly during the past few decades, have we begun to acquire the ability to create ourselves compounds with desired properties, such as binding to and modulating the activity (or expression) of specific proteins (1–15). That being said, even when not using nature as the direct source of new drugs, we still often rely on inspiration from natural product compounds in our search for new medicines (16–18). Studies on the molecular mechanism of action of natural small molecules has also long been a goldmine for identifying the function and importance of proteins from many classes, including membrane and intracellular receptors, signaling proteins, and proteins involved in regulating and carrying out the cell cycle, cell growth, mitosis, DNA replication and repair, transcription, and translation (reviewed in refs 19–21).

A large percentage of compounds of current interest were derived from screens using cell culture or whole organisms and phenotypic or molecular readouts (22–30). While the opportunity for finding novel drugs or drug uses from such assays is high, their primary limitation is that they typically give no indication of the potential drug targets, and finding the direct targets is often the most challenging and time-consuming step of the project. Moreover, drugs screened for or designed to specifically modulate activity of a given protein of inter-

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est are still likely to bind multiple other proteins, many of which may not be predicted simply by sequence or structural homology.

Although mechanistic studies of drug action has a long history, identification of the direct targets of drugs has its roots in the pioneering work on affinity chromatography, biochemical fractionation, and radioactive ligand binding assays. Affinity purification of enzymes with small molecule inhibitors was first developed in the early 1950s by Leonard Lerman (31) and subsequently used for novel identifications by the McCormick and Anfinsen laboratories during the 1960s (32, 33). Around the same time others had begun to use radioisotope-labeled compounds and biochemical fractionation to enrich drug-binding proteins from crude tissue extracts and cell lysates (34–38). Starting with the proof of existence by purification of the *lac* repressor protein by Wally Gilbert, using radioligand binding measured by equilibrium dialysis as an assay (39), radioligand binding quickly became a successful method to identify receptors for hormones and neurotransmitters. Cuatrecasas isolated the insulin receptor, a very low abundance protein, using radioactive insulin binding and insulin covalently linked to agarose beads for affinity chromatography (reviewed in ref 40). Sol Snyder and colleagues discovered the opiate receptors in brain using a radioligand binding assay (41), assisting the demonstration and identification of endogenous opioid peptides and their myriad biological functions. He followed this with rapid identification of the 10 most important neurotransmitter receptors, which hundreds of other workers had been attempting unsuccessfully for years. His success came from identification of suitable high affinity and high specificity ligands and the know-how to employ them properly. The first neurotransmitter receptor to be purified was the nicotinic acetylcholine receptor, using radioactive snake toxin as a binding assay and small molecule affinity chromatography (as well as a tissue source with high expression, electric rays and eels) (42). The first direct identification of amino acids involved in general anesthetic binding (etomidate) in its biotarget, GABA-A receptors in brain, has used photoaffinity labeling with a photoincorporatable radiolabeled aziridine analogue of etomidate that retained anesthetic activity and microsequencing (43). Many prominent natural product target identifications have made use of one of these two techniques (44–52). Today, affinity chromatography, also known as affinity purification, has be-

come the most-used method (53), but radioaffinity and photoaffinity approaches occasionally find use (54).

Affinity Chromatography as a Classical Method for Target ID. Despite the large number of target identification techniques described to date, affinity chromatography remains the most widely used method (55, 56). The typical project begins with structure–activity relationship (SAR) studies in which various functional groups of the small molecule of interest are modified or removed to determine which one(s) are dispensable for drug activity. These nonessential site(s) are then used as points of attachment to an affinity tag (*e.g.*, biotin) or solid matrix (*e.g.*, Affi-Gel agarose beads). Then, much like is performed during immunoprecipitation of specific proteins using an antibody and Protein A/G-conjugated beads, the drug-linked beads are incubated with protein extracts, followed by extensive washing to remove nonspecifically bound proteins. Finally the tightly binding proteins are eluted with excess free drug or under highly denaturing conditions. Most often the eluted proteins are subsequently analyzed by SDS-PAGE and protein bands are identified by mass spectrometry. Given that there are typically many more non-specific binders than actual drug targets, negative control experiments using similarly tagged inactive analogues of the parental compound are performed simultaneously when possible. Once potential targets are identified, subsequent studies must confirm direct binding as well as its biological importance.

The primary limitation of affinity chromatography is the need to derivatize the small molecules of interest. SAR studies are time-consuming and require extensive medicinal chemistry expertise that is often lacking in the academic laboratories performing forward chemical genetics and phenotypic small molecule screens. Even when available, many small molecules cannot be modified without affecting bioactivity, and presumably binding, or cannot be easily obtained or synthesized in quantities large enough to permit SAR and subsequent studies. However, in comparison to other newly developed target identification methods that rely on various phenotypic or molecular signatures to narrow down potential targets, the advantage of affinity-based methods is that they rely solely on binding of the drug to its target protein, rather than specific cellular or biochemical readouts that are only useful for a fraction of compounds (reviewed in refs 57–62).

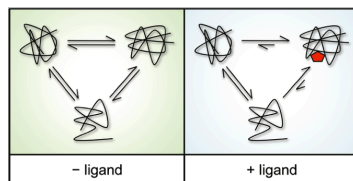


Figure 1. Theoretical model for why DARTS works. Under physiological conditions a protein is in dynamic equilibrium with multiple alternative conformations and may also exhibit some level of local, reversible unfolding (69, 71, 126, 127). Upon saturation with a specific ligand, the equilibrium will shift to highly favor the conformation bound by the ligand as a result of the negative free energy change due to hydrophobic, hydrogen bonding, and/or electrostatic interactions that are formed between the protein and the drug ligand. This leads to a thermodynamically more stable state in which the target protein's conformational fluctuations ("breathing") and unfolding are dramatically decreased and resistance to denaturation and DARTS proteolysis is markedly increased. (The fate of the target protein *in vivo*, on the other hand, cannot be predicted; ligand binding can cause either increase, decrease, or no change in the stability and/or expression of its target depending on the biology (62).)

A recent major advance in the affinity chromatography approach for target identification takes advantage of the quantitative capability of SILAC to drastically increase the sensitivity of this approach for target identification (63). This quantitative proteomic approach eliminates the need to visualize the target protein by gel staining, instead relying upon identification of the target by tandem mass spectrometry, which also allows the identification of multiple targets (polypharmacology). Furthermore, by comparison of the target pull-down with a well-designed control pull-down, true target proteins can be more easily distinguished from the many nonspecific binders due to their unique enrichment in the target pull-down sample. While this development is likely to enhance many target identification projects, it is still reliant upon SAR studies and limited to small molecules with derivatizable moieties. It is also contingent on the availability of a suitable inactive small molecule that can serve as the negative control, which is often not possible to obtain.

In summary, affinity chromatography of small molecules is severely limited due to the vast structural diversity and complexity of biologically active small molecules. Unlike affinity fusion (e.g., to glutathione

S-transferase, or GST) and iodination of peptides, which are carried out routinely by the standard molecular biological (64, 65) and chloramine-T (66, 67) methods, respectively, no universal or simple solution has been obtained to immobilize or label organic compounds.

Drug Affinity Responsive Target Stability (DARTS).

Given the shortcomings of current target identification methodologies, a breakthrough will likely require thinking outside the "chemically modifying the small molecule" box. We imagined that a target identification strategy that only relied on drug-protein binding yet did not require modification of the small molecule would be ideal. Such an approach could potentially identify any protein targets of small molecules with no limitations posed by chemistry or mechanism of action. The idea that a small molecule drug would stabilize its target protein's structure and result in protease resistance offered a possible solution to the problem of target identification, so long as the decreased proteolysis could be readily detected in complex samples. Stabilization of lysozyme structure upon substrate binding is a well-known phenomenon that has been demonstrated by increased resistance to denaturation by heat and chaotropic agents (68). This enhanced stability is postulated to result from a shift in the thermodynamic landscape of the protein to favor the ligand-bound state, which prevents much of the protein's innate flexibility and movement (or protein "breathing") (69–71) from being realized (Figure 1). One recent example that lends support for this model comes from the finding that FK506, which protects FKBP12 in DARTS (62), leads to stabilization of a high-energy backbone conformation that is otherwise minorly populated in the native conformational ensemble of free FKBP12 (72). Ligand-induced stabilization has been exploited by numerous techniques to detect and analyze specific protein-ligand interactions (73–77). It has also been instrumental for purification of natively folded recombinant proteins and subsequent structural determination by protein crystallization (78, 79). Even ligand-induced resistance to proteolysis turns out to not be a completely new idea, as it was amply demonstrated for serum albumin (80, 81), Staphylococcal nuclease and nuclease-T (82), firefly luciferase (83), *etc.* However, very few prior reports detailed small molecule-induced protease resistance, and there certainly had been no realization that it may be general enough to be used as a *discovery approach* for identifying unknown targets. Our work has shown just

TABLE 1. Small molecule–protein interactions confirmed or identified by DARTS

Ligand/Guest (Drug)	Structure	Source	Receptor/Host (Target)	Original target ID method	Binding target discovered/confirmed by DARTS	Binding confirmed by other methods
Didemnin B		Marine tunicate	EF1- α	N-Biotinylbis(c-aminocaproyl)didemnin A, streptavidin–agarose affinity chromatography (84)	Yes (cell lysate mass spec; cell lysate Western) (62)	[3 H]N-Acetyldidemnin A Sephadex G-25 M gel filtration column chromatography (84)
FK506		Bacterium (Streptomyces tsukubaensis)	FKBP12	FK506 Affigel affinity chromatography (45); 32-[1- 14 C]-FK506 and [3 H]dihydroFK506 Sephadex LH-20 column chromatography (45, 46)	Yes (recombinant protein) (62)	Co-crystal structure (85)
			Calcineurin	GST-FKBP12, FK506, glutathione Sepharose affinity chromatography (47)	N/D	Co-crystal structure (86)
Rapamycin		Bacterium (Streptomyces hygroscopicus)	FKBP12	[3 H]dihydroFK506 displacement Sephadex LH-20 column chromatography (87), yeast genetics (88)	Yes (recombinant protein) (62)	Co-crystal structure (85)
			TOR	Yeast genetics (88); GST-FKBP12, rapamycin, glutathione affinity chromatography (48–50); LexA-FKBP12, rapamycin yeast 3-hybrid screen (89)	Yes (cell lysate Western; IVT protein) (62)	Co-crystal structure (90)
Resveratrol		Plant; red wine	Tif1 (yeast eIF4A)	DARTS (cell lysate mass spec) (62)	Yes (TIF1-TAP Western; IVT protein) (62)	N/D; functional confirmation in translation reporter assay and lifespan assay (62)
Curcumin		Plant (Curcuma longa)	mTORC1	Protein complex disruption (91)	Yes (cell lysate Western)	N/D
Celecoxib		Synthetic (Searle)	COX-2	<i>In vitro</i> COX enzyme assay (92)	Yes (cell lysate Western) (62)	Co-crystal structure (93)
SMER3		Synthetic (ChemBridge)	Met30 (yeast)	Expression profiling; DARTS (cell lysate Western) (94)	Yes (IVT protein) (94)	<i>In vitro</i> ubiquitinylation assay; DSF (94)
E4		Synthetic (ChemBridge)	TOR	<i>In vitro</i> kinase assay (62)	Yes (recombinant protein) (62)	N/D

^aN/D: direct binding not determined. IVT: *in vitro* translated. DSF: differential scanning fluorimetry.

how widespread this phenomenon is and demonstrated the potential of utilizing it for target identification (62) (Table 1).

DARTS offers an unprecedented ability to identify new proteins targeted by small molecules. It is similar

to affinity chromatography in that both are affinity-based methods that start with complex protein samples and selectively enrich the target protein(s) while depleting all nontarget proteins. However, whereas affinity chromatography utilizes positive enrichment by selec-

tively pulling out the target proteins and leaving behind nontargets, DARTS uses negative enrichment by digesting away nontarget proteins while leaving behind the target proteins that are rendered protease-resistant (Figure 2). With several decades of affinity chromatography usage, its strengths and limitations have been well characterized. For example, a primary weakness is the often-high level of nonspecific binding of nontarget proteins to the matrix, which confounds isolation of the true targets. Although extensive washing of the matrix can help reduce the number of nonspecific binders, weaker true interactors will often be lost during the process. In contrast, DARTS does not require washing and can be used to analyze lower affinity interactions.

DARTS is also unique in that native small molecules are used without the need for chemical derivatization or even knowing the chemical identity or purity of the compound. As such, DARTS allows bioactive natural product extracts to be used for target ID prior to fractionation, thus enabling research of not only polypharmacology (95) but also polypharmacy (e.g., Chinese herbal medicine). This is a tremendous advantage over affinity chromatography and other prior methods.

Additional Protein-Based Target ID Platforms.

Proteome Chips. The advent of proteome microarrays has revolutionized the global analysis of the biochemical activities of proteins, including the study of protein–

shared with affinity chromatography is the need for small-molecule labeling and the requirement that the labeling moiety can be incorporated without abolishing the compound's bioactivity. The power of this approach will be greatly enhanced by the development of label-free binding detection compatible with the proteome array format (99).

ABPP. A chemical proteomic approach that accesses protein targets *via* the use of reactive small molecule probes also constitutes a powerful means for identifying unknown targets. For example, activity-based protein profiling (ABPP) relies on small molecules containing reactive functional groups (mainly electrophiles) that can covalently attach to catalytic residues in an enzyme active site (100–103) (reviewed in ref 104). Such activity-based probes allow the targeted enzymes to be labeled for purification and analysis. Many flavors of ABPs have been created, including photoactivatable, radioactive, and biotinylated (105, 106), which greatly facilitates both discovering novel enzymes and inhibitor screening (reviewed in refs 54 and 107). Covalent linkage between a reactive probe and its target can thus serve the purpose for target identification (52, 108), although the requirement for derivatization of each small molecule still exists.

SPROX. In the realm of using label-free small molecules for binding target identification, another affinity-based label-free methodology was introduced in 2010 (109). Stability of proteins from rates of oxidation (SPROX) is similar to DARTS in that it detects small molecule-induced changes in the folding and thermodynamic stability of target proteins in complex samples (109). Instead of using differential proteolysis as a readout, SPROX measures ligand-induced changes in the rate of methionine oxidation for target proteins. This approach is similar in essence to hydrogen–deuterium exchange, which has been used extensively to analyze protein–ligand interactions and determine ligand binding domains within individual proteins, except that methionine oxidation levels are more readily measurable in complex protein mixtures (110, 111). Although SPROX clearly has many of the same advantages as DARTS, it has many additional limitations. The major limiting factor of SPROX is that only the most abundant proteins in each sample can be identified and accurately quantified. Unlike affinity chromatography and DARTS, SPROX has no mechanism to enrich the target proteins from nontarget proteins. Therefore, obtaining proteome-wide

KEYWORDS

2D-PAGE: A two-dimensional gel-based method that combines isoelectric focusing and SDS-PAGE to separate proteins by charge and molecular weight.

Affinity chromatography: Traditional method of target identification that uses immobilized small molecules to pull down binding proteins from a complex protein mixture.

Degradomics: The subfield of proteomics devoted to the study of proteases and their biological substrates.

Drug affinity responsive target stability (DARTS): A method for target identification that relies on drug-induced protease resistance.

MudPIT: A gel-free proteomics technique that uses online separation of tryptic peptides by strong cation exchange and reversed-phase HPLC to analyze very complex protein mixtures by mass spectrometry.

SILAC: Stable isotope labeling of amino acids in cell culture.

Target identification: Determination of which proteins a particular small molecule binds in the cell.

coverage using SPROX would require extensive up-front fractionation of the sample. Additionally, only methionine-containing peptides are useful with SPROX analysis, and not all methionine residues exhibit differential oxidation rates informative for determining thermodynamic changes. This is in contrast to the DARTS and affinity chromatography which can utilize information from any identifiable peptides.

Methodology and Practice of DARTS. The simplest procedure for DARTS simply involves separation of compound-treated and control protein samples digested with varying amounts of protease by 1D SDS-PAGE, staining the gel with Coomassie Blue, Sypro Ruby, or silver stain, and analyzing the respective lanes of the gel for bands that are more intense in one sample over the other. Upon finding a band whose abundance differs between the compound-treated and control samples, each band can be cut out, digested with trypsin, and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). After annotating the peptides and proteins identified in each gel band, label-free quantitative analysis using spectral counting, LC/MS extracted ion currents (XIC), or MS/MS total ion current (TIC) can determine which identified protein has been enriched in your DARTS experiment (112–114). Despite its simplicity, we have shown the utility of the 1D gel approach for identifying previously known and novel drug targets with DARTS (62).

The initial success of DARTS with 1D gels is likely due to two factors: the targets of the chosen small mol-

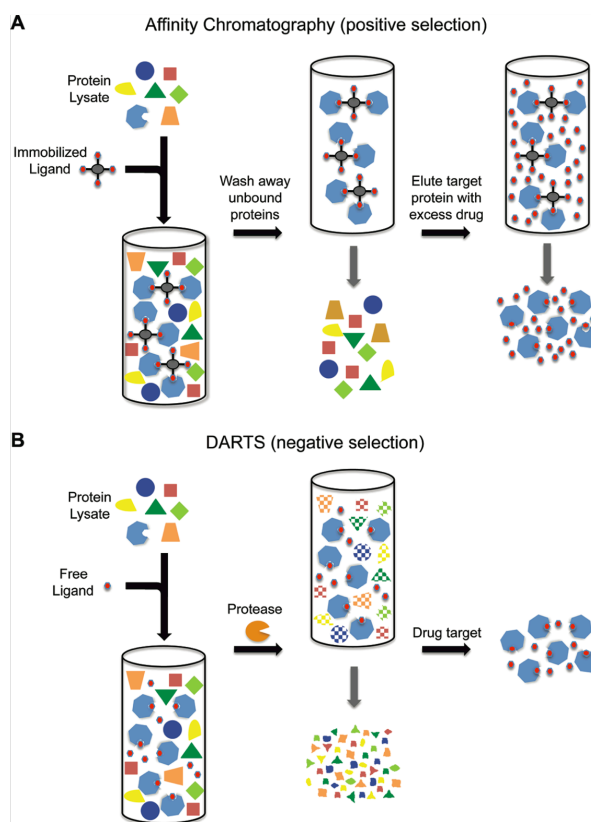


Figure 2. Comparison of affinity chromatography and DARTS. (A) Using small molecule affinity chromatography, protein lysates are incubated with the immobilized small molecule to positively enrich target proteins. Unbound proteins are washed away, followed by elution of the target proteins with excess free drug. (B) With DARTS, protein lysates are incubated with native drug and then treated with proteases. The target proteins are negatively enriched due to their drug-induced protease resistance while nontarget proteins are digested away.

ecules are both high abundance proteins, and neither protein is extremely sensitive or resistant to the proteases used (62). Given the need not only to visualize the target protein on the gel but also to determine that it is more abundant in one lane versus another, it is not hard to imagine that many potential drug targets would be missed by this nonsensitive analytical approach. The target protein could either not be sufficiently abundant

in the cell to be visibly stained, or even if it is abundant enough to see, its enrichment in one sample over another could be masked because the protein comigrates with many other proteins of the same molecular weight on the gel. If just one of these comigrating proteins is much more abundant than your target protein, the fact that it is present at the same amount in both samples will hide the fact that the target protein is highly enriched in one sample. Moreover, the presence of several low or moderately abundant proteins could just as easily mask the differential abundance of the comigrating target protein. Given the limitations of this approach to DARTS, our lab and others are currently pursuing several different proteomics (mainly mass spectrometry) and nonproteomics (mainly cDNA based) platforms that offer vastly increased sensitivity and throughput; only the proteomics approaches will be discussed herein.

Perspective for Future Advances in DARTS.

Proteomics. Perhaps the easiest way to more readily detect changes in protein levels between samples is to perform two-dimensional gel electrophoresis, also known as 2D-PAGE, in which proteins are first separated by charge in the first dimension, followed by SDS-PAGE in the second dimension (115). The two orthogonal separation techniques utilized by 2D-PAGE allows for more sensitive visualization of changes in protein abundance between samples because only proteins with the same isoelectric point and molecular weight will be present in the exact same spot on the gel. Whereas with 1D SDS-PAGE a single band on the gel will likely contain many, potentially dozens of different proteins, most spots on a 2D gel will consist of a single or very small number of proteins. Therefore, abundance differences in single proteins between two samples are much less likely to be masked by other comigrating proteins in 2D-PAGE. Additionally, the development of difference gel electrophoresis (DIGE) allows for even more sensitive analysis of protein abundance differences. With DIGE, two or three samples are analyzed simultaneously by labeling proteins in each sample with differently colored fluorescent tags, which are then combined and separated on a single 2D gel (116). Through automated quantitative analysis of fluorescent signals at each gel spot, similar to a microarray for gene expression analysis, this method eliminates the challenge of comparing all the spots between two or more separate gels, as well as any worries about reproducibility of protein migration patterns between multiple gels. Any spot that exhib-

its differential abundance can then be identified by MALDI-MS or LC-MS to identify the putative target protein.

An alternative proteomics approach that can potentially be paired with DARTS is multidimensional protein identification technology (MudPIT) (117). MudPIT is analogous to 2D-PAGE in the idea that two orthogonal separation techniques are used to provide enhanced coverage of proteins, yet it provides several fold higher sensitivity over 2D-PAGE. Rather than separate intact proteins, however, MudPIT is performed on the tryptic peptides derived from complex protein samples. The peptides are first separated by charge using strong cation exchange (SCX). As the peptides are eluted from the SCX phase, they directly enter the second separation phase consisting of C18 reversed-phase matrix, which then fractionates the peptides based on hydrophobicity just as in traditional LC-MS analysis. After elution from the reversed-phase matrix the peptides are directly sprayed into the mass spectrometer for analysis. This method eliminates the use of gels, as well as the need to visually analyze your samples to find differentially abundant proteins. Two or more samples can be analyzed by MudPIT separately using spectral counting or other label-free methods for quantitative analysis. Alternatively, multiple samples can be labeled using SILAC, ICAT, or iTRAQ and analyzed together in a single MudPIT run for even more sensitive quantitative comparison (118–120).

We have devised a strategy in which the protein samples after DARTS protease treatment are dialyzed to remove all the small peptides and amino acids resulting from digestion, enriching the samples with intact proteins or protein fragments above a given molecular weight (*e.g.*, 10 kDa). After dialysis, the samples are trypsin digested in solution and subjected to MudPIT analysis (Figure 3). The dialysis step is necessary because the large number of small, nontryptic peptides generated during the DARTS protease treatment would compete with tryptic peptides during the MudPIT analysis both for binding to the SCX and reversed-phase matrices as well as for MS/MS analysis by the ion trap mass spectrometer. Their elimination from the sample greatly reduces the sample complexity and increases the likelihood of identifying target proteins of the small molecules under study. This approach is potentially much more sensitive than a gel-based approach and is com-

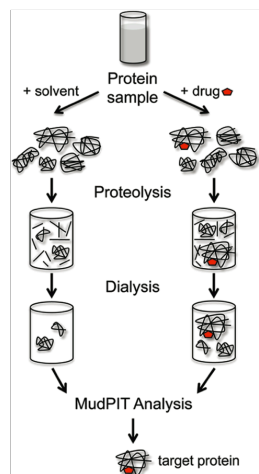


Figure 3. Combined DARTS-MudPIT approach. Protein lysates are split into two and incubated with the small molecule or solvent as a control. After proteolysis, each sample is dialyzed to remove digested peptides, followed by MudPIT analysis to look for enriched proteins in the drug-containing sample. Quantitation can be performed with any isotopic-labeling strategy or using label-free approaches (see text).

patible with all the mentioned label and label-free quantitative methods.

Degradomics. In addition to the arsenal of proteomics tools already described, new developments in a subfield of proteomics called degradomics may also be applicable in DARTS. The degradomics field is devoted to the study of proteases and the identification of all their biological substrates, aka degradomes, and many ingenious techniques have been developed and used to successfully identify hundreds of previously unknown protease substrates (reviewed in refs 121–123). Whereas degradomics aims to identify those few proteins that are proteolyzed against a larger background of undigested proteins, DARTS seeks to find those few proteins that are either not digested or are digested to a lesser extent in one sample versus another, against a larger background of proteins that are equally digested in both samples. Despite these differences, we anticipate that current and future developments in degradomics could have an enormous impact on DARTS by facilitating identification of proteins that are differen-

tially proteolyzed in the presence of small molecule drugs and ligands.

Experimental Conditions. In addition to using more sensitive proteomics techniques, the choice of protease, lysis buffer, and detergents may also influence the likelihood of successfully identifying drug targets with DARTS. The goal of current methods development is to maximize digestion of all nontarget background proteins without compromising the protection of the target protein, as well as to minimize the number of conditions that must be tried for each drug. An ideal DARTS condition would be one in which all proteins are digested at the same rate and extent so that the only intact proteins remaining after proteolysis would be drug targets. However, given the huge diversity of protein sequences, structures, conformational dynamics, complexes and interactions, it is no surprise that proteins vary dramatically in their sensitivity to proteolysis. Furthermore, given that different proteases target specific residues or short motifs for degradation sites as well as have a variety of preferences for folding or location of the recognition site within a polypeptide, proteome-wide susceptibility to proteolysis also varies depending on the protease used.

The initial use of thermolysin and a gentle, nondenaturing lysis buffer meant that only proteins that spontaneously unfold or have native unstructured regions could be digested, despite it being rather nonspecific with regards to substrate sequence. While the protection from thermolysin proteolysis in DARTS experiments was exceptionally robust, presumably because binding of the drugs prevented unfolding of the target proteins, a large fraction of background proteins always remained that were refractory to thermolysin digestion. This complicated proteomic analysis because the remaining samples were very complex, biasing all identifications toward high abundance proteins. Furthermore, if the drug target had been among those proteins not digestible by thermolysin, it would never be identified by DARTS. To overcome this, we first tried another single protease that can digest natively folded and denatured proteins. Using subtilisin we were able to protect target proteins, albeit to a lesser extent, under conditions in which almost all proteins were at least partially digested. Subsequently we reasoned that a mixture of many different proteases with various substrate specificities might work even better than any single protease. This seems to be generally true, as the commercially

available Pronase, a mixture of endo- and exoproteases that digest native and unfolded proteins, can digest all proteins to a similar extent as subtilisin while allowing for a high level of protection similar to that seen with thermolysin. It is conceivable that other homemade protease mixtures could be optimized for DARTS that would not suffer from lot-to-lot variability as does a crude extract like Pronase (which, nevertheless, can be “calibrated” using homemade protein or lysate standards).

Another rather simple approach to maximize sensitivity is to perform subcellular fractionation prior to DARTS analysis. This could particularly be useful when the target of a given drug is believed or known to reside in a specific cellular compartment, such as the nucleus, mitochondria, ER, Golgi, or plasma membrane. Simple and robust methods already exist to purify each of these subcellular structures to near homogeneity from a variety of cell and tissue types. The proteins isolated by most subcellular fractionation techniques are natively folded and fully compatible with DARTS analysis. Indeed, such an approach to improve sensitivity of target identification has been used with other affinity-based approaches (124).

Drug Screening with DARTS. In addition to target identification, protease resistance in DARTS can also potentially be used as a screening readout. Although protein stability measurements made by differential scanning fluorimetry (DSF), differential static light scattering (DSLS), and isothermal denaturation (ITD) are readily performed in microscale and can be rapidly adapted to high-throughput screening of nearly any protein regardless of whether function has been demonstrated, these methods all require relatively large amounts of pure protein in order to screen large compound libraries (125). On the other hand, DARTS is unique in that it does not require using pure proteins. While DARTS can be performed with pure protein as well, its main advantage is that the level of protein purity is not a concern. Moreover, by using miniaturization techniques, high-throughput small-molecule DARTS screening could be performed against any protein in a complex protein mixture. Screening with DARTS, like DSF, DSLS, and ITD, has the potential of identifying compounds that bind to a large number of sites on the target protein. Such molecules may be activators or inhibitors of the protein that can individually or in combination serve as leads for specific probes useful for dissecting its molecular functions across cell types and species.

Conclusions. Drug target identification has been a decades-long quest that until recently was largely dominated by a single technique, affinity chromatography, with minimal improvements. However, with the introduction of many newer affinity-based and affinity-free approaches with diverse chemical and biological mechanisms for identifying drug targets, it may be difficult to choose which methods to use for a particular project. Methods that require extensive research resources or technical expertise have a high “activation barrier”, which often precludes them from being used regardless of their likelihood of success. Furthermore, *a priori* prediction of the methods most likely to work for a given drug is usually not possible.

Although in its infancy, DARTS has the potential to be a major player in drug target identification. The DARTS phenomenon holds over such a wide range of molecules (even for high-micromolar ligands) and conditions (buffers, detergents, proteases, *etc.*) that essentially no (or little) experimental optimization is required. Getting started with DARTS is thus straightforward and can be performed in any lab with basic molecular biology or biochemistry setup. It is also quick to carry out and can be performed with any small molecule and protein samples from any organism. Future improvements in DARTS methodology are expected to increase its sensitivity and decrease the number of proteolysis conditions that must be tested for each small molecule. The use of more sensitive proteomics techniques with DARTS should greatly enhance its capability to identify lower abundance targets, although which exact methods will prove most useful is to be determined. Furthermore, a nonproteomics approach using cDNA libraries provides an alternative means to perform DARTS against the proteins encoded by any genome of interest (62).

Last but not least, DARTS may also be used as a means of target validation. It provides a simple and fast method for verifying direct binding of small molecules to presumed target proteins that were identified by alternative approaches. This may be particularly useful when a list of potential target proteins is suspected by phenotypic analysis or obtained through omic screens or computational prediction (which may implicate proteins of a particular complex or pathway in the compound's mechanism of action but are unable to show which protein if any is directly bound by the drug (94)). DARTS can also be used to identify the binding domain within the target protein because with

more extensive protease treatment often the full-length polypeptide of the target will not survive but a smaller fragment corresponding to the binding domain will retain protease resistance. For both target validation and target domain mapping, the robustness of DARTS with proteins generated from DNA constructs (e.g., expressed in cell lysates or by *in vitro* transcription-translation) becomes extremely handy (62, 94). With such versatility, DARTS is likely to

contribute considerably to the identification and analysis of drug-protein interactions for a long time to come.

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